BIOMARKERS OF ACUTE AND CHRONIC HUMAN HEART ALLOGRAFT REJECTION

by

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Abstract

Background: Cardiac transplantation is considered the primary therapy for patients with end-stage heart failure. However, the detection of chronic cardiac allograft rejection (expressed as cardiac allograft vasculopathy; CAV) remains an important unsettled issue in cardiac transplantation. The current gold standards for the diagnosis and monitoring of acute rejection and CAV are invasive in nature with risk for complications. From a clinical perspective, more accurate, minimally-invasive alternatives are clearly desirable. The goal of my thesis is to identify biomarkers of human heart allograft rejection, and assess their potential clinical utility and biological implications in the respective disease contexts.

Central hypothesis: Peripheral blood-derived molecular biomarker panels provide a means for sensitive and specific diagnosis of acute and chronic cardiac allograft rejection, as well as helping to gain insight into the underlying mechanisms of rejection.

Methods: Genomic biomarkers of acute cardiac allograft rejection (AR) and proteomic biomarkers of cardiac allograft vasculopathy (CAV) were identified via Affymetrix microarray analysis of whole blood samples and iTRAQ proteomic analysis of plasma samples, respectively, from cardiac transplant patients. From the genes differentially expressed between AR vs. Non-rejectors (NR), and differentially expressed proteins between CAV and Non-significant CAV (Non-CAV) subjects, biomarkers panels for AR and CAV were generated using classification methods. AR and CAV biomarkers were further analyzed for their biological implications using bioinformatical tools.

Results: Microarray comparison between the AR and NR subjects revealed over 1000 differentially expressed genes, many of which that were associated with cellular functions involved in innate and humoral immunity. The 12-gene biomarker panel generated based on the differentially expressed candidates demonstrated 83% sensitivity and 100% specificity. Proteomic analysis of CAV versus Non-CAV plasma samples ultimately lead to the generation of an 18-protein biomarker panel which demonstrated 80% sensitivity and 89% specificity for CAV.

Conclusion: Taken together, the work from my thesis shows the potential utility of blood derived 'omic'-based biomarker panels in a clinical setting as diagnostic and monitoring tools for key cardiac post-transplantation conditions. This body of work also demonstrates the value of using 'omics' technologies to gain biological insight into AR and CAV.

Preface

This dissertation contains chapters which are based on published manuscripts.

Chapter 1

Portions of Chapter 1 are based on review articles published in *Circulation Research* [Marchant DJ, Boyd JH, **Lin D**, Granville DJ, Garmaroudi FS, McManus BM: Inflammation in myocardial diseases. Circ Res 2012;110:126-44.] and *Canadian Journal of Cardiology* [**Lin D**, Hollander Z, Meredith A, McManus BM: Searching for 'omic' biomarkers. Can J Cardiol 2009;25 Suppl A:9A-14A.]. I was a co-equal contributor in the first review article; specifically, I wrote the section of the review focusing on cardiac inflammation and cardiac allograft rejection. For the latter review article, I was the primary author and was responsible for the critical review and revision of the manuscript.

Chapter 2

Chapter 2 is an altered version of the manuscript published in *Journal of Cardiac Failure* [**Lin D**, Hollander Z, Meredith A, et al.: Molecular signatures of end-stage heart failure. J Card Fail 2011;17:867-74.]. This study was conceptualized by Dr. Bruce McManus, Ms. Zsuzsanna Hollander and me. With assistance from Dr. Bruce McManus and Ms. Zsuzsanna Hollander (who conducted the statistical analyses), I was responsible for subject review, sample selection and design of analyses. As well, I performed all functional enrichment analyses of the data, interpretation of the genomic and proteomic results and writing of the manuscript. Dr. Bruce McManus, Ms. Zsuzsanna Hollander and Ms. Anna Meredith assisted in editing of the manuscript.

Chapter 3

Chapter 3 is an altered version of the manuscript published in the *Journal of Heart and Lung Transplantation* [**Lin D**, Hollander Z, Ng RT, et al.: Whole blood genomic biomarkers of acute cardiac allograft rejection. J Heart Lung Transplant 2009;28:927-35.]. This study was carried out under the Biomarker in Transplantation (BiT) umbrella, co-lead by Drs. Robert McMaster, Paul Keown and Bruce McManus. This study was conceptualized by Dr. Bruce McManus and me.

With assistance from Dr. Bruce McManus and Ms. Zsuzsanna Hollander (who conducted the statistical analyses on the microarray data), I was responsible for subject review, sample selection and design of analyses. I was also responsible for all Gene Ontology analysis, design, execution and data analysis of the classifier gene validation experiments, in addition to interpreting the results and writing the manuscript. Dr. Bruce McManus and Ms. Zsuzsanna Hollander made intellectual contributions to the design of the study, and assisted in revision of the manuscript.

Chapter 4

Chapter 4 is based on a study that was also carried out under the BiT umbrella. This study was conceptualized by Dr. Bruce McManus and me. With assistance from Dr. Gabriela Cohen Freue (who performed the statistical analyses on the proteomics data), I was responsible for sample selection, design of the study, including all subject review, subject selection and processing of clinical coronary angiography data. I was also responsible for all functional enrichment analysis, interpretation of results, writing and editing of the manuscript. Drs. Gabriela Cohen Freue and Bruce McManus also made intellectual contributions to the design of the study, in addition to final editing of the manuscript.

Ethics Approval

All work described in dissertation, including samples collected and experimented performed, were conducted as part of the BiT project, which was approved by The Providence Health Care Research Ethics Board, certificate number H04-50286.

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List of Abbreviations

Ab – antibody

ABI – Applied Biosystems

ACC – American College of Cardiology

ACE – angiotensin converting enzyme

AHA – American Heart Association

AlloAb – alloantibody

AMR – antibody-mediated rejection

AP-1 – activator protein-1

APC – antigen presenting cells

AR – acute cardiac allograft rejection

ARVC – arrhythmogenic right ventricular cardiomyopathy

AST – aspartate transaminase

AUC – area under the curve

AV – atrio-ventricular

AZA – azathioprine

BiT – Biomarker in Transplantation initiative

BNP – brain natriuretic peptide

CAD – coronary artery disease

CARGO – Cardiac Allograft Rejection Gene Expression Observation

CAV – cardiac allograft vasculopathy

CHF – chronic heart failure

CI – calcineurin inhibitors

CK – creatine kinase

CR – chronic rejection

CRP – C-reactive protein

CSA – cyclosporine

CTL – cytotoxic T lymphocyte

cTnI – cardiac troponin I

cTnT – cardiac troponin T

CVB3 – coxsackievirus B3

DC – dendritic cells

DCM – dilated cardiomyopathy

DS – diameter stenosis

EC – endothelial cell

ECM – extracellular matrix

EDTA – ethylenediaminetetraacetic acid

EMB – endomyocardial biopsy

ESHF – end-stage heart failure

FDA – Food and Drug Administration

FDR – false discovery rate

GO – gene ontology

GR – glucocorticoid receptor

HCM – hypertrophic cardiomyopathy

HF – heart failure

HG – human genome

I/R – ischemia/reperfusion

ICAM-1 – intercellular adhesion molecule-1

ICM – ischemic cardiomyopathy

IFN – interferon

Ig – immunoglobulin

IHD – ischemic heart disease

IL – interleukin

IPI – International Protein Index

ISHLT – International Society for Heart and Lung Transplantation

iTRAQ – isobaric tags for relative and absolute quantitation

IVUS – intravascular ultrasound

LAD – left anterior descending artery

LCX – left circumflex

LDA – linear discriminant analysis

LIMMA – linear models for microarray data

LOOCV – leave-one-out cross-validation

LV – left ventricle

LVAD – left ventricular assist device

MAC – membrane attack complex

MALDI – matrix assisted laser desorption ionization

MBL – mannose-binding lectin

MeSH – medical subject heading

MHC – major histocompatibility complex

MLD – minimum lumen diameter

MMF – mycophenolate mofetil

mTOR – mammalian target of rapamycin

NCF – normal cardiac function

NFH – non-failing heart

NFκB – nuclear factor κB

NICM – non-ischemic cardiomyopathy

NIH – National Institute of Health

NK cells – natural killer cells

NYHA – New York Heart Association

PBMC – peripheral blood mononuclear cell

PC – principal component

PCA – principal component analysis

PG – protein group

QCA – quantitative coronary angiography

qPCR – quantitative real-time polymerase chain reaction

RCA – right coronary artery

RCM – restrictive cardiomyopathy

RD – reference diameter

RMA – robust multi-array average

ROC – receiver operating characteristic

ROS – reactive oxygen species

RT- IVT – reverse transcription-*in vitro* transcription

SAM – significant analysis of microarray

SAS – statistical analysis system

SDA – stepwise discriminant analysis

SIR – sirolimus

SMC – smooth muscle cell

SNP – single-nucleotide polymorphism

SPC – smooth muscle progenitor cell

TAC – tacrolimus

TNF – tumor necrosis factor

TOF – time of flight

VCAM-1 – vascular cell adhesion molecule-1

VO_2 max – maximum oxygen (O_2) uptake

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First and foremost, I would like to thank my supervisor, Dr. Bruce McManus, for his patience and mentorship. Thank you Dr. McManus for your contagious enthusiasm, unwavering support, and timely encouragements. As well, thank you for believing in my abilities but at the same time, being upfront about areas I should improve on. The science and life lessons I have learned under your tutelage I will forever cherish.

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Dedication

To my ah-gong and ah-ma,

And

To my family members, Frank, Linda and Sophia.

I hope you are as proud of me as I am of you.

CHAPTER 1: Introduction

1.1 "Pre-"heart transplantation

1.1.1 Overview of heart failure

Heart failure (HF) is a progressive clinical syndrome characterized by inability of the heart to adequately pump blood to meet metabolic demands of the body.[1,2] The cause of HF is diverse, and can result from a variety of cardiac disorders that affect the structure or function, i.e., contraction/relaxation, conduction, or rhythm of the heart.[3,4]

It is estimated that approximately 2% of the adult population will suffer from heart failure, although the prevalence is considerably higher in the older population, occurring in up to 10% of people 65 years or older.[5] The lifetime risk on average, however, for a 40 year old adult, is approximately 20%.[5-7] Importantly, the prognosis of HF patients also tends to worsen over time,[8,9] and is associated a 30-40% mortality rate within the first year after the initial diagnosis, and a 5-year mortality rate between 48 and 70%.[5,10] Not only does the development of HF greatly affect the quality of life of patients, its prevalence also has a huge economical impact on the health care system. In the United States, more than 39 billion dollars are spent yearly on the care of HF patients, including hospitalization, treatment and associated costs.[5,11]

1.1.2 Conditions leading to heart failure

As described earlier, HF can result from virtually any form of cardiac disorders, and reflect contributions from both genetic and environmental factors.[2] From an etiological point-of-view, two major categories of conditions that can ultimately lead to the development of HF are: i) ischemic heart diseases (IHD)/ischemic cardiomyopathies (ICM), and ii) non-ischemic cardiomyopathies (NICM).[12]

Ischemic heart diseases (IHD) generally arise from coronary artery diseases (CAD) such as atherosclerosis, which is characterized by the narrowing of coronary arteries via intimal plaque formation.[3,13] This occlusive process can lead to a lack of adequate oxygenated blood supply to the heart, generating regions of ischemic heart muscle and ultimately myocardial dysfunction.[3,13] Historically, the term 'ischemic cardiomyopathy' was first introduced in 1970 by

Burch and colleagues in New Orleans, to describe conditions which involve severe myocardial dysfunction thought to be the results of occlusive CAD.[3,14]

Non-ischemic cardiomyopathies (NICM) are a heterogeneous group of conditions, including diseases that are predominantly genetic in nature, e.g., arrhythmogenic right ventricular cardiomyopathy (ARVC) and hypertrophic cardiomyopathy (HCM), mixed/predominantly-non-genetic in nature, e.g., dilated cardiomyopathy (DCM) and restrictive cardiomyopathy (RCM), or acquired, e.g., inflammatory cardiomyopathy due to myocarditis.[15]

Briefly, ARVC is a relatively rare form of inheritable cardiac muscle disease (1:5000), which mainly affects the right ventricle and is characterized by the loss of muscle cells, i.e., myocytes, which are replaced by fatty and/or fibrofatty tissues.[3,15]

HCM is also an inheritable cardiac disease and is more prevalent than ARVC, occurring in 1:500 people in the general population.[3,15] HCM is largely characterized by non-dilated hypertrophy of the left ventricle (LV), in the absence of another disease (e.g., hypertension) which may also cause hypertrophy, or thickening, of the LV wall.[3,15]

DCM is the most common form of NICM,[16] and is characterized by the enlargement of one or both ventricles (although primarily the LV),[17] impaired systolic function, and normal LV wall thickness.[3,15] The origin of DCM can be can be idiopathic, genetic (e.g., familial cardiomyopathy), or environmental (e.g. alcoholic cardiomyopathy), but can all lead to ventricular dilation and associated decreased systolic function (or just systolic dysfunction).[3,15]

RCM is a rare form of cardiac muscle disease that can be either sporadic or familial.[3,15] RCM is characterized by features such as enlargement of both atriums, i.e., biatrial enlargement, ventricular filling dysfunction or abnormal relaxation, presence of restrictive physiology, but normal thickness of LV wall and atrial ventricular (AV) valves, as well as normal (or borderline normal) systolic function.[3,15]

Inflammatory cardiomyopathy is considered an acquired form of NICM, and involves cardiac dysfunction as a result of myocarditis.[3,15] Myocarditis can be either an acute or chronic inflammatory condition, and can be induced by various causes, such as virus (e.g., coxsackievirus CVB3), bacteria (e.g., streptococcus and meningococcus), fungus (e.g.,

aspergillosis), parasites (e.g., toxoplasmosis), as well as toxin (e.g., cocaine), and drug hyper sensitivity reactions (e.g., antibiotics).[3,15] As part of the inflammatory process, myocarditis is typically characterized by progressive and active injury (e.g., infiltration of inflammatory cells leading to myocyte necrosis in the heart), and eventual healing of the damaged heart (e.g., replacement of necrotic tissue with fibrosis).[3,15]

1.1.3 Classification and current therapies of heart failure

Given the complexity of HF, several methods of classification and categorization have been proposed for HF. For instance, HF can be described as either systolic, i.e., involving contraction of the heart, or diastolic, i.e., involving relaxation of the heart. From a more clinical and pathophysiological perspective,[10] the American College of Cardiology (ACC)/American Heart Association (AHA) has proposed a four stage system (Stages A through D), where the first two stages are considered as 'pre-HF', e.g., patients at risk of HF but not yet showing clinical signs of HF, and the latter two stages include patients with clinical HF presently or previously (Stage C), and those who may require more advanced therapy or end-of-life care (Stage D).[18-20] From a more functional-based perspective, the New York Heart Association (NYHA) classification of HF ranges from class I to class IV and is thought to compliment the ACC/AHA system – typically used to further classify patients in stage C or D.[18,19] The NYHA classes, with class I being the least severe and class IV being the most, are largely dependent on clinical symptoms and the degree of functional limitation in normal physical activities for the patients, e.g., climbing stairs.[18-20]

Therapy for heart failure is generally dependent on the clinical symptoms and functional limitations of the patients. The type of therapy can range from lifestyle changes, e.g., increased physical activity, decreased alcohol and salt intake, such as in the case of ACC/AHA Stage A patients, to the use of medications such as angiotensin converting enzyme (ACE) inhibitors and beta adrenergic blocking agents (beta-blockers).[18] Other medications such as angiotensin II receptor blockers and aldosterone antagonists, or even procedures such as resynchronization therapy are also used in selected patients with more severe form of HF, e.g., ACC/AHA stage C.[18]

While the aforementioned therapies are commonly used in attempt to improve the patients symptoms and prevent the progression of heart failure, some patients do eventually advance to end-stage heart failure (ESHF), and will require more extraordinary measures of interventions.[18] Surgical approaches such as implantation of mechanical ventricular assist devices, e.g., left ventricular assist device (LVAD) are often used to support and prolong the life of ESHF patients.[18] Given that adult cardiac myocytes are largely thought to be terminally differentiated cells with limited ability for self regeneration, the human heart is, for the most part, unable to replace injured or dead myocytes to the extent that it significantly reverses the HF progression from the point of ESHF.[3] As such, currently, heart transplantation remains the primary therapy of choice and the definitive long term solution for patients with end-stage vital heart failure.[3,18]

1.2 Heart transplantation

1.2.1 Overview and brief history

The first successful human-to-human heart transplantation was performed on December 3rd, 1967, by Dr. Christiaan Barnard and his colleagues in Cape Town, South Africa.[21,22] Nearly one month later, in January 1968, the first heart transplantation was performed in North America by Dr. Norman Shumway, at Stanford University.[21,22] For the past number of decades, advances in different areas related to heart transplantation, e.g., surgical techniques, patient management and immunosuppressive therapies, have helped improved the survival rate of patients significantly and established heart transplantation as the primary therapy for end-stage vital heart failure patients. To date, it has been estimated that over 85,000 heart transplantations have been performed around the world since the early 1980's.[21-24] According to the latest report by the International Society of Heart and Lung Transplantation (ISHLT), approximately 4000 heart transplantations were reported in 2009 alone.[24,25]

1.2.2 Immunosuppressive therapies

After heart transplantation, recipient's immune system can recognize the transplanted heart, i.e., cardiac allograft, as foreign and react against it.[26,27] This immune (or 'alloimmune') response to nonself antigens expressed by the donor tissue can lead to the injury and dysfunction of the allograft, which can present as rejection episodes.[26,27] During the rejection process, immune cells such as T cells, B cells, natural killer cells and macrophages can undergo activation, proliferation, and infiltrate the cardiac allograft, causing injury to myocytes and tissues via cellular and antibody-mediated mechanisms.[28] The mechanistic details regarding acute and chronic cardiac allograft rejections are described in more details later in this chapter.

In order to suppress and avoid the possible alloimmune response, e.g., immune cell activation and proliferation, and associated inflammation post-transplantation, cardiac transplant patients are routinely put on immunosuppressive regimes, which typically serve several major purposes: 1) to avoid early acute rejection post-transplantation by acting as a

prophylaxis, i.e., induction therapy, 2) to treat acute rejection episodes that does occur, i.e., anti-rejection therapy, and 3) to help maintain the allograft long term as a prophylaxis, i.e., maintenance therapy.[29,30] The specific dosage, type, and duration of immunosuppression is also optimized based on individual patients in order to provide the desired outcome listed above, while minimizing the potential for adverse effects and opportunistic infections.[29-31]

Majority of the immunosuppressive medications used clinically today belongs to one of the following therapeutic groups: i) calcineurin inhibitors, ii) antiproliferative agents / antimetabolites, and iii) steroids.[32] Selected examples of the more commonly used drug from each therapeutic class are discussed below.

Briefly, calcineurin inhibitors (CI) such as cyclosporine (CSA) and tacrolimus (TAC) are able to enter the immune cells through mechanisms such as diffusion, and bind to specific immunophilins, i.e., CSA and TAC to cyclophilin and FC binding protein (FKBP-12), respectively. The resulting immunophilin-drug complex can bind and inhibit calcineurin; this in turn inhibits the transcription of a number of cytokine genes and thus prevents the formation of cytokines such as interleukin (IL)-2 and others.[32,33]

Antiproliferative agents, in general, inhibit the differentiation and proliferation of immunocompetent lymphocytes post-(allo)antigen recognition, thus suppressing the immune response post-transplantation.[27,34] Certain antiproliferative agents can interfere with metabolic pathways or be incorporated in synthesis pathways to generate faulty molecules that are structurally similar to metabolites essential for the differentiation and division of immune cells.[27,34] These specific antiproliferatives are sometimes also referred to as antimetabolites, e.g., azathioprine (AZA) and mycophenolate mofetil (MMF).[27,34] In essence, AZA and MMF inhibits the production of purine and guanine, respectively, which are compounds necessary in cell proliferation.[27,32,34] In blood, AZA is converted by plasma esterases or glutathione to 6-mercaptopurine (6-MP) and eventually to 6-thio-inosine-5'-monophosphate, a purine analog that once incorporated into the cellular DNA can inhibits its synthesis.[27,32,34] By inhibiting DNA synthesis, AZA can effectively interfere with the proliferation of immune cells such as lymphocytes (and in fact, all rapidly dividing cells), particularly during phases of immune

response when these cells require proper nucleotide synthesis to undergo rapid cell division.[27,32,34]

Similar to AZA, MMF also interferes with purine metabolism and inhibits the synthesis of DNA, as well as RNA.[27,32,34] However, unlike AZA which works more broadly on all cells undergoing rapid nucleotide synthesis and division/proliferation, MMF, acts more specifically on lymphocytes, i.e., T and B cells.[27,32,34] In the body, MMF is metabolized to mycophenolic acid, which inhibits inosine monophosphate (IMP), a key enzyme involved in regulation of guanosine monophosphate (GMP) and de novo pathway for synthesis of purines, e.g., guanine.[27,32,34] With the exception of lymphocytes, most cells in the human body are able to utilize two pathways, i.e., de novo *and* salvage, for the production of purine nucleotides.[27,32,34] As such, MMF affects primarily the DNA replication and proliferation of lymphocytes.[27,32,34] Another example of antiproliferative agent is sirolimus (SIR). Similar to calcineurin inhibitors (CI) such as CSA and TAC, SIR is also able to bind to immunophilins.[27,32,34] In contrast to CSA and TAC which inhibits calcineurin, the immunophilin-SIR (i.e., FKBP-sirolimus) drug complex binds and inhibits the mammalian target of rapamycin (mTOR) instead.[27,32,34] mTOR is a key kinase downstream of IL-2 receptor (IL2R) signaling pathway that assists in the production of proteins which regulate cell cycle progression. Thus, through inhibition of mTOR, SIR can suppress IL-2R signaling mediated activation and proliferation of immune cells, e.g., T and B cells.[27,32,34]

Corticosteroids (or referred to simply as steroids) are commonly given to cardiac transplant recipients because of their immunosuppressive and anti-inflammatory properties.[27,32,34] One of the more frequently used types of corticosteroids, glucocorticoids (e.g., prednisolone, active metabolite of prednisone), are able to enter cells via diffusion and intracellularly bind to cytoplasmic glucocorticoid receptors (GR).[27,32,34] The resulting GR-glucocorticoid complexes can translocate to the nucleus, act as a transcription factor via binding to different regulatory elements, e.g., glucocorticoid response element, and transcriptionally regulate expression of different genes that will ultimately affect the immune and inflammatory response. One example of this is the IκB gene, which can be transcriptional upregulated by the GR-glucocorticoid complex. This can eventually lead to increased production of IκB enzyme complex, which is able to inhibit nuclear factor (NF) κB and activator protein-1 (AP-1), both of which considered key players in the activation of lymphocytes.[27,32,34]

Given the diversity of mechanisms, pathways and effects associated with each type of immunosuppressive agents, a combination of drugs is typically used in the management of cardiac transplant recipients.[27,30] Currently, the most commonly used immunosuppressive regime for cardiac transplant recipients post-transplantation, i.e., maintenance immunosuppression, is widely referred to as the triple drug therapy, which involves a calcineurin inhibitor, e.g., CSA, a corticosteroid, e.g., prednisone, and an antiproliferative/antimetabolite agent, e.g., MMF.[27,30]

1.2.3 Survival rate of heart transplantation patients

Advancements in our understanding and utilization of immunosuppressive agents have helped improve the clinical outcome for heart transplant patients. In particular, the short-term survival rate (i.e., 1-year) for cardiac recipients has now surpassed 85%,[24,35,36] where as the 5 year survival rate is roughly 75%.[37] According to the ISHLT 2011 report, the half life, defined as time at which half of the cardiac recipients are still alive, is approximately 10 years.[24] However, despite the substantial progress in allograft and patient survival, the detection of allograft rejection (described later) remains to be one of the most important yet unsettled areas of cardiac transplantation.[38]

1.3 "Post-"heart transplantation hurdles

1.3.1 Overview of cardiac allograft rejections

As noted earlier, the cardiac allograft rejection process can be generally described as the result of recipient's immune response to nonself antigens expressed by the donor tissue, i.e., cardiac allograft.[39] Moreover, cardiac rejection can be further distinguished, in part based on the timeframe of events and histological evidence, into three categories: hyperacute, acute, and chronic.[26,27]

Hyperacute rejection refers specifically to the process in which preformed donor-specific antibodies from the recipient's body react rapidly and intensely, usually within minutes to hours post-transplant, against the cardiac allograft.[26,27] This is almost always a lethal process but fortunately, with the use of blood-type matching and crossmatch assays to ensure there are no donor-specific cytotoxic antibodies present in the recipient's serum prior to transplantation, hyperacute rejections are largely preventable.[26,27]

In contrast, acute rejection is a predominantly cell-mediated process that can also involve the presence of acquired antibody-mediated response. Although acute rejection episodes typically occur within 6 months to 1 year post-transplant, it can also take place at later timepoints.[26,27,37]

Chronic rejection, or more specifically, development of cardiac allograft vasculopathy (CAV) as an expression of chronic rejection, is a relatively more insidious process that tends to develop over months and years after transplantation.[27,40] It thought to be mediated by immune and non-immune processes that can ultimately lead to obliterative vasculopathy and failure of the cardiac allograft.[41-45]

In line with the premise of my research work, the following sections will further describe acute rejection and CAV as an expression of chronic rejection.

1.3.2 Acute cardiac allograft rejection

1.3.2.1 Occurrence and diagnosis of acute cardiac allograft rejection

It has been estimated that between 20% to 50% of patients may experience acute cardiac allograft rejection (AR) at least once within one year post-surgery, despite the use of immunosuppressive therapy.[46] Nevertheless, improvements in surgical management, as well as refinement in combination immunosuppressive regimes and post-transplantation patient management have helped decreased the rate of acute rejection over the last number of years.[24,25] Between 1994 and 2000, approximately 59% of cardiac transplant recipients required hospitalization for rejection treatment within 5 years post-transplant. According to the most recent report by the ISHLT, this percentage has dropped to approximately 45%, i.e., incidence of rejection within 5 years post-transplant that ultimately required patient hospitalization.[24,25]

Clinically, cardiac recipients undergoing acute cardiac rejection may be asymptomatic or present with a range of symptoms, depending on the severity and duration of the rejection.[26,27] These symptoms can be as mild as shortness of breath, to arrhythmias that could transpire to syncope and in some cases, cardiac arrest.[26,27]

Given the high likelihood of AR (particularly during the first year post-transplantation) and the potential severity of allograft dysfunction, routine screening for detection and diagnosis of AR remains one of the most important areas of cardiac transplantation research. The current gold standard for definitive diagnosis of acute cardiac allograft rejection relies primarily on endomyocardial biopsies (EMB).[3]

Routine EMBs are generally performed every week during the first month post-transplant, then once every two weeks/bi-weekly during the second month, followed by once every month until the end of year one.[26] Depending on the transplantation program, the subsequent EMBs after the first year post-transplant can range from every 6 months to every year.[26] EMBs are also indicated when patients are suspected of having acute cardiac rejection based on clinical symptoms. The EMB procedure is invasive, and involves the use of cardiac catheterization and a flexible bioptome, which is then guided transvenously, typically via the right internal jugular vein (sometimes the femoral vein), into the right ventricle of the

allograft.[3,4,26] Once inside, the bioptome excise generally a minimum of 3, to preferably 4 or more, 1-mm^3 pieces of the myocardium.[47] The collected EMBs are then processed for the histological evaluation by pathologists, and graded based on the 2004 ISHLT grading system.

Based on the grading system that was originally adopted by the ISHLT in 1990, the revised 2004 version classify acute (cellular) cardiac allograft rejection into three grades (0R to 3R, with 3R being the most severe).[47] Briefly, Grade 0R represents no rejection. Grade 1R is considered mild rejection, evident by presence of immune cell infiltrates in the interstitial and/or perivascular space, with no more than 1 focus of myocyte damage.[47] Grade 2R, or moderate rejection, is characterized by the presence of two or more foci of cellular infiltrate with associated myocyte damage. In grade 3R, or severe rejection, diffuse cellular infiltrate along with multiple foci of myocyte damage can be observed; vasculitis, edema or hemorrhage may also be present.[47]

In the same ISHLT consensus report ("Revision of the 1990 Working Formulation for the Standardization of Nomenclature in the Diagnosis of Heart Rejection"), a separate histological evaluation guideline and grading nomenclature was also recommended for the recently emphasized form of cardiac rejection, called antibody-mediated rejection (AMR).[47,48] There remain some controversies with regards to the specific definition of cardiac AMR, although a consensus statement has been recently described by the ISHLT.[48,49] Furthermore, the significance of mixed acute cellular rejection and AMR remains to be elucidated.[49] The details regarding diagnostic challenges and management of AMR episodes have been extensively described in a recent review by Kittleson and Kobashigawa.[50] Currently, however, the diagnosis of acute *cellular* rejection based on EMB is still considered the more effective and widely adopted surrogate for the outcome and wellbeing of transplanted hearts.[49]

For the purpose of this dissertation and the research projects described within, the term "acute rejection" (AR), unless stated otherwise, refers specifically to acute *cellular* rejection as defined by the criteria for 2004 ISHLT grade 2R and above (or grade 3A and above based on the older 1990 ISHLT grading system; see Figure 1).

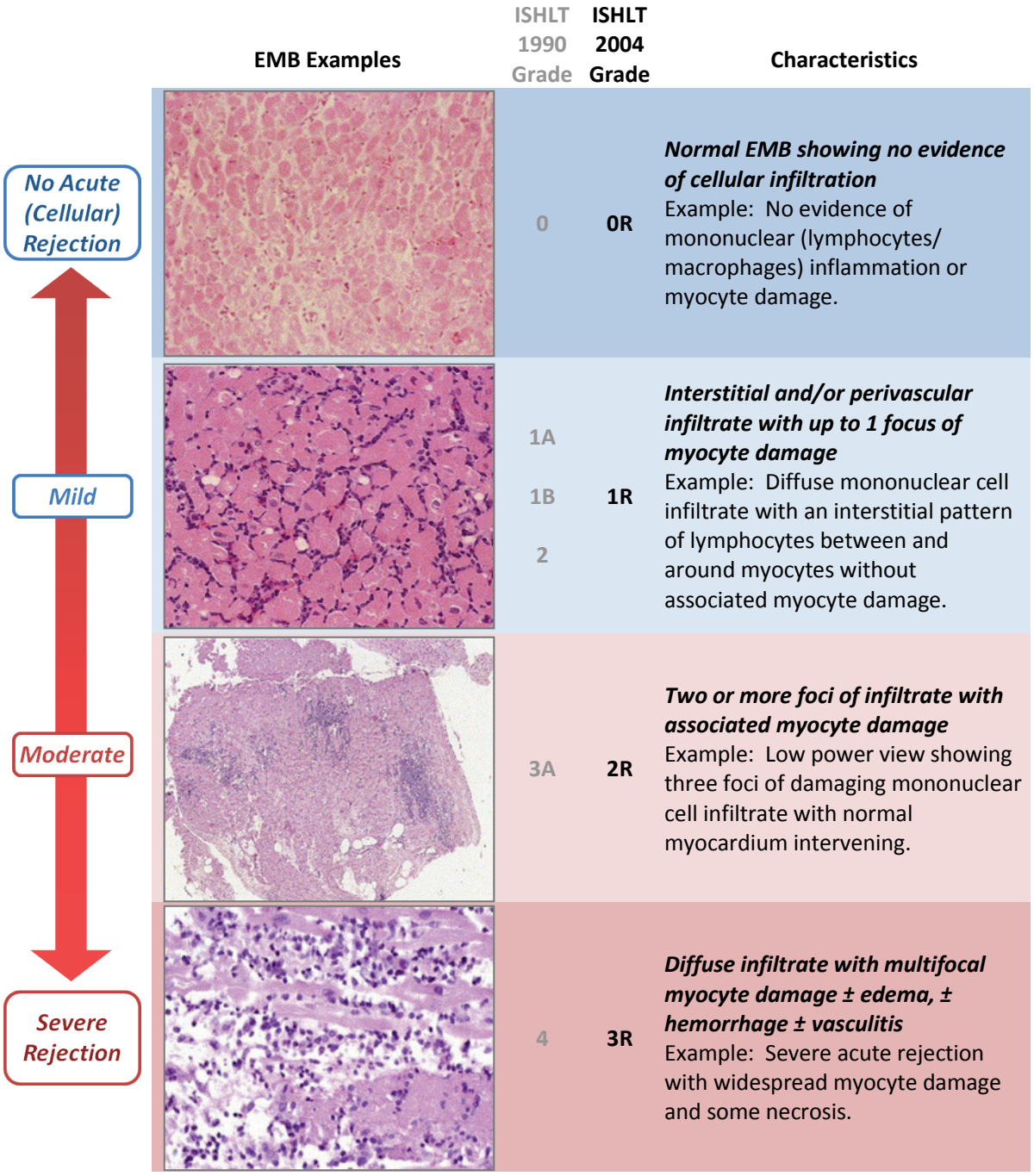

Figure 1. Histological diagnosis and grading of acute cardiac rejection.

Examples of EMBs corresponding to the different ISHLT rejection grades are shown. Images shown are reproduced from the official ISHLT guideline,[47] with permission from the publisher and the *Journal of Heart and Lung Transplantation*. All EMB images shown are based on Hematoxylin and Eosin (H&E) staining.

1.3.2.2 Pathogenesis of acute cardiac allograft rejection

As noted earlier, histologically, acute cellular rejection, i.e., ISHLT grading 2R or above, is typically characterized by the presence of inflammatory cellular infiltrates with associated myocyte damage.[47] The cellular infiltrates are generally comprised of lymphocytes, macrophages, and occasionally eosinophils.[47,51]

Mechanistically, acute cardiac rejection is thought to involve both cellular and humoral processes.[52,53] After the heart transplantation, donor and recipient-derived antigen presenting cells (APCs; e.g., dendritic cells) can trigger direct and indirect allorecognition, respectively. In direct allorecognition, the intact foreign donor MHC antigens and peptides presented on the surface of *donor* APCs are recognized by recipient T cells. Specifically, the donor organ-derived APCs can migrate from the allograft to the recipient's lymphoid tissues, where they activate, through the direct pathway, CD4+ and CD8+ T cells.[45,52,53] On the other hand, in indirect allorecognition, the *recipient* APCs first uptake and process the donor MHC antigens, before presenting the donor-derived allopeptides to recipient T cells.[45,52,53] While both direct and indirect pathways are activated as part of the alloimmune response post-transplant, it is thought that the direct pathway is primarily responsible for initiating the acute cellular rejection process, whereas the indirect pathway has been linked more so to the development of CAV and chronic rejection.[45,52,53]

After the initiating allorecognition event, activated T cells can undergo clonal expansion, as well as produce cytokines, e.g., IL-2, IL-4, IL-5, IFN-γ, and chemokines, thus creating an inflammatory milieu which further promotes the recruitment and activation of additional immune cells.[45,52,53] Activated immune cells, e.g., T cells, B cells, macrophages and dendritic cells (DC), can all interact dynamically with the complement system.[54] A brief description of the different immune effectors/regulators, including complement system, and their roles in the context of cardiac allograft rejection is summarized in Table 1.

Table 1. Roles of immune effectors and complement system in allograft rejection

Key immune Effectors and Regulators		Examples of roles and involvement in cardiac rejection
T cells	CD8+ cytotoxic T cells	• Cytotoxic T cell-mediated direct lysis of target cardiac allograft cells via perforin and granzyme-mediated pathways • Production of inflammatory cytokines
	CD4+ T helper cells	• Involved in activation of CD8+ T cells and B cells • Production of inflammatory cytokine, e.g., IL-4, IL-5
	T helper 17 cells	• Production of IL-17, as well as IL-21 and IL-22; IL-17 is considered to have proinflammatory properties
	Regulatory T cells	• Homeostatic controllers of inflammation partly through production of anti-inflammatory cytokines, e.g., IL-10 and TGF-β
B cells		• Production of alloantibodies • Alloantibody(alloAb)-mediated and alloAb-independent mechanisms, e.g., regulation of T-cell mediated inflammation, cytokine production
Neutrophils, Mast cells, NK cells, DC and Macrophages		• Early responders as part of the innate immunity, e.g., infiltration of recipient NK cells and macrophages into cardiac allograft • Production of cytokines, e.g., IL-1, IL-6, TNFα, IFNγ • Antigen presentation and activation of other immune cells • Perforin and granzyme-mediated attack, i.e., NK cells
Complement system		• Brain death and ischemia/reperfusion (I/R), both part of the organ procurement/transplant process, favor a proinflammatory condition and activation of the complement system cascade • Active complement fragments can act as inflammatory mediators, e.g., C3a and C5a, and modulate immune cell response • Formation of membrane attack complex (MAC), which can trigger subsequent damage and destruction of the targeted (foreign) cells, e.g., myocytes of the cardiac allograft

Together, the major pro-inflammatory mechanisms and immunological events that are triggered post-transplantation can result in inflammation and immune cell infiltration in the myocardium, as observed in EMBs taken during acute rejection episodes.[37,45,52,53] Consequently, insults to the local myocardial tissue, through mechanisms such as cytotoxic T cell-mediated direct lysis, complement cascade activation and B cell alloantibody production, can cause myocyte damage and necrosis, ultimately leading to functional impairment of the cardiac allograft, or "cardiac rejection" (summarized in Figure 2) .

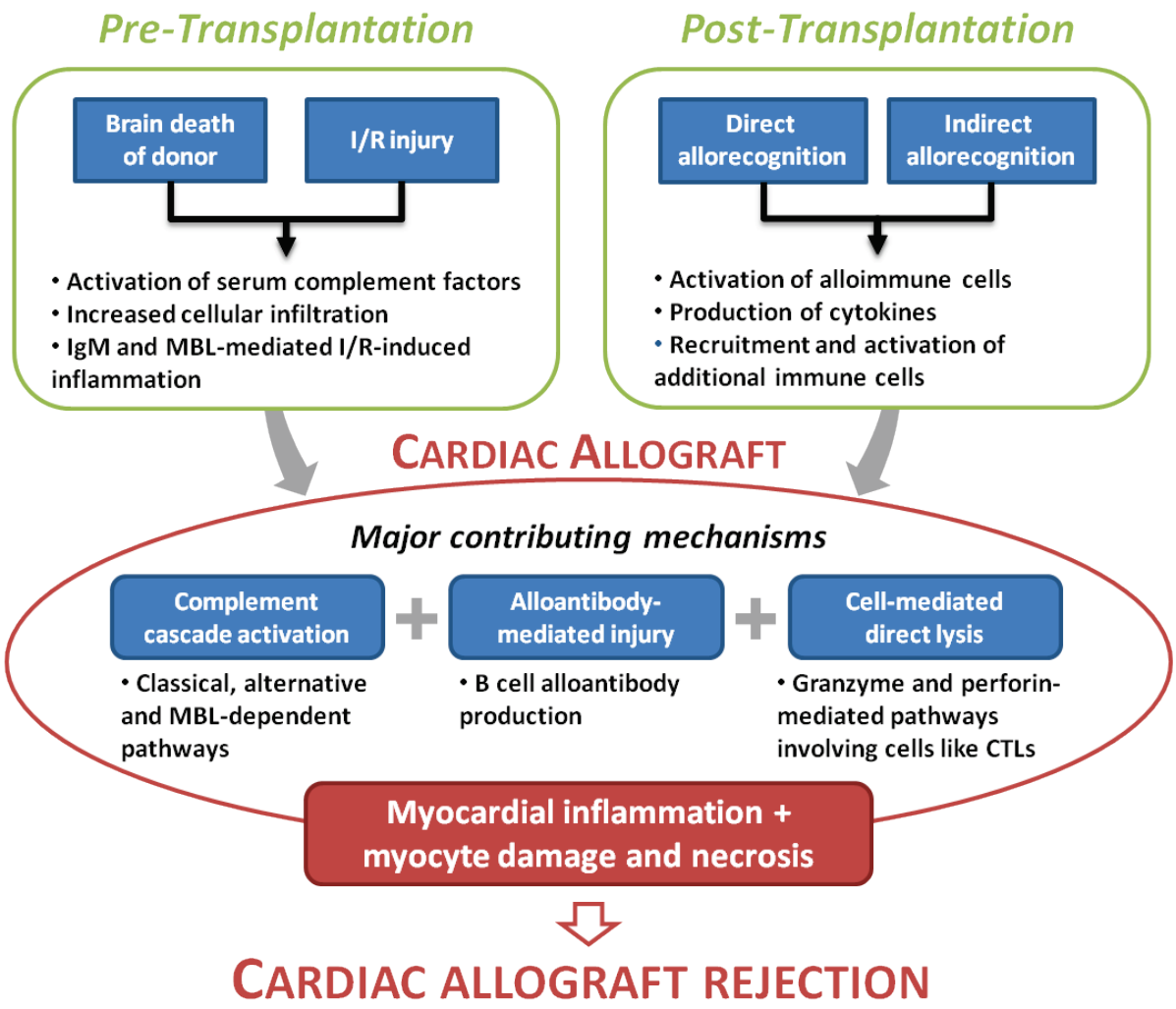

Figure 2. Major contributing mechanisms of myocardial inflammation in the context of cardiac allograft transplantation.

Both pre- and post-transplantation events can contribute as initiators of downstream complement-, alloantibody- and cell-mediated pathways. These major pathways can act synergistically to create an inflammatory milieu and eventually lead to the necrosis and apoptosis of cells in the cardiac allograft, e.g., endothelial and parenchymal cells. This can further exacerbate the myocardial inflammation observed in acute and chronic cardiac allograft rejections. Abbreviations: CTL=cytotoxic T lymphocytes; MBL=mannan-binding lectin; I/R=ischemia/reperfusion.

1.3.3 Cardiac allograft vasculopathy (CAV)

1.3.3.1 Occurrence and diagnosis of CAV as an expression of chronic rejection

The long term (i.e. 10-year) survival rate of the heart recipients is only about 50%,[35,55] and is largely limited by the development of cardiac allograft vasculopathy (CAV) as an expression of chronic rejection (CR). CAV affects approximately half of cardiac transplant recipients within the first several years post-transplantation, and is responsible for up to 15% of deaths in cardiac allograft recipients after they have survived the first year post-transplant.[56] Given the prevalence and the significance of CAV, cardiac transplant recipients undergo routine tests, typically *at least* once a year, to monitor the health of the transplanted heart.

Currently, the most widely used modality for the diagnosis of CAV remains coronary angiography. In this procedure, a cardiac catheter is inserted into the patient's artery, commonly through femoral artery in the groin area, and threaded into the coronary arteries of the cardiac allograft. A radio-opaque contrast material is then injected through the catheter, and X-rays images are taken via fluoroscopy to allow visualization and evaluation of the allograft coronary blood vessels, e.g., epicardial coronary arteries and braches. The screening and diagnosis of CAV is largely based on the detection of narrowing, or stenosis, of coronary arteries, i.e., blood vessels which supply oxygenated blood to the heart.[45,57,58] The severity of stenosis is often expressed as percentage diameter stenosis (%DS),[59] as shown in Figure 3.

In contrast to the diagnosis of acute cellular cardiac rejection, where specific histological criteria and grading guideline have been established and widely adopted internationally for years, there is relatively less consensus on the classification of CAV. A working formulation of nomenclature for CAV has recently been put forth by the ISHLT.[60] However, the cut-off values used in the proposed guideline to define the mildest form of CAV are quite high, e.g., angiographic evidence of stenosis in the primary coronary arteries up to 70% diameter reduction.[60] From a research perspective, a lower stenosis threshold to define 'significant' CAV may be required in order to identify highly sensitivity and specific biomarkers for screening and diagnostic purposes. This 'fit-for-purpose' approach in the context of biomarker development,[61] e.g., using more 'extreme' patient phenotypes for biomarker discovery, is further demonstrated and explained in later research and discussion chapters throughout this dissertation.

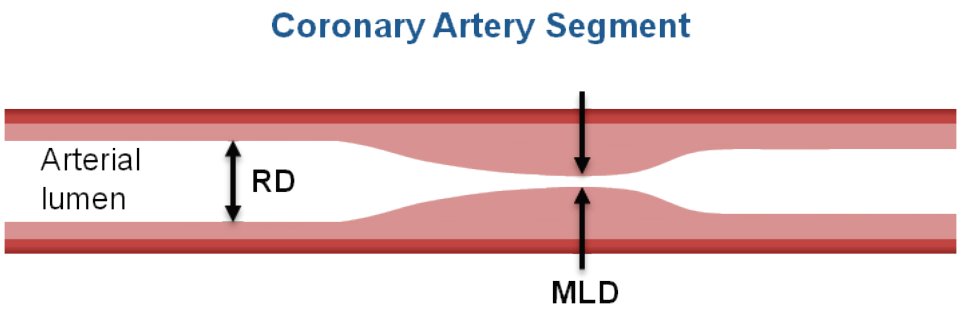

Figure 3. Percentage diameter stenosis of coronary artery

The illustration above represents a simplified view of a longitudinal section of a coronary artery segment. The severity of coronary artery stenosis is often measured and expressed as percentage of diameter stenosis (%DS), as defined by the following formula:

%DS = [(RD – MLD) / RD] x 100

where RD is the reference diameter (an average of the normal region), and MLD is the minimum lumen diameter.

1.3.3.2 Pathogenesis of cardiac allograft vasculopathy

The histopathological features of CAV were first described in human cardiac allografts in 1970 as a diffuse, occlusive vascular condition involving coronary intimal proliferation and obliterative changes in the coronary arteries.[62-66] Decades later, it has been recognized that CAV is a complex, multifactorial condition involving both immunologic and non-immunologic contributing factors and mechanisms.[40,45,67,68]

Although the exact pathobiology remains unclear, it is widely accepted that CAV is initiated by a combination of allogeneic response to the allograft and ischemia/reperfusion (I/R) injury, resulting in damage to the endothelial cells (ECs).[40,69-73] In the progression of CAV, the initial activation, injury, dysfunction or destruction of ECs is typically followed by the subsequent activation, migration and proliferation of vascular SMCs, along with elaboration of cytokines and extracellular matrix (ECM) protein.[40,44,45,74] These events are thought to be central to the onset and development of progressive luminal narrowing and eventual impaired vascular function of the allograft.[40,44,45,74]

Multiple processes can contribute to the injury of ECs during the cardiac transplantation process, e.g., surgery-related mechanical damage, hypoxia and I/R-induced complement system mediated injury.[40,45,74,75] Post-transplantation, immunologic responses to the donor vasculature, via activated immune cells and circulating inflammatory cytokines and complement fragments, can further exacerbate the damage and activation of the endothelium.[40,45,74] Activated ECs can up-regulate the expression of adhesion molecules (e.g., intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and P-/E-selectins),[74,76-81] as well as secrete cytokines such as IL-1, IL-6, and TNF-α.[55,74,82,83] This can further promote the recruitment and attachment of leukocytes to ECs, and create an inflammatory milieu.

The same immune mechanisms described earlier, e.g., cell-, antibody- or complement-mediated mechanisms, can also lead to the apoptosis of target cells in the allograft, including ECs.[84] Apoptosis of ECs, either through acute alloimmune response (i.e., 'acute rejection') or low grade, persistent inflammatory response (i.e., 'chronic rejection'), are also thought to play a pivotal role in the development of CAV.[84] In human heart transplants, apoptosis of ECs are observed in graft coronary arteries showing early signs of CAV.[84,85]

Another key feature seen in the development of CAV is the progressive neointimal accumulation of vascular SMCs.[74,86-88] Parallel to this, a decrease in medial SMCs can also often be observed.[74,89,90] This reduction is believed to be, in part, due to the injury and/or death of SMCs through similar mechanisms that affects the ECs, e.g., I/R injury, cytotoxic soluble mediators or receptor-based apoptosis.[69,74,91-95] Several processes are thought to contribute to neointimal hyperplasia: i) activation of medial SMC in response to soluble mediators such as growth factor TGF-β, and cytokines such as IFN-α and IL-1, ii) migration of SMC (in response to chemokine gradients), and iii) proliferation of SMC in the intima.[74,96,97]

Further, it has been suggested that SMCs undergoing response to injury can transform from a more (differentiated) contractile state to a more (dedifferentiated) synthetic one.[40,44,68] The synthetic state SMCs are capable of migrating from the media to the intima, proliferate and synthesize ECM components, e.g., proteoglycans.[40,73] The latter two effects are considered major contributors of obliterative intimal thickening seen in the vessels with CAV.[40,67,98,99] More recently, it has been proposed that circulating smooth muscle progenitor cells can be recruited to the injured vascular sites, and are also able to contribute to the progression of CAV through similar effects.[40,43,45,72] Key events central to the onset and progression of CAV based on current literature are summarized in Figure 4.[40,45,74,77-81,100-105]

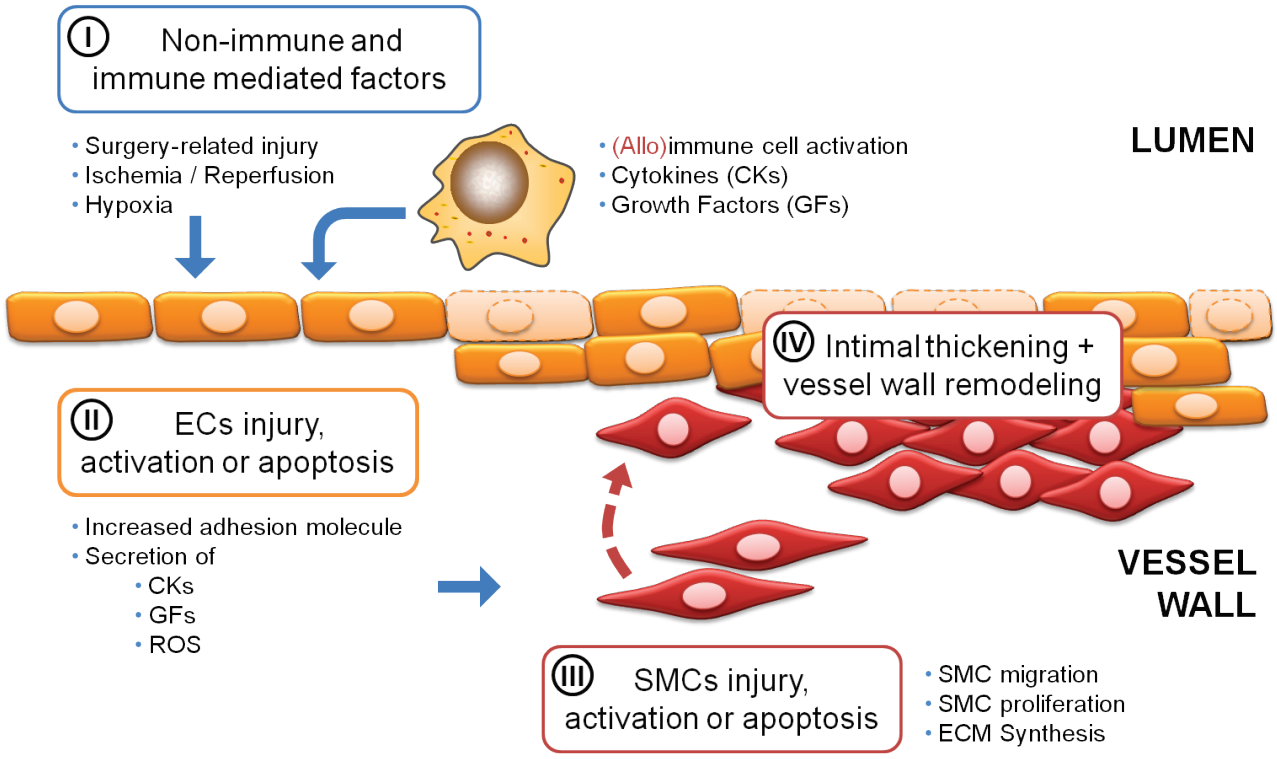

Figure 4. Major events central to the onset and development of CAV.

It is widely accepted that the development of CAV is contributed by both immune and non-immune factors. **(I)** Peri-transplantation effects, e.g., surgical-, I/R, or hypoxia-mediated injury, as well as post-transplantation effects involving cell-, antibody- and complement-mediated mechanism, can damage the cardiac allograft vasculature, e.g., endothelium, lead to the generation of an inflammatory milieu. **(II)** ECs that are activated injured, or undergoing apoptosis can secrete additional soluble mediators, which can act in a paracrine fashion and affect neighboring ECs, or induce neighboring medial SMCs. **(III)** The inflammatory environment can lead to the injury, activation or apoptosis of SMCs in the allograft. Once activated, SMCs can migrate to the intimal layer, proliferate, as well as synthesize ECM such as proteoglycans. **(IV)** The cumulative effects by the cells in the vasculature (e.g., activation and proliferation of ECs, fibroblasts and SMCs; excess production and deposition of ECM), as part of the response-to-injury process, help contribute to the thickening of the intima, remodeling of the vessel wall and consequently, progression of CAV. Abbreviations: EC=endothelial cell; SMC=smooth muscle cell; ECM=extracellular matrix; CK=cytokine; GF=growth factor; ROS=reactive oxygen species

1.4 Overview of biomarkers

The term biological marker was first popularized by the *United States National Library of Science* in 1989 when it was introduced as a *Medical Subject Heading* (*MeSH*; such as that used by *PubMed* article database; www.pubmed.com) to help categorize and index journal articles.[106] At the time, biological markers (or now referred to simply as 'biomarkers'), was defined as "measurable and quantifiable biological parameters (e.g., specific enzyme concentration, specific hormone concentration, specific gene phenotype distribution in a population, presence of biological substances) which serve as indices for health- and physiology-related assessments, such as disease risk, psychiatric disorders, environmental exposure and its effects, disease diagnosis, metabolic processes, substance abuse, pregnancy, cell line development, epidemiologic studies, etc". [106]

Decades later, a similar definition was adopted by the United States Food and Drug Administration (FDA) and National Institute of Health (NIH) working group, and biomarker was defined in the pharmacogenomics guidance published in 2001 as "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".[107]

Similarly, according to Health Canada, a biomarker is described as any "measurable characteristic that is an indicator of normal biologic processes, pathogenic processes, and/or response to therapeutic or other interventions."[108] It is interesting to note that, by the current definitions of biomarkers, it has been argued that one can approach the biomarker discovery process in an existential fashion. Such a perspective means that, to a significant extent, we do not necessarily need to know the detailed identity, function or biological relationship of a given biomarker or biomarker set to be reproducibly and objectively useful, and clinically valuable.[61,109]

Historically, biomarkers have been identified, sometimes serendipitously, through targeted studies of physiological processes. Many biomarkers discovered decades ago in the pre- 'omics' era are still being used in medical practice, such as aspartate transaminase (AST), C-reactive protein (CRP), and cardiac troponin I (cTnI).[61] Presently however, given the recent

explosion of high-performance 'omic' technologies – genomics and proteomics, among others – the rate at which biomarker candidates are being discovered is now faster than ever. Specific examples of the 'omic' platforms utilized in the scope of this dissertation and the research projects within are described in the Appendices – Assays and 'Omics' Methodologies section.

In this dissertation, biomarkers are simply defined as distinctive, objectively measured biological or biologically derived indicator of a process, event, or condition.

CHAPTER 2: Dissertation overview

2.1 Rationale of research proposal

Currently, cardiac transplantation is considered the primary therapy for patients with end-stage vital heart failure. In the context of pre-transplantation research, studies have suggested that unique molecular expression profiles are associated with different conditions i.e., cardiomyopathies, that can lead to HF and the eventual need for a cardiac transplantation. However, it is not clear whether these molecular expression profiles remain unique at the point *just* before transplantation (i.e., end-stage heart failure).

Once the patient has received the cardiac allograft, the detection of acute and chronic rejections (as cardiac allograft vasculopathy; CAV) remains to be some of the most important yet unsettled areas of cardiac transplantation research. As described earlier in the introduction, the current gold standards for the diagnosis and monitoring of acute rejection and CAV are invasive in nature with risk for complications. From a clinical perspective, more accurate, minimally-invasive alternatives for the diagnosis of AR and CAV are clearly desirable.

While traditional (molecular), blood-based cardiac biomarkers such as Creatine Kinase / CK-MB or Troponin I and T (cTnI and cTnT) can be quantitatively measured to reflect the severity of cardiac injury,[4,110,111] they are not specific to the *cause* of the injury. Classic markers such as troponins are also thought to lack diagnostic sensitivity during the early post-operative period.[35,112,113] As such, renewed efforts to improve long-term survival through enhanced monitoring and diagnosis of AR episodes and development of CAV have directed attention towards the search of better biomarkers.[114]

The availability of a diverse range of high-performance 'omic' technologies has meant new and exciting opportunities for biomarker discovery. It has been suggested that, rather than finding a single 'silver bullet' biomarker of rejection, multiple biomarker may be necessary to achieve the level of diagnostic power that could potentially be clinically useful one day.[36] Indeed, recent studies have shown evidence in this regard.[115,116] However, more work is required to examine the use of a high-throughput, unbiased approach to identify biomarkers for the diagnosis of acute and chronic human heart cardiac allograft rejection.

2.2 Overview of research proposal

In this dissertation I focus on the discovery and examination of biomarkers, using current state-of-the-art 'omics' technologies, at three specific timepoints and relevant conditions of interest in the context of heart transplantation, including ESHF, AR and CAV. Specifically, I explore the potential utility of these biomarkers from a clinical point of view, as well as investigate their biological plausibility and implications from a pathobiological perspective.

I start the research by examining the molecular profile of patients at the 'end-stage' of their chronic heart failure, in order to determine whether there is any unique 'omic' profile that associates with them. In particular, I want to determine i) whether HF of non-ischemic or ischemic origin present with unique biomarker profiles and, ii) what molecular and biological processes are perturbed in these CHF patients that underlie the need for eventual heart transplantation?

In my next two research aims, I transition from the pre- to the post-transplantation phase. The major portion of my work then focuses on two critical events after transplantation that are detrimental to the wellbeing and long-term survival of patients – acute rejection and CAV as an expression of chronic rejection. The main questions I attempt to answer here are: i) are there biomarkers that are significantly different between patients undergoing these conditions, *versus* those who are not? ii) can a specific combination or panel of biomarkers work together and be potentially utilized for the diagnosis or monitoring of AR and CAV? iii) what can the identified biomarkers inform us regarding the underlying pathobiology of these conditions? Finally, I will also examine and discuss the caveats and potential challenges of biomarker research and translation of bench findings to the clinic.

2.3 Central hypothesis

Peripheral blood-derived molecular biomarker panels provide the means for sensitive and specific diagnosis of acute and chronic cardiac allograft rejection, as well as help gain insight into the underlying mechanisms.

2.4 Specific aims

1) To compare the blood-based molecular profiles between patients with heart failure of ischemic or non-ischemic origin, and determine the major biological processes perturbed in these patients at the 'end-stage' of their chronic heart failure.

2) To determine if there is a unique gene expression profile in the whole blood that is specific to the acute cardiac allograft rejection; and if such expression profile is present, determine its clinical utility and biological plausibility.

3) To determine if there is a unique proteomic signature in the plasma that is able to reflect the presence of significant coronary artery stenosis, a strong indicator of CAV development, and examine its implications for CAV screening and monitoring.

CHAPTER 3:
Molecular signatures of end-stage heart failure patients prior to cardiac transplantation

3.1 Background

Heart failure (HF), a progressive clinical syndrome characterized by inability of the heart to adequately pump blood to meet metabolic demands of the body,[1,2] can result from virtually any form of cardiac disorders.[2] Yet, despite being a multifactorial pathological condition with diverse etiologies, initial HF can persist and progress into chronic heart failure (CHF) and ultimately 'end-stage' heart failure (ESHF). The latter is often the final pathway leading to death or the need for cardiac transplantation.[1,117] According to the 2011 report from the registry of the ISHLT (based on data between January 2005 to June 2010), the two major etiologies of heart disease that preceded the need for heart transplantation in adults are in fact, ischemic heart disease/cardiomyopathy (37.7%) and non-ischemic cardiomyopathy (53.3%).[24]

Conditions such as ischemic heart disease (IHD) and non-ischemic cardiomyopathy (NICM) may follow different timelines leading to the onset of HF and eventual progression to CHF.[1] While CHF primarily impacts the cardiovascular system, it is often considered a multi-system disorder due to its interplay with musculoskeletal, neurohormonal, metabolic, immunological, and other systems of the body.[118-120] The complexity and systemic nature of CHF has made it an intriguing and attractive candidate for 'omic' investigations. Since the first human microarray analysis of ESHF,[121] research to date have generally involved comparing gene expression changes in failing *versus* non-failing hearts (NFH),[122-124] pre-left ventricular assist device (pre-LVAD) *versus* post-LVAD implantation,[125,126] or in different causes of HF (i.e., cardiomyopathies).[127,128] These published work have typically been based on cardiac tissue samples.[2] However, previous studies have reported high concordance between (gene) expression profiles derived from disease tissue and PBMC,[129] and proposed the use of whole blood and PBMC as viable substitutes.[130]

3.2 Rationale

It is currently unclear whether unique whole blood-derived signatures exist in patients with end-stage HF of either non-ischemic or ischemic origin. Although some pilot studies have documented significant differences in genomic signatures, albeit derived from cardiac tissues,

between the different etiologies of HF,[128] others have had less success in similar efforts.[131] It is also unclear what molecular and biological processes are perturbed in these CHF patients that underlie the need for eventual heart transplantation

In this chapter, I first compare IHD and NICM using a high-throughput holistic approach and attempt to shed light on the issues noted earlier, and answer the following questions: Is there a significant difference in the peripheral blood genomic and proteomic profiles between IHD and NICM patients at the time of ESHF? As well, what major biological processes and networks are dysregulated in CHF patients in general, relative to individuals with NCF?

3.3 Materials and methods

3.3.1 Subjects and specimens

The experiments in this chapter were conducted under the Biomarkers in Transplantation (BiT) initiative, which was approved by the Providence Health Care Research Ethics Board.[132] Peripheral blood samples used in this research chapter were collected within, on average, two weeks *just* prior to transplantation (i.e., "end-stage") from 29 cardiac transplant patients. Blood samples were also collected from 20 healthy individuals with normal cardiac function (NCF).

The cardiac transplant patients were divided into two groups based on the original clinical/pathological diagnoses: 1) Ischemic heart disease (IHD; n=16), or 2) Non-Ischemic Cardiomyopathy NICM (n=13). The IHD and NICM patients were grouped as the CHF cohort for the analysis comparing CHF and NCF (Table 2).

Whole blood and plasma samples were collected in PAXgene and EDTA tubes, and analyzed using Affymetrix microarrays and iTRAQ proteomics, respectively. Additional descriptions of each of these techniques are provided in the Appendices – Assays and 'Omics' Methodologies section.

Table 2. Demographics of subject cohorts (CHF and NCF)

Patient Demographics		CHF		NCF
		IHD	NICM	
Age	Range	52-70	26-64	26-65
	Average	60	46	42
Percent Men		94	62	60
Race (Percent)	Caucasian	94	100	80
	Asian	6	-	15
	Other	-	-	5
Ejection Fraction (Percent)	Range	13-35	15-40	-
	Average	20	20	-
New York Heart Association Class	Range	2-4	2-4	-
VO2 Max	Range	6-15	10-18	-
	Average	10.0	13.4	-
Length of Time with Heart Failure (Years)	Range	0.4-20	0.7-15	-
	Average	7.6	7.3	-
Co-morbidities (Percent)	Chronic Kidney Disease	31	8	-
	Hypertension	31	8	-
	Dyslipidemia	38	0	-
	Diabetes Mellitus	13	8	-
	Cirrhosis	6	8	-
	Others	19	15	-
	No co-morbidities	25	69	-

3.3.2 Sample and data processing

3.3.2.1 Genomics

A total of 26 CHF patient PAXgene whole blood samples (i.e., 15 IHD plus 11 NICM) and 20 NCF samples were available and used for the microarray analysis. Total RNA was isolated using PAXgene™ RNA Kits as previously described.[116,132] RNA samples were processed via reverse-transcription-*in vitro* transcription (RT-IVT) to generate labeled cRNA which were then fragmented for hybridization on the microarray for analysis. The microarray analysis was performed at the Microarray Core Laboratory at Children's Hospital, Los Angeles using Affymetrix Human Genome U133 Plus 2.0 chips. The microarrays were checked for quality problems using affy v1.22.0 and affyPLM v1.20.0 BioConductor packages.

3.3.2.2 Proteomics

In total, 22 CHF patients' plasma samples (14 IHD and 8 NICM) were available for proteomic analysis. Plasma samples from 16 healthy individuals were pooled and served as the normal reference sample for each iTRAQ experimental run. Sample processing, data acquisition and analysis were carried out as previously described.[133] Briefly, samples were processed via immuno-affinity chromatography (Genway Biotech; San Diego, CA), to deplete the 14 most abundant plasma proteins (albumin, fibrinogen, transferrin, IgG, IgA, IgM, haptoglobin, α2-macroglobulin, α1-acid glycoprotein, α1-antitrypsin, apolipoprotein-I, apolipoprotein-II, complement C3 and apolipoprotein B).[133] Depleted plasma protein samples were labelled with iTRAQ reagents according to manufacturer's protocol (Applied Biosystems; Foster City, CA). iTRAQ labelled peptides were analyzed by a 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems; Foster City, CA). Data was analyzed using ProteinPilot™ software v2.0 with the integrated Paragon™ Search and Pro Group™Algorithms searching against the International Protein Index (IPI HUMAN v3.39) database.

3.3.3 Analysis

The statistical analysis of the genomic and proteomic data was performed using R version 2.9.0 and BioConductor version 2.4.[135] The details described below are applicable to both comparisons in the study: IHD *versus* NICM and CHF *versus* NCF.

3.3.3.1 Genomics

Background correction, normalization and summarization of the microarrays corresponding to the 29 CHF and 20 NCF were performed with Robust Multi-array Average (RMA) technique (affy BioConductor package v1.22.0), including log base 2 transformation of the data. To reduce noise, probe sets with relatively constant expression values across all samples, i.e., interquantile range <0.5, were eliminated from further analysis. The remaining probe sets were analyzed using a robust moderated *t*-test available in the limma BioConductor package, v2.18.0. Probe sets with a False Discovery Rate (FDR) <5% were considered differentially expressed.

3.3.3.2 Proteomics

The data was log base two transformed. Protein group code algorithm was used to allow subsequent comparison and analysis of proteins, as protein 'groups' (PGs), across different iTRAQ experiments. Only protein groups (PG) detected in at least 2/3 of the samples in each comparison group were included in the analysis, as described previously.[133] In the second analysis (CHF *versus* NCF), since the data for each of the CHF samples represent abundance expressed relative to the pooled normal reference sample (NCF), the PGs were analyzed using a one group robust moderated *t*-test for the null hypothesis that the mean relative abundance equals one. Tests results with FDR <5% were considered statistically significant.

3.3.3.3 Functional enrichment

Functional enrichment of the statistically significant genes, proteins and metabolites was pursued using MetaCore (GeneGo Inc., www.genego.com), a web-based suite of tools designed for functional analysis of (-omics) experimental data. Through MetaCore, Gene Ontology (GO) and GeneGo process networks-based analyses were carried out. The functional ontologies used in the two analyses are unique and complementary; the former utilizes a

publicly available database (www.gene-ontology.org) and GO terms, while the latter uses a manually curated and annotated GeneGo process networks database of human gene/DNA and protein interactions. These networks reflect the interplay of molecules, as a group, in particular biological/molecular processes or pathways. The GO terms, GeneGo process networks and pathways identified with FDR <5% were considered significant.

3.4 Results

3.4.1 Ischemic heart disease (IHD) versus Non-ischemic cardiomyopathy (NICM)

3.4.1.1 Genomics

After normalization, pre-filtering was carried out to eliminate probe sets with relatively constant expression levels across all samples. A total of 14,047 probe sets, representing 7,450 genes, remained and were analyzed via robust moderated *t*-test. Differentially expressed probe sets were not found between IHD and NICM at FDR <5%.

3.4.1.2 Proteomics

A total of 125 PGs were detected in at least 2/3 of the samples within each group and included in the subsequent robust moderated *t*-test analysis. None of the PGs (FDR <5%) were differentially expressed between IHD and NICM samples.

3.4.2 Chronic heart failure (CHF) versus Normal cardiac function (NCF)

3.4.2.1 Genomics

Following normalization and pre-filtering, a total of 14,047 probe sets remained and were subjected to a robust moderated *t*-test analysis. 7,426 probe sets included in the moderated *t*-test were found to be differentially expressed between CHF and NCF samples (FDR <5%), with 2,364 being up-regulated in CHF relative to NCF. The top 100 of these probe sets, corresponding to 75 genes, were visualized using a *heatmap* (Figure 5).

Enrichment analysis was also carried out on the 7,426 probe sets, using GO-based and GeneGo process network-based functional ontologies to uncover differentially regulated biological/molecular processes and functions, as well as networks. To gain a complete, unbiased, *global* perspective on the processes and networks that are significantly regulated in CHF and NCF subjects, *all* the genes which we found to be differentially expressed were analyzed. The top 10 significantly enriched GO terms and GeneGo process networks (FDR <5%) are summarized in Figure 6 and Table 3.

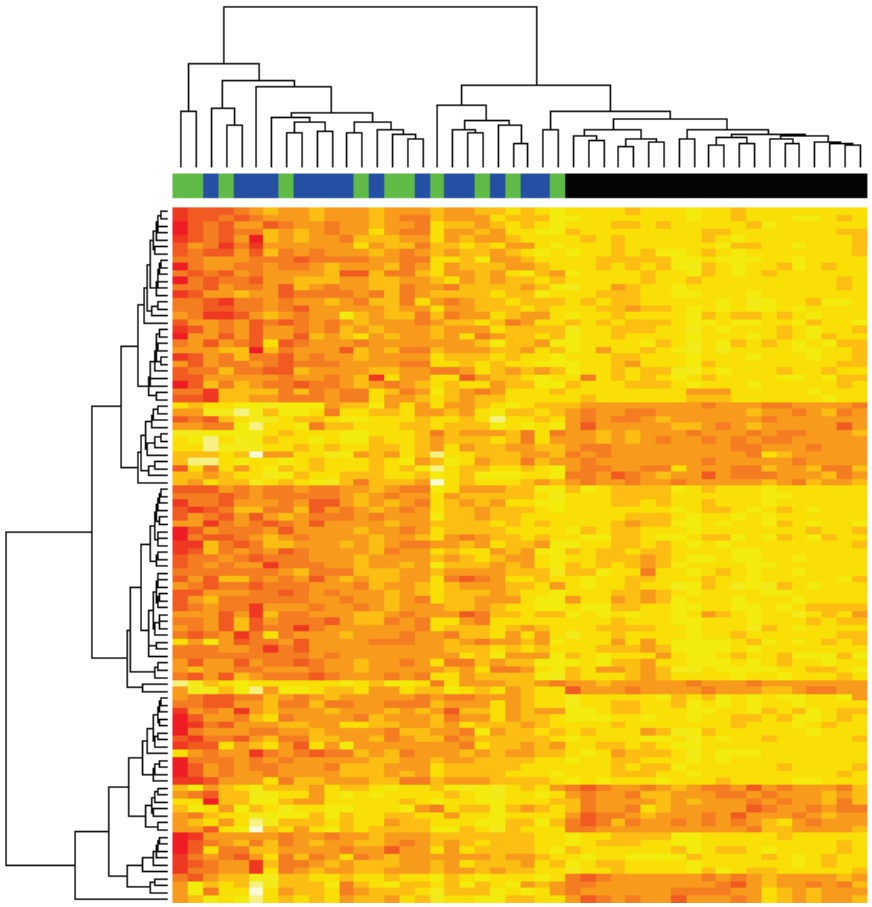

Figure 5. Heatmap of the top 100 differentially expressed probe sets between chronic heart failure (CHF) and normal cardiac function (NCF).

Each row represents 1 probe set and each column represents 1 sample. The CHF cohort is comprised of two groups of samples: ischemic heart disease (blue) and non-ischemic cardiomyopathy (green). NCF samples are shown in black.

Table 3. Top 10 process networks identified by MetaCore as statistically significant, based on the 7,426 differentially expressed probe sets between CHF and NCF.

Process Networks
Transcription mRNA processing
Immune TCR signalling
Signal transduction androgen receptor nuclear signalling
Cytoskeleton regulation of cytoskeleton rearrangement
Proteolysis/ubiquitin-proteasomal proteolysis
Cytoskeleton actin filaments
Apoptosis/apoptotic nucleus
Proliferation lymphocyte proliferation
Cell cycle G1-S interleukin regulation
DNA damage checkpoint

Abbreviation: TCR = T cell receptor

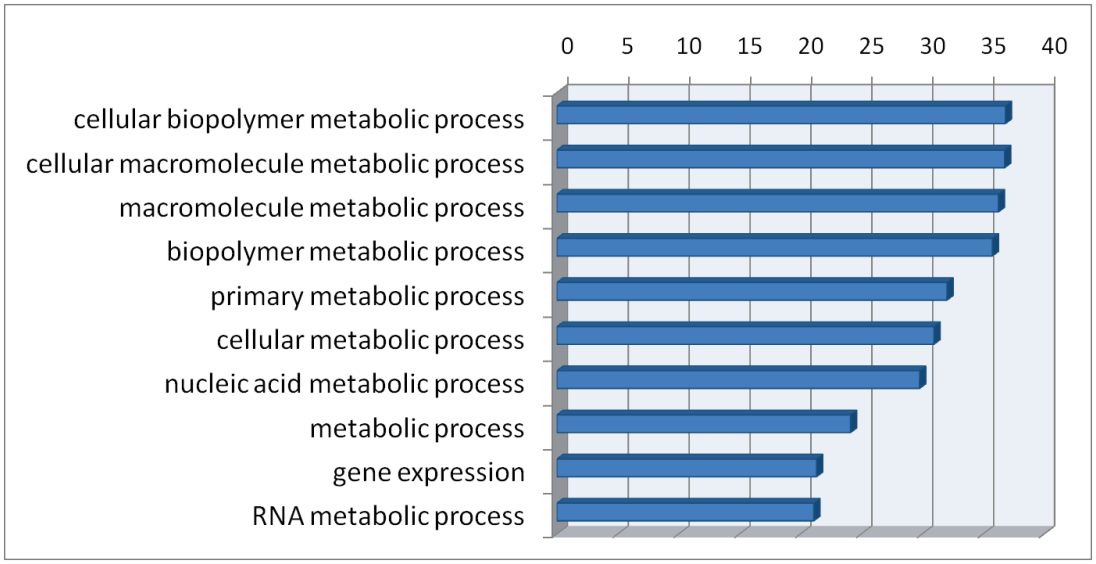

Figure 6. Top 10 GO terms based on functional enrichment analysis of the 7,426 probe sets differentially expressed between CHF and NCF.

The x-axis represents the statistical significance of each Gene Ontology term: -log(*P* value)

3.4.2.2 Proteomics

125 PGs were detected in at least 2/3 of the CHF samples. Of these, 71 PGs had a mean ratio significantly smaller or larger than one, relative to a pooled normal sample (FDR <5%), suggesting these PGs may be differentially abundant in CHF samples. Of these, 17 were observed at higher levels of abundance in CHF relative to the pooled normal reference sample. All statistically significant PGs were subjected to the same functional enrichment analyses as in the Genomics section. The significantly enriched GO terms and GeneGo process networks have been summarized in Table 4 and Figure 7 (FDR <5%).

Table 4. Top 10 process networks identified as statistically significant by MetaCore, based on the differentially expressed proteins between CHF and NCF.

Process Networks
Blood coagulation
Inflammation kallikrein-kinin system
Inflammation protein C signalling
Inflammation complement system
Cell adhesion / platelet-endothelium-leucocyte interactions
Immune phagosome in antigen presentation
Proteolysis ECM remodelling
Cell adhesion integrin priming
Immune phagocytosis
Inflammation IL-6 signalling

Abbreviation: ECM=extracellular matrix.

The analysis was based on 71 PGs differentially expressed between CHF and NCF

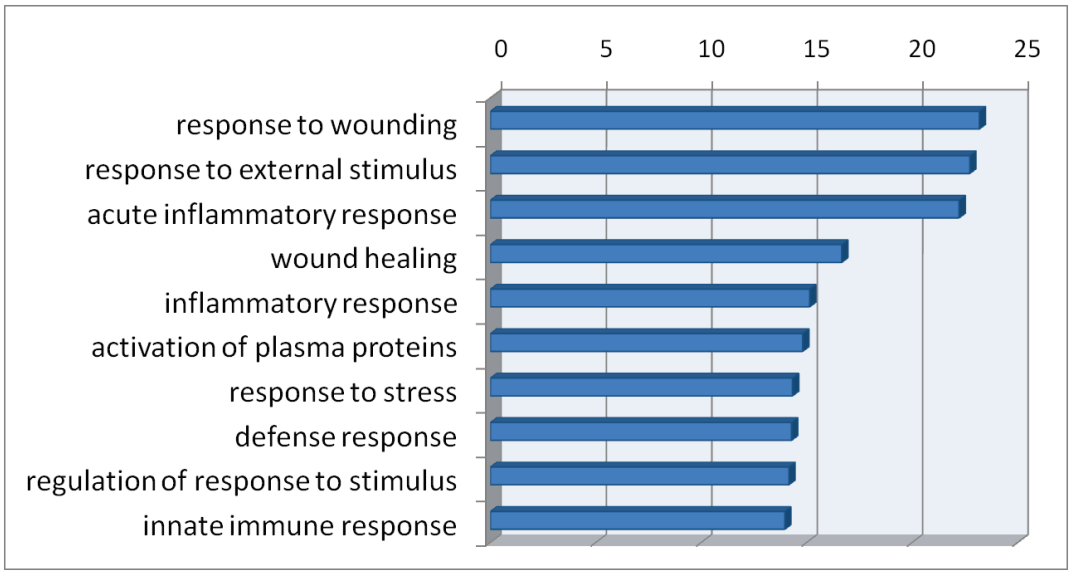

Figure 7. Top 10 GO terms based on functional enrichment analysis of the 71 PGs which showed differential concentrations between CHF and NCF.

The x-axis represents the -log(*P* value)

3.5 Discussion

The first key finding based on results from this study is that there was a lack of evidence of a difference in 'omic' profiles between end-stage IHD and NICM subjects. Using genomic and proteomic platforms, no differentially expressed genes *or* proteins between the IHD and NICM whole blood and plasma samples were observed. These results suggest that there may be a high level of similarity between the IHD and NICM subjects from an 'omics' profile perspective.

Previous work by others has suggested substantial similarities in transcriptomic patterns of ICM and NICM.[117,131] In a study by Steenman et al., no differentially expressed genes were found between ICM and NICM tissues, although a larger sample size would have been desirable.[117] A prevailing view in the literature is that, irrespective of the distinct underlying etiology that initiated HF, the expression signatures in advanced/end-stage HF may be dominated by a final common pathway.[123,130,131] Thus, it is possible that what I have observed is the result of a convergence of perturbations, reflected in the peripheral blood, obscuring initial upstream differences between IHD and NICM. Although this result may not necessarily represent convergence of myocardial events, gene expression changes in peripheral blood have been shown to correlate with the histological and functional status of the heart as demonstrated in the context of cardiac transplantation.[136-139]

In light of such findings, the next logical steps are to further utilize the results generated from the same platforms, and examine what major biological processes and networks are perturbed in CHF patients in general, relative to individuals with NCF.

In contrast to the highly similar molecular signatures between IHD and NICM during end-stage of CHF, analysis of CHF versus NCF revealed significant differences. A total of 7,426 probe sets were found to be differentially expressed in the CHF relative to the NCF blood samples in the genomics analysis. Although this result is not completely surprising, it fully illustrates molecular dysregulation present in end-stage CHF patients relative to healthy controls, at least at the genomic level. Unsupervised hierarchical clustering was performed on the top 100 of these differentially regulated probe sets, and a heat map was created to visualize relative expression levels between CHF and NCF samples. As shown in Figure 5, based on 100

probe sets alone, there is a greater resemblance between IHD and NICM etiologies of HF than between CHF of either etiology versus NCF. Significant differences between CHF and NCF were also observed in the proteomic results, as 71 proteins were found to be being differentially expressed between the two groups.

3.5.1 Integration of biological information and interpretation

The pathophysiology of HF involves systemic disturbances in a variety of biological processes. Using multiple discovery platforms and ontological databases has provided a global perspective on the interplay of diverse pathological processes underlying CHF. When functional enrichment of ''-omic'' profiles was carried out using MetaCore, the top 10 significant GO terms identified (Figure 6) based on the 7,426 differentially expressed probe sets suggest metabolic dysregulation manifest in the peripheral blood of CHF patients. Perturbations in cardiac energy metabolism are generally accepted to play a role in the progression of HF.[140,141] Other significantly regulated GO functions and GeneGo networks (Figure 7; Table 3 and Table 4) also reinforce existing knowledge regarding CHF. Specifically, the functional enrichment results demonstrated that majority of the differentially expressed genes and proteins found in the CHF samples fall within one of the following categories:

3.5.1.1 Response to cardiac damage/wound healing response (wound healing, extracellular matrix (ECM) remodeling, cytoskeleton regulation)

A key feature of HF progression is adverse structural remodeling of the myocardium. I noted a prominent overrepresentation of differentially expressed genes and proteins associated with cytoskeleton regulation and the remodeling process in CHF patients (Figure 7; Table 3 and Table 4). As discussed by Liew and colleagues, damage to the cardiac ECM and cytoskeleton is common during the remodeling process.[2] The differentially expressed plasma proteins detected are potentially derived from multiple organs, including the failing heart and other organs affected during the course of the HF development.[142] These plasma proteins can reflect soluble factors arising from remodeling, as well as inflammation and immune responses (Table 4). Braunwald, in a recent review, suggested the use of ECM breakdown and remodeling-related molecules as biomarkers of HF.[118] In fact, the linkage between CHF and matrix/

cytoskeletal, as well as proteolysis/stress, genes and proteins has also been described in studies comparing failing and nonfailing human hearts.[143]

It is also worth noting that circulating cellular components of the peripheral blood are contributors to the expression profiles detected on microarrays. Given that inflammation and immune activation are also thought to play a major role in CHF,[120] PBMC-derived gene expression changes are likely to be significant. Thus, it is possible that some of the differentially expressed genes related to cytoskeleton regulation were a consequence of immunological events underlying CHF arising from immune cell activation and cytoskeletal rearrangement in the circulating blood cells of CHF patients.

3.5.1.2 Inflammation/immune response (IL-6 signaling, kallikrein-kinin system)

Involvement of immune system and inflammatory mediators in CHF has been previously suggested in the literature by Fildes et al. and others.[144] The IL-6 signaling network was revealed as statistically significant in this study (Table 4). IL-6 is known to play a multitude of roles, whereas it has been associated with myocardial dysfunction and muscle wasting, it has also been shown to induce myocyte hypertrophy and inhibit cardiac myocyte apoptosis.[145] Increased concentrations of IL-6 in the plasma and myocardium of CHF patients have also been observed.[146] Activation of kallikrein-kinin system (Table 4) has been shown to be involved in the intramyocardial inflammation process.[147] As described earlier, many of the cytoskeletal regulatory genes identified may be a result circulating immune cell activation. In the context of stroke and acute cardiac rejection, circulating blood cell-derived gene profiles have already been implicated in immune events at the systemic and organ levels.[137,148]

3.5.1.3 Blood coagulation/cell adhesion (protein C signaling, platelet-endothelium-leukocyte interactions)

Development of CHF has been linked to endothelial and blood coagulation abnormalities,[149] as well as interactions between platelets, endothelium, and leukocytes.[150] Further, marked increase in plasma level of soluble adhesion molecules has been shown in CHF patients.[151] Upregulation of adhesion molecules in the myocardium of failing hearts has also been associated with chronic low grade inflammation.[152] The identification of protein C signaling as a significant network (Table 4) is also interesting. Protein C, an extracellular serine

protease important in anticoagulation, is also known to exhibit anti-inflammatory and anti-apoptotic activities through its interaction with endothelial protein C receptors, which are found on the endothelium and some white blood cells;[153] however, the exact role of protein C in CHF remains largely unclear at the present time.

3.5.1.4 Apoptosis/DNA damage checkpoint

During the cardiac remodeling process, a gradual but substantial reduction of myocytes can be seen after initial hypertrophy.[154] The contribution of cardiomyocyte apoptosis as a mechanism of progressive myocardial dysfunction and CHF has been suggested by Narula et al. [154] However, it is also likely that the apoptosis-related events highlighted by the enrichment analysis are contributed by immune cells involved in the chronic inflammation process. Thus, it remains to be elucidated whether the observations here are due to apoptosis of myocytes, peripheral blood cells, or both, and whether they are a cause or effect of CHF.

In summary, to highlight certain salient features of the CHF vs. NCF results from this chapter: 1) there is a high level of agreement in biological processes and networks between significant markers identified across multiple '-omics' technologies, namely genomics and proteomics, and 2) multiple '-omics' strategies uncovered a cohesive set of markers involved in themes currently accepted to be involved in CHF. The high concordance between results generated from multiple platforms reinforces our current understanding of the central mechanisms involved in CHF. Additional studies will be required to decipher the functional nuances of individual biomarkers and determine whether they are a cause or consequence of CHF. Such work will add significantly toward a better understanding of cardiac injury and repair, and its therapy.

3.5.2 Potential applications, caveats to the study, and future direction

The possible impact of clinical variables such as gender and age on the present study warrants mention. Boheler et al. have demonstrated that HF gene expression profiles can differ considerably among patients of different age and sex.[122] It is not clear to what extent this may have influenced the observations in the study – the most noticeable difference being the higher percentage of men and average age in the IHD group. However, it is important to note that the

analysis by Boheler et al. focused primarily on failing *versus* nonfailing hearts, and has not been extended to IHD versus NICM comparisons.[122]

One other caveat of the present study relates to the use of end-stage CHF samples. In contrast to an earlier study wherein Kittleson et al. identified a 90-gene expression profile that was able to differentiate ICM and NICM based on myocardial tissue-derived RNA.[128] None of these 90 genes was statistically significant (FDR <0.05) in the IHD versus NICM peripheral blood data (results not shown). There are a few obvious explanations for the observed difference – one relates to sample collection time points. Kittleson et al. sampled at different disease stages (eg, at LVAD placement or cardiac transplantation, after LVAD support, or at endomyocardial biopsy in newly diagnosed HF patients), whereas all of the CHF blood samples used in this study were collected days before cardiac transplantation (i.e., end-stage). As such, the results observed are likely to specifically relate to the converging biological mechanisms at the end of IHD and NICM development. That said, had unique genomic or proteomic profile been observed with either IHD or NICM group at the end-stage of heart failure, these molecular profiles can have interesting implications for follow-up studies. For instance, one could examine whether the unique molecular profiles associated with IHD and NICM are still detectable and distinguishable in blood post-transplantation. One may also consider comparing these unique profiles with the NCF subjects pre- and post-transplantation, and examine whether the level of difference changes. In other words, from the 'omics' perspective – how does cardiac transplantation influence the ESHF patients? And, do the IHD or NICM patients respond similarly or differently to the cardiac transplantation process?

Another factor to consider for future study design, of course, is the sampling site, blood versus heart tissue. It would be of great interest in future studies to analyze the 90 genes described by Kittleson et al., in blood samples collected at earlier time points before transplantation. An interesting question arises as to whether one can establish when the biological effects of CHF dominate the causes, and whether there is a ''point-of-no-return'' in the development of CHF. For these reasons, future studies should consider incorporating analysis of additional blood and biopsy samples from subjects at earlier stages of HF to better understand the full spectrum of mechanisms involved in the development of CHF.

CHAPTER 4:
Biomarkers of acute cardiac allograft rejection

4.1 Background

As described earlier, it has been estimated that up to 50% of patients will experience at least one episode of acute cardiac allograft rejection (ISHLT grade 2R or above) during the first year post-transplantation, despite the use of immunosuppressive therapies.[46] Currently, the definitive diagnosis of allograft rejection relies primarily on the endomyocardial biopsy (EMB), an invasive and inconvenient procedure.[35,155] EMB is also hindered by sampling errors and inter-observer variability despite the availability of international guidelines such as those set by the International Society for Heart and Lung Transplantation (ISHLT).[38,115]

Considering cardiac transplant recipients in many programs undergo at least 12-13 surveillance EMBs in the first year post-transplantation, and each procedure poses low but definite risks for complications to the patients – pneumothorax, cardiac perforation and even death – more accurate, precise, and less invasive alternatives are clearly desirable.[35,156,157]

4.2 Rationale

In recent years, the availability of high-performance platforms has provided researchers an alternative unbiased approach to discovering biomarkers, and the use of 'omics' technology has been explored as a potential tool to help enhance the diagnosis of acute and chronic rejection.[114] Of the current 'omics' technologies available, genomics is the relatively more recognized, studied and used platform.

Rejection is a complex process that involves several critical leukocyte-mediated events (i.e., recognition of alloantigen on the allograft, release of effector molecules, initiation of the inflammatory response, and activation/recruitment of circulating immune cells). As such, numerous research groups have made efforts to examine the peripheral blood (mononuclear cells) [PBMC] expression profile in relation to allograft rejection.[137,158-164] This approach has shown some promise, and recent studies involving microarray and qPCR analysis of peripheral blood gene expression profiles have provided evidence that they may be closely correlated with biopsy-proven acute cardiac allograft rejection.[115,137,139]

These early successes reported in the literature are encouraging, and demonstrate the promise in quantitative assessment of peripheral blood gene expression. In order to further elucidate the potential and possible pitfalls of 'omics', i.e., genomics technology, in the context of acute cardiac allograft rejection, additional studies and more evidence are clearly needed.

In this chapter, I will focus on the discovery and examination of acute rejection biomarkers using a genomics-based approach, via Affymetrix microarrays. First, I will explore the peripheral blood to determine if there are any biomarkers within that are differentially expressed in patients undergoing acute cardiac allograft rejection, relatively to those who are not. If these biomarkers are indeed present, I will then investigate if i) a specific combination of them can potentially be utilized for the diagnosis or monitoring of cardiac transplant recipients, and ii) what the individual biomarkers may represent, from a pathobiological aspect, in the context of acute cardiac allograft rejection.

4.3 Materials and methods

4.3.1 Subjects and specimens selection

The experiments in this chapter were conducted under the Biomarkers in Transplantation (BiT) initiative, which was approved by the Providence Health Care Research Ethics Board.[116] Subjects who received a cardiac transplant at St. Paul's Hospital, Vancouver, British Columbia between March 2005 and February 2008 were invited to participate. Subjects who agreed and signed consent forms were enrolled in the study. Transplant subjects received basilimax induction followed by standard triple immunosuppressive therapy (cyclosporine, prednisone, mycophenolate mofetil). Cyclosporine was replaced by tacrolimus for women and by sirolimus in the setting of renal impairment. Subjects with a 2R or 3R rejection episode within 3 months post-transplant received methylprednisolone.

For the purpose of this chapter, the focus was to initially identify biomarkers of acute cardiac allograft rejection of ISHLT grade 2R or above, i.e., moderate rejection or worse (characterized by the presence of two or more foci of cellular infiltrate with associated myocyte damage based on the EMB). As such, the two groups of patients (blood samples) that were analyzed and compared were "acute rejection" (AR; defined as ISHLT grade ≥2R), and "non-rejection" (defined as ISHLT grade = 0R).

A total of 28 subjects with blood samples corresponding to at least one AR (12 subjects) *or* one NR episode (16 subjects) were selected for microarray analysis. The subjects were divided into two independent cohorts (Figure 8). The first was a training cohort, (Figure 8, left) consisting of 6 AR and 12 NR samples collected from subjects with no serious complications (e.g., prolonged peri-transplant ischemia, infection, non-responsiveness to AR treatment). The training AR samples were collected *prior* to treatment for rejection and corresponded to the first AR episode of the subject. The second was a test cohort (Figure 8, right) consisting of 6 AR and 4 NR samples from subjects not included in the training set. All AR samples were collected within two days of biopsy-proven rejection episodes. All biopsies were over-read in a blinded manner by an experienced transplant cardiac pathologist using the revised ISHLT grading

scale.[47] Patient demographics were comparable between training and test cohort subjects (Table 5).

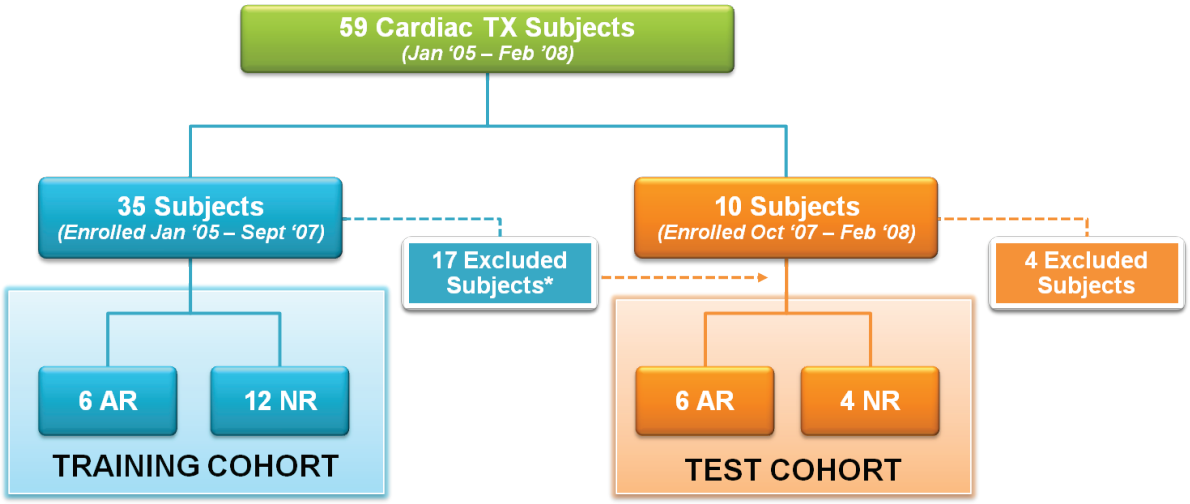

Figure 8. Division of subject samples into training and test cohorts.

Subjects enrolled between January 2005 and September 2007 who satisfied the selection criteria were considered training samples and used for biomarker discovery. The inclusion and exclusion criteria are as follow: only include NR samples collected from patients with no AR episode in first 6 months post-transplantation; exclude subjects who did not respond to AR treatment and/or had major/multiple complications within the first 6 months post-transplant; exclude NR samples which were taken during an acute rejection treatment. Two NR samples were mapped to each AR sample, collected at approximately the same timepoint. One AR and 3 NR subjects who did not satisfy the aforementioned criteria were excluded from the training cohort and included in the test cohort in addition to 5 five AR subjects and 1 NR subject enrolled between October 2007 and February 2008. Four subjects, enrolled in this time-period, were excluded due to death or lack of blood sample collection at the time of biopsy-proven AR.

Table 5. Demographics of cardiac transplant subject cohorts.

	Training Cohort (n=18)	Test Cohort (n=10)
Age (mean, standard deviation in years)	52±15	48±13
Gender (n male)	14	7
Ethnicity (n)		
Caucasian	16	9
Asian	1	1
Other	1	-
Primary Disease (n)		
Ischemic Heart Disease	9	4
Non-ischemic Cardiomyopathy	7	4
Other	2	2

4.3.2 Sample and data processing

Blood samples were collected in PAXgene tubes. The 28 subjects blood samples selected for microarray analysis were processed to isolate total RNA, using PAXgene™ Blood RNA Kits as previously described.[116] RNA quality was checked using an Agilent BioAnalyzer. RNA samples were then processed via reverse-transcription-*in vitro* transcription (RT-IVT) to generate labeled cRNA which is then fragmented for hybridization on the Affymetrix GeneChip® Human Genome (HG) U133 Plus 2.0 array. Microarray analysis was performed at the Microarray Core Laboratory at Children's Hospital in Los Angeles, California. The microarrays were checked for quality using affy (version 1.16.0) and affyPLM (version 1.14.0) BioConductor packages,[165,166] and Mahalanobis Distance Quality Control (MDQC).[167]

4.3.3 Analysis

4.3.3.1 Identification of biomarkers

Statistical analysis was performed using a "funnel" approach (Figure 9) with SAS System for Windows version 9.1.3,[168] R version 2.7.0, [134 134 134 133 132 132 132 132 132 131131] and BioConductor version 2.2.[135] In step 1, the Robust Multi-array Average (RMA)[169] technique was used for background correction, normalization and summarization (affy BioConductor package version 1.18.1). To reduce noise, probe-sets with consistently low expression values across all samples were eliminated from further analysis. The remaining probe-sets were analyzed using three moderated *t*-tests (Figure 9, step 2). Significance Analysis of Microarrays (SAM)[170] was performed using samr R package version 1.25 (http://cran.r-project.org/web/packages/samr/index.html). Limma BioConductor package (version 2.14.3)[171] was used for performing the other two moderated *t*-tests.[172] To ensure stringency, only probe-sets with a False Discovery Rate (FDR) <5% in all three moderated *t*-tests and a fold change >2 were considered statistically significant.

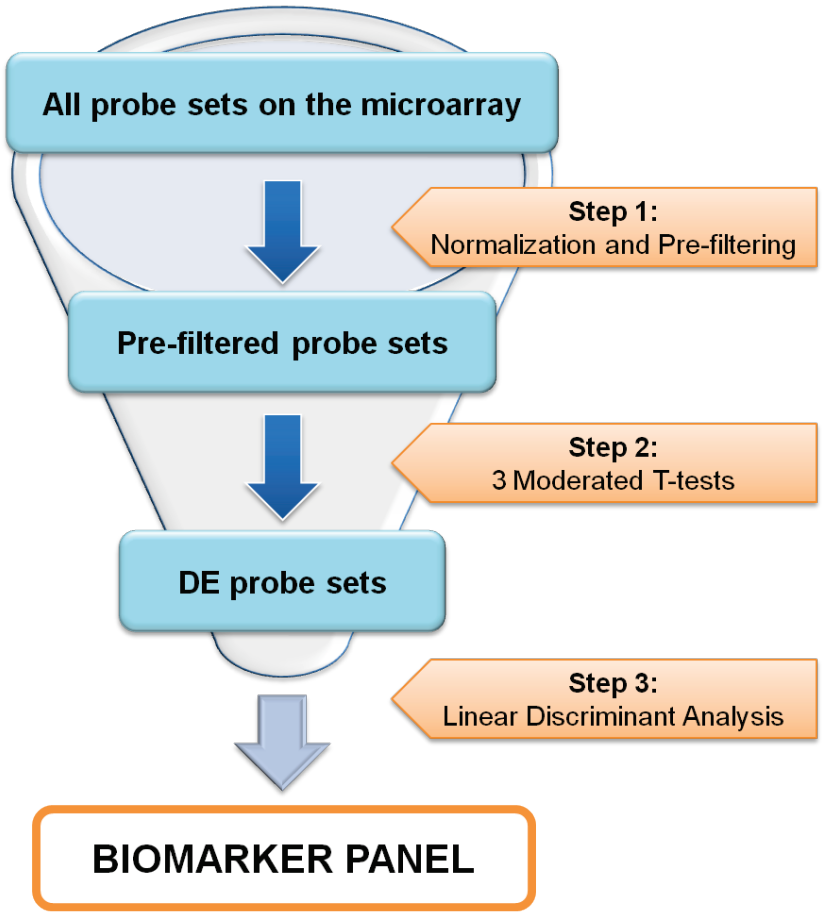

Figure 9. Overall workflow of the data analysis.

DE = differentially expressed.

4.3.3.2 Functional enrichment analysis

Functional enrichment of the differentially expressed genes (identified as described earlier) was examined using FatiGO,[173] available within version 3 of Babelomics,[174] a suite of web-based tools designed for functional analysis.

4.3.3.3 Generation and evaluation of the AR biomarker panel

Biomarker panel genes were pinpointed using Stepwise Discriminant Analysis (SDA) with forward selection on the statistically significant probe-sets (Figure 9, step 3). The classifier was built and tested with Linear Discriminant Analysis (LDA).

The biomarker panel genes were assessed by quantitative reverse-transcription-polymerase chain reaction (qRT-PCR; or simply as qPCR) using whole blood RNA from 16 of the training samples. RNA samples were first reverse transcribed to cDNA using SuperScript III First-Strand Synthesis System according to the manufacturer's protocol. qPCR was performed using gene-specific primers and Applied Biosystems (ABI) TaqMan Gene Expression Assays, on the ABI 7900HT Fast Real-Time PCR System. The qPCR data was analyzed using qBase v1.3.4.[175] Expression levels of the biomarker genes were normalized against β-actin gene.

The performance of the biomarker panel was also assessed through a six-fold cross-validation and an internal validation using the test cohort (Figure 8, right column). In both validations, the threshold between AR and NR samples was chosen to maximize the negative predictive value.

4.4 Results

4.4.1 Differentially expressed genes in AR patients

After normalization and pre-filtering, 25,082 probe-sets remained and were included in analyses (Figure 9, step 2) using the training samples (Figure 8, left side). A total of 1295 probe-sets were identified as having an FDR <5% in three moderated *t*-tests (SAM, robust and non-robust moderated *T* statistics) and a fold change >2.

4.4.2 Dysregulation of molecular and cellular processes in AR patients

4.4.2.1 Gene ontology (GO) analysis

Of the 1295 biomarker candidates that were differentially expressed in the training cohort, 1208 were downregulated and 87 were upregulated in AR relative to NR samples. Using FatiGO, these 1295 candidates were mapped to gene ontology (GO) terms through functional enrichment analysis. Over-represented, statistically significant GO terms were reviewed and are summarized in Table 6. Many downregulated probe-sets found in AR were associated with molecular and cellular functions such as regulation of enzymatic activities and protein metabolic processes. Conversely, numerous upregulated probe-sets found in AR were linked to innate and humoral immunity, response to wounding, and hypoxia. These functions and cellular processes have been linked to allograft rejection.[176,177]

Table 6. Relative expression levels and associative GO terms over-represented in the 1295 statistically significant probe sets.

Regulation in AR vs. NR	Number of probe Sets	GO Term Type	Exemplary GO terms corresponding to the significant probe sets
Down	1,208	Biological processes	- signal transduction - biopolymer metabolic processes - cellular protein metabolic process - cellular component organization and biogenesis
		Molecular functions	- GTPase regulator activity - RNA binding - ion binding - enzyme inhibitor activity
Up	87	Biological processes	- innate / humoral immune response - response to wounding - response to hypoxia - acute inflammatory response
		Molecular functions	- creatine transporter activity - transcription factor binding / activity - tumor necrosis factor binding - actin binding

4.4.3 AR biomarker panel genes

SDA was applied on the 1295 differentially expressed probe-sets. Twelve probe-sets (corresponding to 12 genes) were identified that, together, best differentiate between AR and NR samples (Table 7). These 12 biomarker panel genes showed a 2 to 3.3 fold change in expression levels between AR and NR samples. Ten of the 12 biomarker panel genes were downregulated in AR.

Table 7. Acute cardiac allograft rejection biomarker panel.

Probe Set ID	Gene Symbol	Gene Name	Fold Change	Regulation of AR versus NR
207883_s_at	TFR2	Transferrin receptor 2	2.1	up
229067_at	SRGAP2P1	SLIT-ROBO Rho GTPase activating protein 2 pseudogene 1	3.3	down
221841_s_at	KLF4	Kruppel-like factor 4	2.7	down
214659_x_at	YLPM1	YLP motif containing 1	2.0	down
204493_at	BID	BH3 interacting domain death agonist	2.0	down
201669_s_at	MARCKS	Myristoylated alanine-rich C-kinase substrate	2.8	down
1556209_at	CLEC2B	C-type lectin domain family 2, member B	2.3	down
235412_at	ARHGEF7	Rho guanine nucleotide exchange factor (GEF) 7	2.2	down
226851_at	LYPLAL1	Lysophospholipase-like 1	2.2	down
202749_at	WRB	Tryptophan rich basic protein	2.1	down
1556283_s_at	FGFR1OP2	FGFR1 oncogene partner 2	2.6	up
209580_s_at	MBD4	Methyl-CpG binding domain protein 4	2.0	down

4.4.4 Evaluation of the AR biomarker panel

qPCR was performed for the 10 classifier genes with commercially available primers on 5 AR and 11 NR training samples. Three exemplary genes are illustrated in Figure 10. Seven of the 10 genes were consistent in the direction of regulation of AR relative to NR between the microarray and the qPCR platforms.

The cross-validation sensitivity was 100% and specificity was 75%. The biomarker panel was then applied to the test cohort. One AR (out of possible 6) and one NR (out of possible 4) sample were misclassified, corresponding to a sensitivity of 83% and specificity of 75% for the 12-gene classifier. Alternatively, when the classifier was trained using all 37 AR and NR samples collected within the first five months post-transplant from the 18 training cohort subjects, the internal validation results improved to 100% specificity (Table 8).

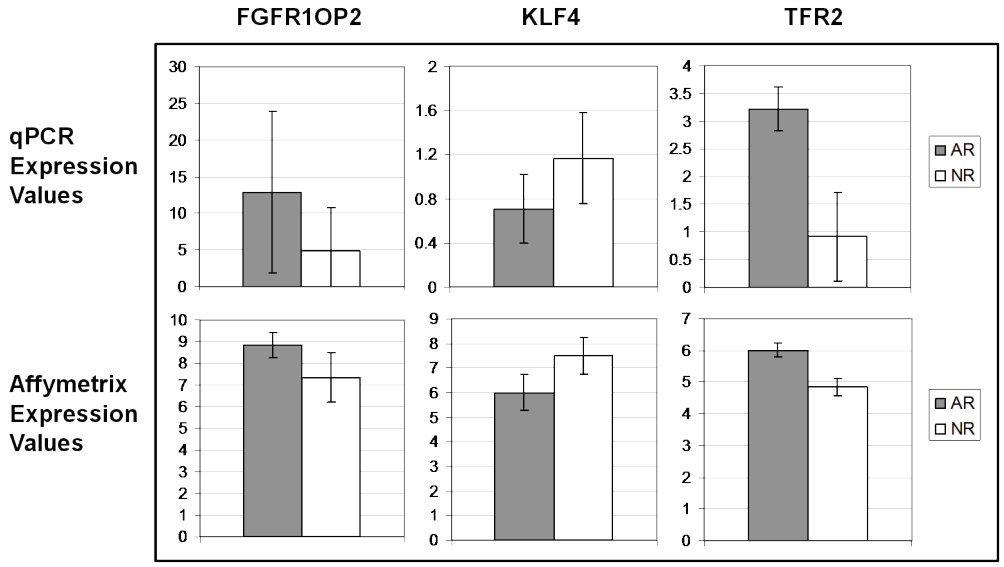

Figure 10. AR biomarker expression evaluation.

Average gene expression values of AR and NR samples from qPCR (top row) and microarrays (bottom row) are displayed for each gene (columns). The error bars represent the standard deviation within each group.

Table 8. AR biomarker performance evaluation.

	Cross-Validation	Internal Validation [10 Test Samples]	
		Training Set [18 Samples]	Training Set [38 Samples]
Sensitivity	100%	83%	83%
Specificity	75%	75%	100%

During cross-validation, the data was randomly split into six parts, each part containing 1 AR and 2 NR samples. In each fold, a different part of the data served as the test set, while the remaining (five of six) parts were used as the training set. This process was repeated six times (each 'part' served as the test set once), and each time, analysis steps 2 and 3 (Figure 9) were performed on the training set, and the obtained biomarker panel was tested on the test set. The internal validations were performed on the test cohort samples

4.5 Discussions

4.5.1 Integration of biological information and interpretation

With high performance platforms now available, a new door has opened to allow discovery of molecular signatures. In the previous research chapter, the 'omics' technology was applied with the goal in mind to gain insight into the underlying pathobiology and molecular profile disturbances in the context of end-stage heart failure. In contrast, a major focus in this chapter is identifying specific 'panels' of differentially expressed genes to serve as *classifiers* of disease presence or absence, i.e., monitoring and diagnostics.

It should be noted that the main measure of a classifier's excellence is based on classification accuracy with independent data, rather than biological plausibility.[178] While the interpretation of biological context behind biomarker classifiers is not always straightforward,[178] biomarkers that fit currently accepted biological and physiological paradigms are more readily accepted by the research and clinical communities.[179] Therefore, interpreting the biological plausibility of classifier genes is worthwhile as it increases the value of a microarray dataset.[178]

In this study, 1295 probe-sets demonstrated expression levels that differ in AR *versus* NR patients. This is perhaps not surprising, given the profound disturbance that allograft rejection-related processes can have at a cellular and molecular level. These gene expression changes in the peripheral blood reflect responsive and adaptive mechanisms to the early events underlying rejection (i.e., inflammation, alloimmune activation/response). From the 1295 probe-sets identified as statistically significant, 12 genes were identified together as the most effective biomarker panel. I then examined the published literature for information regarding the biological functions of these 12 and found that nine have been relatively well studied. Observations of potential relevance to transplantation have been summarized in Table 9.

Table 9. Summary of the biological functions of the AR biomarker panel genes based on previous literature.

Gene Symbol	Described Biological Functions
TFR2	• Transferrin receptor 2 • Involved in iron homeostasis [180], disruption in iron homeostasis observed in lung allograft patients [181] • TFR2 levels upregulated in activated T cells [182]; T cell activation is an expected phenomenon during allograft rejection
KLF4	• Kruppel-like factor 4 • Linked to regulation of B cells; overexpression suppresses cell proliferation [183] • B cell activation is associated with downregulation of KLF4 mRNA and protein [184]
BID	• BH3 interacting domain death agonist • Involved in perforin and granzyme B induced apoptosis [185,186] • Involved in hypoxia/reoxygenation induced lymphocyte apoptosis [187] • Antagonizes apoptosis in certain circumstances [187]
MARCKS	• Myristoylated alanine-rich C-kinase substrate • Is a major substrate of PKC [188] • MARCKS and PKC implicated in many cell growth, differentiation, metabolic and functional pathways in all immune cell types [188-199]
CLEC2B	• C-type lectin domain family 2, member B • Also called Activation-induced C-type lectin, a transmembrane receptor on monocytes, granulocytes, B and T cells [200,201] • Is the ligand for the NKp80 receptor on NK cells and monocytes; interaction regulates activity of these cells [201] • Reverse signalling induced by NKp80 binding of CLEC2B on monocytes result in TNFα production which has been suggested to play a role in acute and chronic lung rejection [202]
ARHGEF7	• Rho guanine nucleotide exchange factor (GEF) 7 • Is a positive regulator of Rho family of small molecular weight G-proteins [203,204] • IL-2 stimulation of T cells result in upregulation of ARHGEF7 mRNA, and is thought to be involved in Rho mediated cellular changes (cytoskeletal rearrangements) [203,204]
WRB	• Tryptophan rich basic protein • Gene first identified as mapping to 21q22.3, a locus associated with congenital heart disease in Down's syndrome [205,206] • WRB has been shown to be downregulated in endothelial cells in response to C-reactive protein [207]
FGFR1OP2	• Fibroblast growth factor receptor oncogene partner 2 • First described as a fusion partner of fibroblast growth factor receptor 1 in the setting of 8p11 myeloproliferative syndrome (EMS), which is characterized by eosinophilia and T/B cell lymphoma prior to transformation into acute myeloid leukemia [208,209]
MBD4	• Methyl-CpG binding domain protein 4 • Encodes a DNA repair protein [210,211] • Plays critical role in genome stability/integrity, repair and cell cycle response to DNA damage [212] • Found to be mutated in various carcinomas [213-215]

The process of graft rejection involves activation and proliferation of immune and inflammatory cells. In this context, the upregulation of TFR2 and FGFR1OP2, and downregulation of KLF4 and BID in AR versus NR patients is consistent with cell activation and proliferation during an AR episode. TFR2 is a transferrin receptor important in iron uptake into cells and its levels rise in activated immune cells.[182] TFR2 is also important in regulating iron homeostasis, and interestingly, dysregulated iron homeostasis has been observed in lung allograft patients.[181] The FGFR10P2 gene has also been associated with proliferation as it was first identified in a myeloproliferative syndrome that involves eosinophils as well as T and B cells, [208,209] but the normal function of FGFR10P2 is not yet known.[209] Some genes are known to be downregulated in cells undergoing activation or proliferation, including the transcription factor KLF4[184] and the pro-apoptotic protein BID,[187] so their decrease in AR versus NR patients is consistent with activation of immune cells.

The other genes in the classifier panel identified have also been implicated in biological processes that may have relevance to the process of graft rejection. Additional biological studies are needed to determine which specific peripheral blood cells express these genes and why many of their levels are decreased in AR patients. The Rho guanine nucleotide exchange protein ARHGEF7 is important in regulating cytoskeletal changes necessary for maintenance of cell morphology and migration, and has been reported to be upregulated in activated T cells.[204] The observed downregulation of ARHGEF7 mRNA in AR patients would seem contradictory. However, many possibilities exist to explain this observation; for example, perhaps the most activated and migratory T cells have left the circulation to enter the tissues of the graft. Similar arguments could be made for the intracellular signalling molecule MARCKS and transmembrane ligand/receptor CLEC2B. Finally, two of the genes in the biomarker panel have described functions, but their role in graft rejection will have to be defined. MBD4 is a DNA repair enzyme,[210,211] while WRB is a gene that has been described to be downregulated in non-blood cells by inflammatory mediator C-reactive protein.[207]

4.5.2 Assessment and validation of the AR biomarker panel

The 12 genes belonging to the biomarker panel have been subjected to preliminary biological investigation, and hypotheses regarding their involvement in acute rejection can be formulated. Three genes in the panel, SRGAP2P1, YLPM1 and LYPLAL1, have not yet been characterized in any biological studies. Their sequence, cellular location and predicted biochemical or biophysical characteristics are available in databases such as Genecards [www.genecards.org] and NCBI [www.ncbi.nlm.nih.gov].

While the identified biomarkers appear to be biologically plausible in the context of acute cardiac rejection, as described earlier, the other major focus in this study is the assessment and validation of the classifier (i.e., biomarker panel consisting of specific combination of genes) of acute rejection that has been generated based on genes that were the differentially expressed between the AR and NR subjects.

In this particular study, qPCR was performed for 10 classifier genes on 16 training patient samples (5 AR and 11NR) available, using commercially available primers, i.e., Applied Biosystems (ABI) TaqMan Gene Expression Assays. The motivation for performing this set of experiment was to assess the expression levels of the classifier genes using an alternative technological platform and in turn, investigate the potential utility and transition to using qPCR as a possible clinical assay. For discovery purposes, microarrays have shown great potential as a tool for screening a large number of potential candidates to discover the key (combination of) biomarkers that is correlated with the disease of interest. From a pragmatic perspective however, it has been suggested that biomarker panel/classifier gene signature analysis that requires the simple use of qPCR may be more ideal, since such platforms are more readily available in clinical laboratories; in some cases they may already be validated for clinical diagnostic tests.[178,216]

Of the 10 classifier/biomarker panel genes evaluated, seven were consistent in the direction of regulation of AR relative to NR between the microarray and the qPCR platforms. Potential factors contributory to differences between qPCR and microarrays have been detailed in the work of Morey et al.[217] Indeed, disagreement or lack of correlation between microarray and qPCR data is quite common and has been reported by others.[217,218] Beyond the explanation

of others,[217,218] the binding of commercially available *gene-specific* primers to gene regions wherein polymorphisms or splice variations may exist could differ from those regions detected by the probe-sets on a microarray. Ultimately, internally developed *probe-set-specific* primers may help avoid the aforementioned gene region binding discrepancy, provide better correlations between qPCR and microarray result, and address this widely recognized issue.

As noted earlier, the *main* measure of a classifier's excellence is based on classification accuracy with independent data.[178] In this study, the subjects were first divided into two independent cohorts for this purpose (Figure 8), thus allowing internal validation of the biomarker panel, in addition to cross-validation. As shown in Table 8, the biomarker panel, which consists of 12 genes, was able to classify the internal validation samples with 83% sensitivity and 100% specificity. In comparison, the sensitivity and specificity of EMB (the current gold standard for the diagnosis of acute cardiac allograft rejection) has been reported to vary between approximately 75-90% and 80-90+%, respectively; these values are also thought to be heavily dependent on the number of (right ventricular) biopsy samples taken for evaluation.[219-221] Taken together, the initial results based on this study suggest that peripheral blood genomic molecular profile, such as the 12 gene classifier panel identified in this study, hold considerable potential in discriminating acute rejection from non-rejection in heart transplant recipients.

4.5.3 Current study results versus CARGO results

The Cardiac Allograft Rejection Gene Expression Observation (CARGO) study warrants a mention here, as it is perhaps the most widely recognized and discussed microarray study in the context of acute cardiac allograft rejection diagnosis in the recent years.

The CARGO study used microarray analysis and real-time PCR to examine and validate gene expression profiles of allograft recipients' peripheral blood mononuclear cells. Deng and colleagues[115] reported 11 genes that distinguish ISHLT grade 0 rejection (quiescence) from moderate/severe rejection (1990 ISHLT grade ≥3A; see Figure 1). These 11 genes were compared to the 12-gene biomarker panel identified in this study, and no genes were found to be in-common. There are several reasons for such an apparent difference in results.

First, the microarray platforms used were different between the two studies – CARGO employed a custom array with 7370 genes represented, while this particular study used Affymetrix HG U133 Plus 2.0 microarray with 47,000 transcripts which correspond to at least 25,000 human genes. A key factor in different results relates to the fact that only 3 of the 12 biomarker classifier panel genes from this study were present on the custom array used in CARGO. In other words, 9 of the biomarker panel genes found in this study had no chance being detected on the array that CARGO investigators used.

Second, the diagnostic timeframe (i.e., time post-transplant) for which the CARGO biomarker panel is able to diagnose rejection is different from that observed with the biomarker panel generated in this study. In the CARGO published study, analyses were carried out on samples collected ~60+ days post-transplant. Thus, the generalizability and diagnostic utility of the classifier developed, based on the samples used in CARGO, is aimed towards samples collected *after* 2 months post-transplant [www.xdx.com/allomap].[115] In this study, the samples analyzed were collected between week 1 and month 5 post-transplant. Thus, the classifier reflects differential gene expression detectable as early as week 1 post-transplant.

Lastly, the sample sources used were different in between the two studies –the CARGO study focused on peripheral blood mononuclear cells (PBMC), while in the current study whole blood PAXgene samples were used for microarray analysis. The gene expression evaluated in

this study, therefore, is reflective of *all* peripheral blood circulating cells during acute rejection, and is not necessarily restricted to those transcripts arising from PBMCs.

Regardless of particular differences in the biomarker panels that distinguish rejection from non-rejection in this initial study and that of CARGO, there was commonality at a higher, biological process level. In-common gene ontological (GO) terms (based on the AR biomarkers from both studies) include biological processes such as such as cell motility, signal transduction and immune response.

4.5.4 Potential applications, caveats to the study, and future directions

Based on the internal- and cross-validation results, the classifier panel developed in this study appeared to be able to discriminate between AR and NR samples collected as early as one week post-transplant and as late as five months post-transplant. This characteristic, along with the advantage of whole blood approach that is minimally invasive, gives the biomarker panel the potential to serve as a complementary, pre-screening tool to help determine which patients *really* need the EMB.

There are several additional considerations pertinent to these studies that deserve comment. First, larger training and testing cohorts would be desirable. However, the statistical approach chosen was designed to be sufficiently robust to accommodate a smaller sample size. Second, patients enrolled in this study were primarily of Caucasian ethnicity, from a single institution and largely on a consistent local immunosuppressant regimen. To increase the generalizability and broader applicability of the biomarker panel, inclusion and testing of independent, external cohorts would be desirable for future studies. Third, this particular study examined rejection episodes that occurred within the first 5 months post-transplant, but given that most acute rejections occur within this timeperiod (usually during the first 6 months),[37] such a classifier panel can still potentially benefit the care of most cardiac transplant patients. Further, this initial study has focused on the discovery of biomarkers of acute cardiac allograft rejection defined as ISHLT grade 2R or above. It may also be of interest in future studies to include mild rejection patients (i.e., ISHLT grade 1R), and examine whether the biomarker

panel/molecular signature described here also work for a wider spectrum of patients and correlate with different severity of rejection. Last but not least, a universal limitation faced in biomarker studies aimed at classification of AR *versus* NR patients is the reliance on EMB. As noted in the introduction, the different ISHLT rejection grades are subject to variability under the eyes of expert pathologists, which creates somewhat of a predicament in trying to identify a biomarker panel that outperforms the current "gold standard", the EMB, in the diagnosis of acute heart rejection.

The primary focus of this study was to create a classifier which can help discriminate between AR and NR samples, regardless of the underlying mechanisms that cause the rejection episode. The complexity of the rejection process, including cellular and soluble factors,[222] remains a challenge to better understand how to care for patients and to interpret any biomarker panel results. This intriguing problem will remain the focus of many research groups in the biomarker arena. Certainly, among variables that influence the potential applicability of molecular signatures identified in this study in guiding care, the time post-transplant is among the most important for many reasons. Immunosuppressive regimens, including the nature of transient augmentation in the face of rejection, will continue to evolve. These therapeutic changes no doubt, will modify signatures in ways yet to be discovered. The enigmas that remain about how to monitor for human cardiac allograft rejection may be partly resolved through additional work in the future and examine alternative sources for biomarkers, such as the plasma proteome and the serum and urine metabolome.

CHAPTER 5:
Biomarkers of cardiac allograft vasculopathy

5.1 Background

Whereas the occurrence of acute cardiac rejection is considered one of the main short term obstacles, the development of Cardiac allograft vasculopathy (CAV) is a major hurdle in the long term survival of cardiac transplant recipients.[45,56,58] Each year, approximately 4000 cardiac transplantations take place around the globe.[56] Of these, almost 50% will develop CAV in the first several years post-transplant.[56] It has been estimated that CAV is responsible for up to 15% of deaths in cardiac allograft recipients after they have survived the first year post-transplant.[56]

Considering the prevalence and the significance of CAV, cardiac transplant recipients typically undergo routine tests *at least* once a year to monitor the health of the transplanted heart. The screening and diagnosis of CAV is largely based on detection of narrowing, or stenosis, of coronary arteries, i.e., blood vessels which supply oxygenated blood to the heart.[45,57,58] Unfortunately, the most widely used modality for the diagnosis of CAV remains coronary angiography, an invasive technique that is costly and uncomfortable for patients. Further, this procedure is associated with definite risks for complications.[45,57,58]

5.2 Rationale

Given the nature of the current gold standard, an alternative, minimally invasive method for detecting CAV that is both sensitive and specific is highly desirable. A simple blood test that is based on specific molecular biomarker signatures to help screen, diagnose or monitor CAV has the potential to alleviate discomfort of cardiac transplant patients and improve their wellbeing.

Similar to the scenario in acute cardiac allograft rejection, biomarkers such as C-reactive protein (CRP) and brain natriuretic peptide (BNP), as well as other gene expression tests,[223] have all been suggested as having utility in the diagnosis of CAV.[45] However, with the increasing availability 'omics' technologies, it is now possible to examine multiple molecular biomarkers of risk or disease in a high-throughput, unbiased manner. As noted in the previous chapters, this holistic approach to biomarker discovery has yielded promising data, such as in the case of

acute kidney rejection diagnosis[133] and acute cardiac rejection diagnosis, which resulted in identification of biomarker panels that are more sensitive and specific for the disease of interest, ultimately leading to better translation into the clinic.[115]

Currently, proteomics technology has yet to be examined in the context of detecting allograft coronary artery stenosis as a strong indicator of CAV development and expression of chronic rejection. As such, there were several goals in this work. First, I wanted to identify plasma-derived biomarkers that are differentially expressed between patients with and without significant CAV using an unbiased, data-driven approach. Second, I want to assess the diagnostic performance and the potential clinical utility (e.g., for CAV development screening and monitoring) of the biomarker panel generated based on the biomarker candidates identified. Lastly, I also examined the biological plausibility and the possible implications of these biomarkers in the context of CAV development.

5.3 Materials and methods

5.3.1 Subjects and specimens

This study was conducted under the Biomarkers in Transplantation (BiT) initiative, which was approved by the Providence Health Care Research Ethics Board.[132] Subjects who received a cardiac transplant at St. Paul's Hospital, Vancouver, British Columbia, were approached by our research coordinators, and those who consented were enrolled in the study.

5.3.1.1 Screening and identification of CAV and Non-CAV patients

Screening for CAV as an expression of chronic rejection was routinely performed using dobutamine stress echocardiography, coronary angiography and intravascular ultrasounds (IVUS) according to the 'Protocol for Long-term Surveillance of Cardiac Allograft Vasculopathy' guidelines [http://www.heartcentre.ca/CADsurveillance2007.pdf.pdf][224] as established by St. Paul's Heart Centre. Angiograms were assessed in a core lab using quantitative coronary angiography (QCA) as previously described.[59] Percentage diameter stenosis (%DS) was calculated based on the following formula: %DS = [(RD – MLD)/RD] X 100, where RD is reference diameter (an average of the normal region of the blood vessel), and MLD is the minimum lumen diameter. Whenever possible, the proximal, mid, and distal portion of the coronary arteries are assessed. Physicians, nurses and technicians who are involved in the collection of the coronary angiography data were blinded to the molecular study protocol and other data.

Coronary angiographic criteria were used to characterize patients in the current study as QCA is the most widely-available and consistently measured endpoint for evaluating CAV at our institution. Presence of biologically significant cardiac allograft vasculopathy (CAV) was defined in the study as maximum percentage of diameter stenosis (Max %DS) in the left anterior descending artery (LAD) ≥ 40%. Non-significant CAV development (Non-CAV) was defined as Max %DS in LAD ≤20%. Possible CAV was defined as 20%< Max %DS <40%.

5.3.2 Sample selection and data processing

Blood samples used for the current study were those collected in EDTA tubes at the nearest (earlier) timepoint corresponding to the date when coronary angiography was carried out – typically at least 1 year post-transplant, during routine post-transplantation check-ups.

40 cardiac transplant patients samples were selected for the proteomic analysis (10 CAV and 9 non-CAV used for generating the biomarker panel/classifier; additional 21 possible CAV for principal component analysis). Plasma samples from healthy individuals were pooled and served as the normal reference sample for each iTRAQ experimental run. Sample processing, data acquisition and analysis were carried out as described in previous studies.[133] Briefly, samples were processed via immuno-affinity chromatography (Genway Biotech; San Diego, CA), to deplete the 14 most abundant plasma proteins (albumin, fibrinogen, transferrin, IgG, IgA, IgM, haptoglobin, α2-macroglobulin, α1-acid glycoprotein, α1-antitrypsin, apolipoprotein-I, apolipoprotein-II, complement C3 and apolipoprotein B).[133] Depleted plasma protein samples were labelled with iTRAQ reagents according to manufacturer's protocol (Applied Biosystems; Foster City, CA). iTRAQ labelled peptides were analyzed by a 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems; Foster City, CA). Data was analyzed using ProteinPilot™ software v2.0 with the integrated Paragon™ Search and Pro Group™ Algorithms, and subsequent searching against the International Protein Index (IPI HUMAN v3.39) database.

5.3.3 Analysis

5.3.3.1 Identification of CAV biomarkers and functional enrichment

The statistical analysis was performed using R version 2.10.1.[225] The data was log base two transformed. Protein groups (PG; described earlier and in the Appendices) detected in at least 75% of the CAV and Non-CAV samples were included in the subsequent analysis. A robust moderated *t*-test was used to find proteins with differential relative levels between CAV and Non-CAV samples. Elastic Net model[226] was applied to consider the most significant proteins (robust moderated *t*-test *p*-value <0.1) to identify the biomarker panel/classifier.

Functional enrichment was performed on the candidate protein biomarkers (identified by Robust-LIMMA and Elastic Net) using MetaCore (GeneGo Inc; *www.genego.com*). Gene Ontology (GO)-based analyses were carried out through MetaCore, using publicly available (*www.geneontology.org*) database, to assess the functional significance of the proteins of interest. GO terms with FDR <5% were considered statistically significant.

5.3.3.2 Validation of CAV biomarker panel

Cross validation, i.e., leave-one-out cross-validation (LOOCV), was used to evaluate the performance of the analytical pipeline (and the biomarker panel). In leave-one-out cross-validation, one sample is left out as a test sample and the remaining 18 samples are used to discover and build a classifier score. The resulting score is then used to classify the sample left out. This procedure is repeated until all samples are left out once, and the performance is estimated by the average of the results. The classification of test samples based on their plasma samples was performed using the Elastic Net classifiers built based on the training set in each fold of the cross-validation. Specifically, the biomarker classifier score (i.e., a value generated based on the combined contribution of the biomarker panel proteins and their expression levels) was used to classify test patient samples from the cross-validation as either having significant CAV or not.

Principal component analysis was also performed on all patients samples selected for this study, based on the biomarker panel proteins. Receiver operating characteristic (ROC) curve was also constructed for the CAV biomarker panel, based on the probabilities estimated by the cross-validation. The ROC curve and the area under the curve (AUC) were computed using the ROCR package.[227]

5.4 Results

5.4.1 Coronary angiography and patient characteristics

The heart transplantation population included in the current analysis comprised of consented patients for whom both coronary angiography data and corresponding plasma samples were available (Table 10). In the 40 patients available for the study, out of the three major coronary arteries assessed (LAD, left anterior descending; LCX, left circumflex; RCA, right coronary artery), the most severely stenosed (Max %DS) vessel was typically the LAD, in approximately two-thirds of the patients (n=26; 65%) (Table 11). The proteomic biomarkers (Table 12) were identified in the training cohort, which was selected based on the more 'extreme' phenotype according to the CAV definition employed in the current study.

In the training cohort, the Max %DS in the LAD vessel in the CAV subjects ranged from 41% to 70% and averaged around 53%, whereas the Non-CAV subjects ranged from 0% to 20% and averaged at 10% (Table 11; Figure 11). It is also interesting to note that in almost all Non-CAV subjects, all three major coronary arteries, i.e., LAD, LCX and RCA, were relatively clear compare to the CAV subjects, and generally did not show angiographic signs of moderate/severe coronary artery stenosis (Figure 11). Additional summarization of the coronary angiography data is provided in Table 11.

Table 10. Cardiac transplant patient demographics.

		Training Cohort Samples		Additional Samples
		CAV	Non-CAV	Possible CAV
		n = 10	*n* = 9	*n* = 21
Sex	Male, *n* (%)	9 (90%)	6 (67%)	16 (76%)
	Female, *n* (%)	1 (10%)	3 (33%)	5 (24%)
Age	Years, (mean ± SD)	54.5 ± 12.1	50.1 ± 13.6	52.2 ± 12.0
Ethnicity	Caucasian, *n*	9	7	19
	Asian, *n*	0	2	1
	Others, *n*	1	0	0
Primary Disease	Ischemic heart disease, *n*	3	3	8
	Non-ischemic cardiomyopathy, *n*	2	4	8
	Others*, *n*	5	2	5

*Others include cardiogenic shock, hypertrophic cardiomyopathy and unspecified cardiomyopathy. CAV, significant cardiac allograft vasculopathy (Max %DS ≥40%); Non-CAV, non-significant cardiac allograft vasculopathy (Max %DS ≤20%), Possible CAV (20%<Max %DS<40%); SD, standard deviation

Table 11. Summary of angiographically-assessed data regarding coronary artery stenosis.

Maximum percentage diameter stenoses (Max %DS) are shown for the three primary coronary arteries, i.e., left anterior descending (LAD), left circumflex (LCX), and right coronary artery (RCA)

		Training Cohort Samples		Additional Samples
		CAV	Non-CAV	Possible CAV
	Max %DS	***n* = 10**	***n* = 9**	***n* = 21**
LAD	mean ± SD	53 ± 11	10 ± 9	29 ± 5
	median (range)	51 (41-70)	14 (0-20)	29 (21-39)
LCX	mean ± SD	23 ± 9	14 ± 9	18 ± 12
	median (range)	20 (11-36)	15 (0-27)	15 (0-44)
RCA	mean ± SD	31 ± 21	17 ± 19	14 ± 13
	median (range)	34 (0-68)	16 (0-60)	16 (0-37)

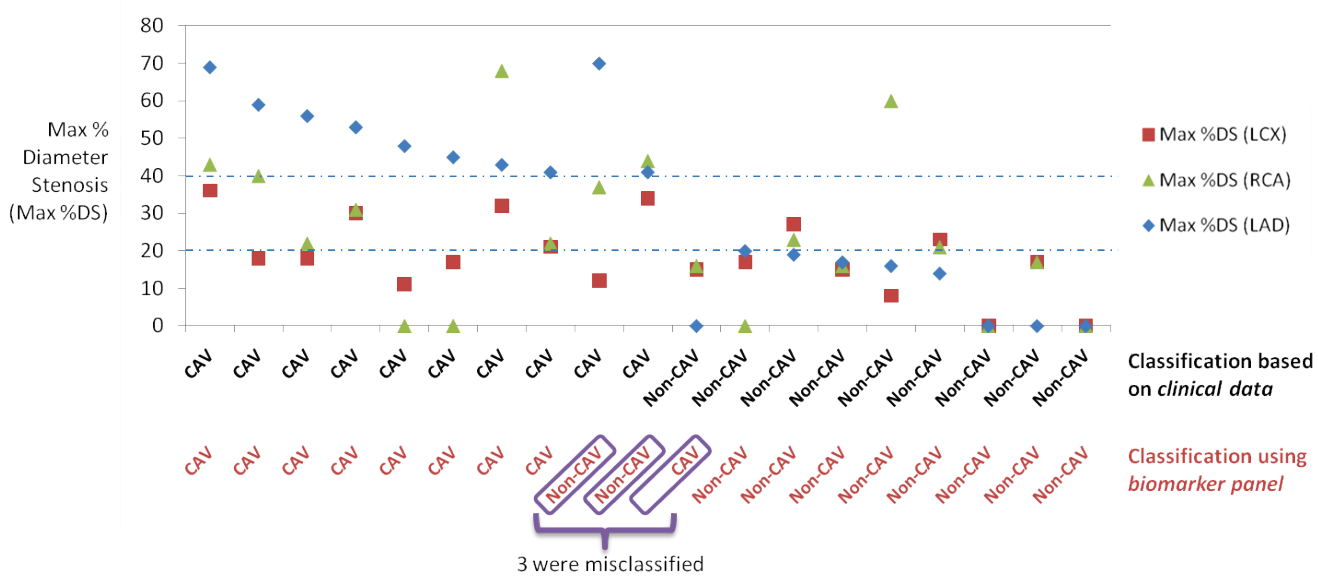

Figure 11. Classification performance of the CAV biomarker panel.

Each vertical row represents individually unique patients and their corresponding Max% DS values for the three major coronary arteries: left anterior descending (LAD; blue diamond), left circumflex (LCX; red square), and right coronary artery (RCA; green triangle). The blue horizontal dotted lines represent the cut-offs used for defining CAV (Max %DS ≥40%) and Non-CAV (Max %DS ≤20%). The classification of patients based on clinical data (shown below the x-axis in black), as discussed in manuscript, was based on the angiographically-assessed Max %DS in the left anterior descending (LAD) artery. Plasma sample-derived, proteomic data-based classifications using the biomarker panel are shown below the x-axis in red. 8 out of 10 CAV and 8 out of 9 Non-CAV patients were correctly classified, which corresponds to 80% sensitivity and 89% specificity.

5.4.2 Differentially expressed proteins and CAV biomarker panel

Approximately 2500 protein groups (PGs) were found in at least one of the 19 samples included in the training cohort. After prefiltering, a total of 131 PGs were detected in at least 75% of the 10 CAV and 9 Non-CAV samples and used for subsequent statistical analysis. Of the 131 PGs analyzed, 29 proteins with the most significant differential relative concentrations between the CAV and Non-CAV samples (p-value < 0.1) were considered for biomarker panel generation. After applying Elastic Net to these 29 proteins, an 18-protein biomarker classifier was generated (Table 12). Of the 18 candidate protein biomarkers, 7 were downregulated, while the rest were upregulated in CAV relative to Non-CAV subjects.

Table 12. Proteomic biomarker panel reflecting cardiac allograft vasculopathy.

PGC	Accession	Gene Symbol	Protein Name	FC
3	IPI00922744.1	C4B	Complement protein C4B frameshift mutant (Fragment)	2.19↑
	IPI00654875.1	C4B	Complement C4-B	
	IPI00032258.4	C4A	Complement C4-A	
	IPI00892547.1	LOC100294156	Complement component 4A	
	IPI00384419.4	TDRD9	tudor domain containing 9	
	IPI00418163.3	C4B	complement component 4B preproprotein	
	IPI00930091.1	-	147 kDa protein	
	IPI00887154.2	C4B	Complement component 4B	
	IPI00889723.2	C4A	C4A protein	
	IPI00892604.1	C4B;C4A	Complement component C4B	
	IPI00643525.1	C4A	Putative uncharacterized protein C4A	
	IPI00843913.3	LOC100292046	Complement component 4A	
	IPI00413613.4	TDRD9	Isoform 2 of Putative ATP-dependent RNA helicase TDRD9	
	IPI00935601.2	LOC100293534	similar to complement component 4B, partial	
	IPI00937598.2	LOC100294156	similar to C4A protein isoform 2	
	IPI00943009.1	TDRD9	103 kDa protein	
18	IPI00026314.1	GSN	Isoform 1 of gelsolin	1.23↓
	IPI00796316.4	GSN	cDNA FLJ53327, highly similar to gelsolin	
	IPI00647556.2	GSN	Gelsolin isoform c	
	IPI00646773.2	GSN	Isoform 2 of Gelsolin	
20	IPI00296608.6	C7	Complement component C7	1.16↓
42	IPI00760962.1	KANK2	Isoform 2 of KN motif and ankyrin repeat domain-containing protein 2	1.48↑
	IPI00021842.1	APOE	Apolipoprotein E	
	IPI00878953.1	APOE	MRNA for apolipoprotein E	
	IPI00013537.3	KANK2	cDNA FLJ20004 fis, clone ADKA02391	
	IPI00442843.1	HMGA1	CDNA FLJ26517 fis, clone KDN07769	
	IPI00470559.2	KANK2	Isoform 1 of KN motif and ankyrin repeat domain-containing protein 2	
	IPI00760928.1	KANK2	Isoform 3 of KN motif and ankyrin repeat domain-containing protein 2	
	IPI00879368.1	APOE	Apolipoprotein E	
	IPI00879456.1	APOE	25 kDa protein	
45	IPI00022426.1	AMBP	Protein AMBP	1.19↑
61	IPI00292946.1	SERPINA7	Thyroxine-binding globulin	1.14↓
68	IPI00940099.1	SHBG	Isoform 2 of sex hormone-binding globulin	1.25↓
	IPI00023019.1	SHBG	Isoform 1 of sex hormone-binding globulin	
	IPI00884913.2	SHBG	sex hormone-binding globulin isoform 4 precursor	
	IPI00929685.1	SHBG	sex hormone-binding globulin isoform 2 precursor	
	IPI00219583.3	SHBG	sex hormone-binding globulin isoform 3 precursor	
	IPI00884958.1	-	Sex hormone binding globulin (Fragment)	
	IPI00885112.2	SHBG	Sex hormone binding globulin	
72	IPI00020986.2	LUM	Lumican	1.26↓
	IPI00794403.1	LUM	23 kDa protein	
	IPI00796888.1	LUM	26 kDa protein	

PGC	Accession	Gene Symbol	Protein Name	FC
	IPI00884981.1	PZP	Isoform 2 of Pregnancy zone protein	
74	IPI00025426.2	PZP	Isoform 1 of Pregnancy zone protein	1.17↓
	IPI00748437.2	PZP	Putative uncharacterized protein PZP	
	IPI00787434.1	BANK1	Isoform 3 of B-cell scaffold protein with ankyrin repeats	
	IPI00010471.5	LCP1	Plastin-2	
	IPI00646259.1	LCP1	Lymphocyte cytosolic protein 1	
85	IPI00909658.1	LCP1	cDNA FLJ52759, highly similar to plastin-2	1.53↓
	IPI00179337.5	BANK1	Isoform 1 of B-cell scaffold protein with ankyrin repeats	
	IPI00787141.1	BANK1	Isoform 2 of B-cell scaffold protein with ankyrin repeats	
	IPI00895834.1	BANK1	Isoform 4 of B-cell scaffold protein with ankyrin repeats	
103	IPI00022394.2	C1QC	Complement C1q subcomponent subunit C	1.19↓
110	IPI00419744.4	FCN3	Isoform 2 of ficolin-3	1.22↑
	IPI00293925.2	FCN3	Isoform 1 of ficolin-3	
	IPI00657670.1	APOC3	Apolipoprotein C-III variant 1	
138	IPI00021857.1	APOC3	Apolipoprotein C-III	2↑
	IPI00657715.1	APOC3	Putative uncharacterized protein APOC3	
140	IPI00643948.2	C1QB	Complement component 1, q subcomponent, B chain	1.21↓
	IPI00477992.1	C1QB	complement component 1, q subcomponent, B chain precursor	
	IPI00294713.4	MASP2	Isoform 1 of mannan-binding lectin serine protease 2	
151	IPI00871597.1	MASP2	21 kDa protein	1.22↓
	IPI00306378.5	MASP2	Isoform 2 of mannan-binding lectin serine protease 2	
169	IPI00027482.1	SERPINA6	Corticosteroid-binding globulin	1.14↓
	IPI00914948.1	APOL1	apolipoprotein L1 isoform c precursor	
	IPI00186903.4	APOL1	Isoform 2 of apolipoprotein L1	
	IPI00514475.5	APOL1	Isoform 1 of apolipoprotein L1	
	IPI00877654.1	APOL1	Putative uncharacterized protein APOL1	
236	IPI00877765.1	APOL1	Apolipoprotein L, 1	1.53↑
	IPI00877915.1	APOL1	Apolipoprotein L, 1	
	IPI00878099.1	APOL1	Apolipoprotein L, 1	
	IPI00878633.1	APOL1	Putative uncharacterized protein APOL1	
	IPI00940501.1	APOL1	Putative uncharacterized protein APOL1 (Fragment)	
303	IPI00022731.1	APOC4	Apolipoprotein C-IV	1.42↑

PGC, protein group code; FC, fold-change. Fold change shown in red represents higher expression in CAV relative to Non-CAV, those shown in blue are higher in Non-CAV relative to CAV.

5.4.3 Principal component analysis of the proteomic CAV biomarker panel

The CAV biomarker panel was further examined using the Principal Component Analysis (PCA) plot. The PCA plot demonstrated a clear separation between the CAV and the Non-CAV phenotypes based on the joint contribution of the biomarker panel proteins (Figure 12). The possible CAV subjects were scattered throughout and was not clearly separated from the extreme phenotype groups based on the angiography measures *or* the molecular biomarkers identified in the study (Figure 12).

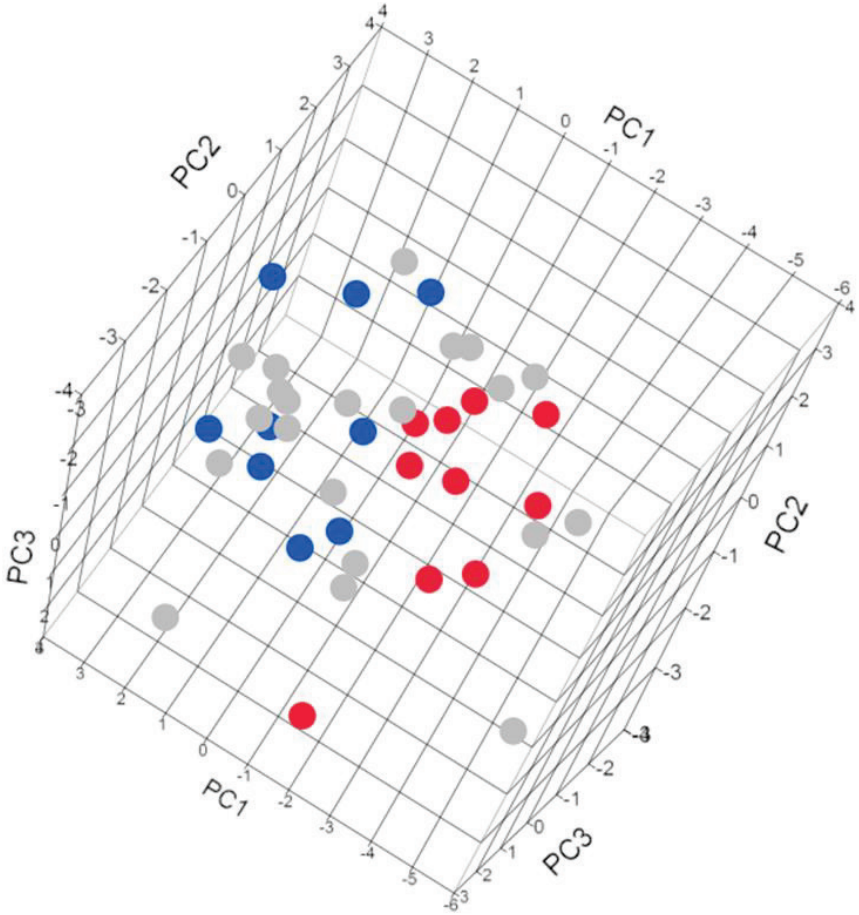

Figure 12. 3D scatter plot of the principal component analysis (PCA) results.

The PCA results was based on the 18 protein identified as part of the biomarker classifier. Each axis (x, y, z) represents the first 3 principal components (PC) illustrating the joint contribution of the biomarker panel proteins. Each dot represents individual subjects used in the analysis: CAV (shown in red), Non-CAV (blue), or possible CAV (grey).

5.4.4 Functional enrichment of the protein CAV biomarkers

Enrichment analysis was performed on all proteins in the biomarker panel/classifier, using Gene Ontology (GO)-based functional ontologies, to elucidate potential biological and molecular processes that are differentially regulated in CAV versus Non-CAV subjects. The top 10 significantly enriched GO terms (FDR<5%) are summarized in Figure 13.

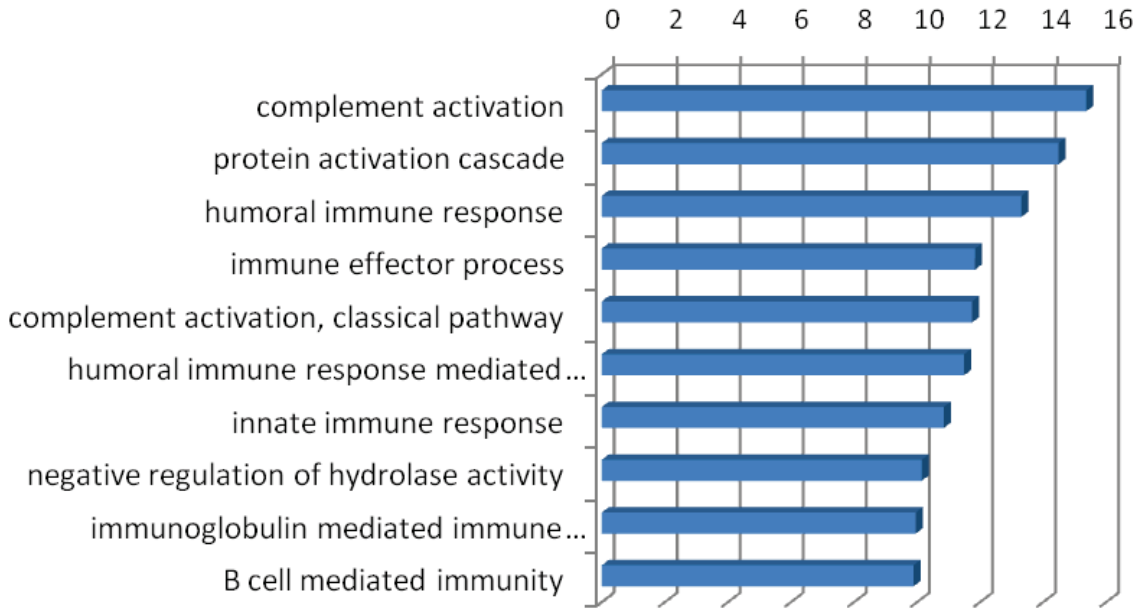

Figure 13. Top 10 Gene Ontology (GO) terms based on functional enrichment analysis of the proteins on the CAV biomarker panel generated.

The x-axis represents the statistical significance of each GO term: -log (P value).

5.4.5 Performance estimation of the of CAV biomarker panel

The clinical classification of test samples based on their plasma samples was performed using the Elastic Net classifiers built based on the training set in each fold of the cross-validation. Specifically, the biomarker classifier score was used to classify test patient samples from the cross-validation as either having significant CAV or not. Of the 19 samples tested (10 CAV and 9 Non-CAV left out one at a time in the cross-validation), 8 out of 10 CAV and 8 out of 9 Non-CAV patient samples were correctly classified. This result translates to a sensitivity of approximately 80% and a specificity of 89% (Figure 11) using a probability cut-off of 0.5 to classify samples as CAV. To further examine the performance of our biomarker panel, a receiver operating characteristic (ROC) curve was constructed based on the 18-protein biomarker panel/classifier, which demonstrated an area under the curve (AUC) of 0.94 as shown in Figure 14.

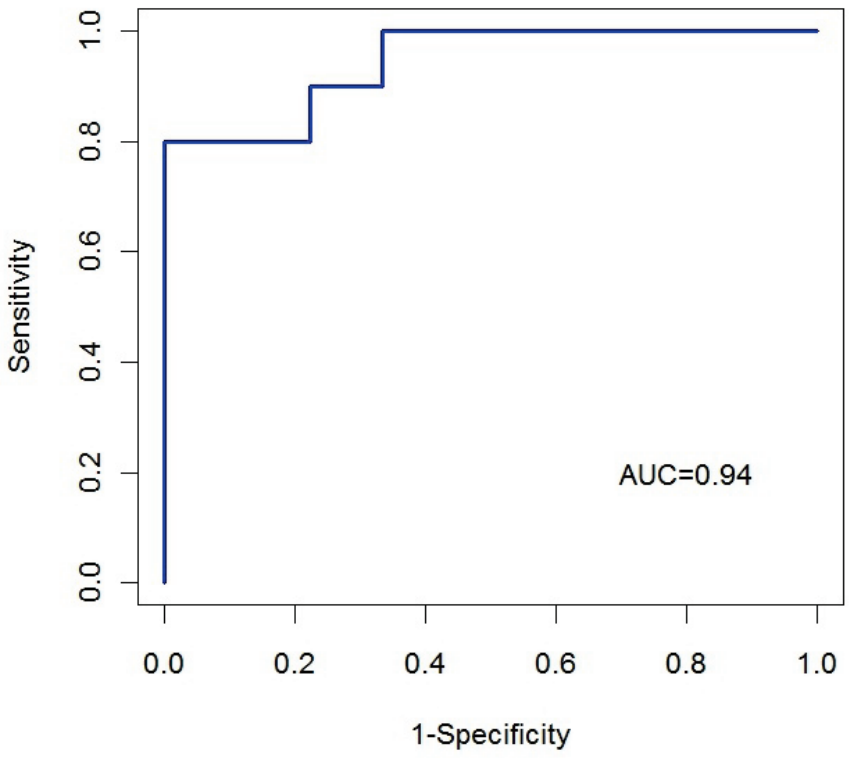

Figure 14. Receiver operating characteristic (ROC) curve for the CAV protein biomarker panel identified.

The ROC curve was constructed based on the probabilities of the test samples from each fold of the cross-validation. Area under the curve was 0.94.

5.5 Discussion

In the context of heart transplantation research, numerous efforts have been made to pinpoint biomarkers of acute cardiac allograft rejection. However, the potential utility of proteomics technology to assess coronary artery stenosis post-transplantation, a strong indicator of CAV development and an expression of chronic rejection, has yet to be examined. In this initial study, an 18-protein biomarker panel was identified using heart transplant patients' plasma samples that may help discriminate between patients with significant development of CAV and those without (Non-CAV).

5.5.1 Establishing definition of CAV for the study

The complexity of CAV has lead to a variety of criteria for defining the disease in a research setting. The coronary angiographic definition of CAV has ranged from coronary artery stenosis from as low as 30% to as high as 70%.[60] For the purposes of this study, I have focused initially on angiographically-measured stenosis in the LAD and used it as the criterion for defining CAV (Max %DS ≥ 40%). Indeed, stenosis of 40% or greater in the epicardial coronary artery, e.g., LAD, LCX and RCA, has been used to define the presence of definite CAV.[228] Further, it has been previously reported that detection of moderate or severe CAV with 40% or greater coronary artery stenosis was able to predict a mortality rate of more than 50% at 2 years post-transplantation.[60,229] By selecting patients with more 'extreme' phenotypes, i.e., Max % DS ≤20% or ≥40%, the first goal in the study, via the two-group analysis, was to first discover proteins that are significantly up- or down-regulated in the CAV *versus* Non-CAV groups. Once these proteins have been pinpointed, the next goal was to uncover the best combination of proteins from this list of candidates, and identify a biomarker panel/classifier that can most effectively differentiate between the two groups.

5.5.2 Integration of biological information and interpretation

Through examination of proteins that are differentially abundant between the CAV and Non-CAV subjects, I was also able to gain insight, using functional enrichment analysis, into potentially significant biological and molecular processes that may be implicated in CAV

development (Figure 13). While the functional enrichment results largely point towards immune-mediated processes, further investigation of the biomarkers identified using existing literature suggest a more complex picture (Figure 15).

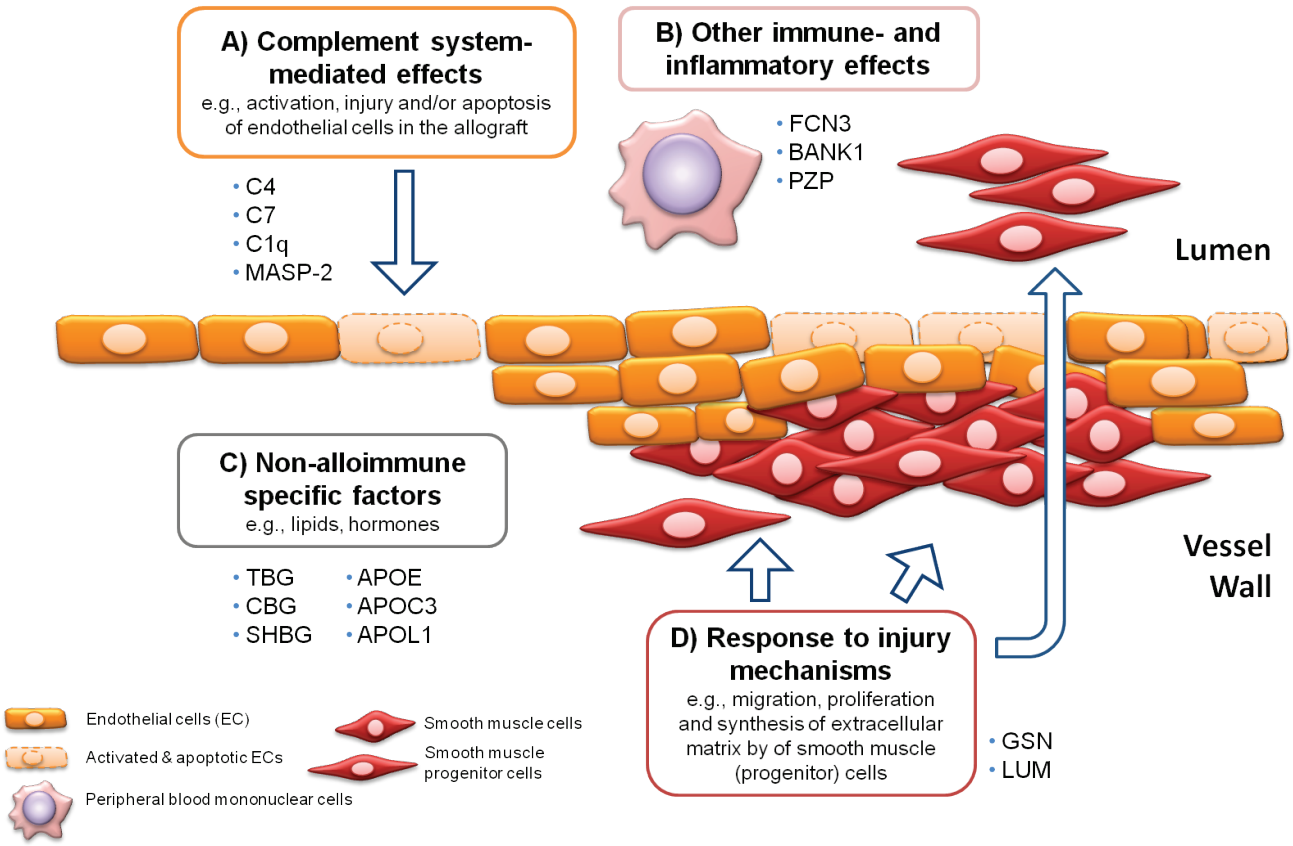

Figure 15. Potential implications of biomarkers identified in the context of cardiac allograft vasculopathy (CAV).

The figure provides a simplified view of the cardiac allograft arterial wall. Majority of biomarkers from the biomarker panel have been linked to either immune-mediated factors thought to contribute to the development of CAV, such as **A)** complement-system mediated reactions and **B)** other immune- and inflammatory effects, or **C)** non-immune related factors, and **D)** mechanisms involved in response to injury.

Recent studies have underscored the complex pathophysiology underlying chronic rejection and CAV, which has been described as a low-grade chronic inflammatory condition.[28,45] Although there is currently no consensus on the exact pathogenesis of CAV, it is thought that several immune- and non-immune components are involved.

5.5.2.1 Complement system-mediated effects

The potential roles of antibody and complement in the context of CAV has been previously suggested.[230] As indicated in Table 3, numerous protein biomarkers belonging to the panel are associated with the complement system activation and regulation. C1q and MASP-2 molecules are known to be involved in the initiation of classical and lectin pathways, respectively.[230-232] Activation of these pathways can lead to the cleavage of complement component C4. As observed in this study, fragments of C4 activation/cleavage, e.g., C4a and C4b, were significantly higher in the CAV *versus* Non-CAV subjects. In light of the apparent C4 cleavage fragment generation, the observation of the downregulation of C1q, MASP-2 and C7 in the CAV samples (relative to Non-CAV) would appear contradictory. However, it is possible that this observation may be indicative of increased localization from the peripheral blood effectors to the targeted cells in the cardiac allograft and expenditure/utilization of these molecules in binding complexes, or a possible negative feedback mechanism to limit the level of complement activation and associated inflammation, or both.

5.5.2.2 Other immune- and inflammatory effects

A number of biomarkers identified in this study, although relatively unknown in the context of CAV, have been associated with immune function regulation and other inflammatory conditions. FCN3 protein, which was upregulated in CAV vs. Non-CAV, is considered a collagen-like molecule and is believed to play a role in the innate immune system.[233] BANK1, which was downregulated in CAV relative to Non-CAV, is a scaffold protein that is mainly expressed in B cell.[234] The role of B cells and their production of alloantibodies in CAV remain controversial at this point.[28] PZP was originally detected as a pregnancy-associated protein but is now known to be present in both male and female.[235] PZP, which has been suggested as a link between innate and adaptive immunity,[236] has demonstrated immunosuppressive effect and prolonged allograft

survival in mouse model of heart transplantation.[237] PZP was upregulated in the Non-CAV relative to the CAV subjects in this study.

5.5.2.3 Non-alloimmune specific factors, e.g., lipid and hormone transport

Several biomarkers from the panel involved in hormone and molecule transport, including SERPINA7 (or thyroxine-binding globulin; TBG), SHBG, and SERPINA 6 (corticosteroid-binding globulin; CBG), were all downregulated in CAV relative to Non-CAV subjects. In the context of inflammatory reactions, decrease in plasma TBG and serum CBG concentration has been observed.[238-240] SHBG is thought to influence conventional cardiovascular risk factors through regulation of hormone availability but its role in inflammation and heart transplantation remains controversial. AMBP, which was found to be upregulated in CAV relative to Non-CAV subjects, has been previously recognized as a biomarker of acute rejection in liver transplant patients.[241]

Several apolipoproteins were also identified as part of the CAV biomarker panel (all higher in CAV relative to Non-CAV). Hyper- and dyslipidemia are thought to be contributing factors of CAV.[45] ApoE has been implicated in both allograft vasculopathy as well as atherosclerosis.[40] Both ApoC-III and Apo L-1 have been suggested as protein biomarkers of cardiovascular disease and stroke.[242] On the other hand, the role of ApoC-IV in cardiovascular diseases is relatively unknown.

5.5.2.4 Response to injury mechanisms

A key feature in the development of CAV, according to the 'response to injury' paradigm, is the vascular remodeling process.[40,45] This process typically involves the initial injury and apoptosis of endothelial and parenchymal cells, and the subsequent migration and proliferation of smooth muscle (progenitor) cells, with eventual intimal thickening and vessel occlusion in the cardiac allograft.[40,45] Gelsolin (GSN) is an actin-binding protein that has been shown to have both pro- and anti-apoptotic activity, and has been suggested to participate in cardiac remodeling, albeit in the context of post-myocardial infarction repair.[243-245] Lower GSN was observed in CAV relative to Non-CAV (Table 12). Intriguingly, plasma GSN has also been described as a potential protector against patient's own inflammatory response. [246] Increased presence of circulating smooth muscle progenitor cells (SPCs) has been correlated with severity

of CAV.[247] Relative to differentiated smooth muscle cells (SMC), expressions of lumican (LUM), a proteoglycans and a matrix protein, is downregulated in SPCs *in vitro.*[248] Plasma lumican level was downregulated in CAV relative to Non-CAV subjects.

The diversity of biomarkers identified as part of the panel/classifier further highlights the complexity behind the biology and pathology of CAV. Although many of these biomarkers are biological plausible in the context of CAV, clearly additional *in vitro* and *in vivo* work would be required to follow-up on the significance of individual biomarkers, i.e., whether they are contributing factors, effectors, or by-products of the pathology, as well as how differential biomarker regulation may fit together as part of the larger puzzle.

5.5.3 Evaluation of the CAV biomarker panel performance

The biomarker panel for distinguishing CAV versus Non-CAV consists of 18 PGs. As shown in

Figure 11, classification of patients' blood samples using the proteomic biomarker panel consistently matched those made based on clinically measured angiographic data in the cross-validation test. Of the 10 CAV and 9 Non-CAV samples tested, 8 and 9 were correctly classified, respectively. This corresponds to approximately 80% sensitivity and 89% specificity.

The initial results from this study suggest that the current biomarker panel may be useful in differentiating extreme CAV phenotypes, while it remains to be seen the impact on 'possible' CAV subjects. As noted in the PCA plot (Figure 12), there was a clear separation between the CAV and the Non-CAV phenotypes based on the joint contribution of the biomarker panel proteins (Figure 12). However, as anticipated, the possible CAV subjects were scattered throughout and were not clearly separated from the extreme phenotype groups (not only based on the angiography measures, but also based on the identified molecular biomarkers). I believe this observation further underscores the widely known difficulty in stratifying CAV patients in both clinical and research setting.

Nevertheless, the biomarker panel described in this proof-of-concept work has demonstrated the potential to help guide the use of coronary angiography. As shown in Figure 14, the AUC for the CAV biomarker panel's ROC curve is 0.94. In a clinical setting, the proteomic

panel score cut-off would be optimized to achieve the desirable performance characteristics, e.g., using the test as a screening tool to avoid potentially unnecessary angiographies by maximizing the specificity and minimize the number of false positives, or vice versa.

5.5.4 Potential applications, caveats to the study, and future directions

Although these initial results are encouraging, several limitations and caveats of this study warrant mention. First, the patients with either CAV or Non-CAV available for the study were predominantly male, and given the current sample size, an internal cross-validation was performed to ensure the robustness of the analysis. The ROC curve and correlation analysis provided promising results; however, a larger, more gender-balanced, external validation cohort would be desirable for follow-up studies. Second, I have initially focused on the LAD vessel as it is the most severely stenosed vessel in majority of patients in the subject cohort (n = 26; 65%), and previous studies have used comparable cut-off criteria for defining CAV. It will be of interest in the future to examine a full spectrum of CAV patients, including those who have relatively clear LAD vessels but stenosed RCA and/or LCX. Lastly, the inherent error associated with coronary angiography cannot be excluded, even when assessed by digital techniques under the blinded supervision of an expert in this arena. Although more sensitive techniques such as intravascular ultrasound (IVUS) are available, albeit not as universally implemented and data less frequently collected, future studies may also benefit from the use of IVUS-defined CAV patient cohorts.

Ultimately, the results presented in this chapter provide evidence in support of the potential utility of proteomic-based biomarker panels in the context of post-cardiac transplantation monitoring and screening for CAV. Specifically, the blood-based biomarkers identified in this study may provide an alternative, minimally invasive mean to determine patients who have significant, angiographically-detectable stenosis in their cardiac allograft arteries, and differentiate them from those without significant CAV. It may also be of interest in future studies to explore whether proteomic-based biomarker panel, such as the one described here, can be further refined and tailored to not only stratify patients between different classes, but also to reflect *specific* clinical values, e.g., %DS, that may otherwise require invasive techniques based on current routine techniques.

CHAPTER 6: Conclusion

6.1 Closing remarks

The work presented in this dissertation describes the discovery and examination of biomarkers, using genomics and proteomics technologies, to study three key stages and events that are of interest in the context of pre- and post- heart transplantation – ESHF, AR and CAV. Through the course of these projects, I also evaluated the potential utility of the biomarkers identified from a clinical point of view, and investigated their plausibility and implications from a pathobiological perspective.

In the current literature, both the genomics and proteomics technology have been suggested as powerful new tools for searching clinically useful biomarker of cardiovascular conditions;[36,114,242] however, there has been a limited number of studies available, particularly in the context of cardiac transplantation research. Further, more evidence is needed to fully realize the potential and possible pitfalls associated with these high-throughput technologies, especially if the ultimate goal is to use the data from 'omics'-based research in creating a clinically useful diagnostic or monitoring tool.

In the first part of this dissertation, I explored the molecular profiles of heart failure patients just prior to receiving cardiac transplantation. While some studies have reported differences in genomic signatures, albeit derived from cardiac tissues, between HF of different etiologies,[128] others have had less success in similar efforts.[131] The major finding in the work presented here was that there was little evidence of a significant difference between end-stage IHD and NICM subjects' 'omic' profiles. This result was consistent with a theory which has been proposed in the literature, which suggested that despite the difference in underlying etiology of HF, progression to advanced/end-stage heart failure (ESHF) may be dominated by a final common pathway.[123,130,131] It is important to note however, that this work suggested a convergence of 'omic' profiles in the *peripheral* blood, and this does not necessarily represent convergence of events in the *myocardial* tissue. Nevertheless, this study brings evidence to suggest that gene expression changes (or lack thereof) in peripheral blood may be useful in helping to reflect myocardial events, as previously shown in the context of cardiac transplantation.[136-139] In contrast, when patients with ESHF of ischemic and non-ischemic origin were grouped as one and compared to control subjects with normal cardiac function, there was

a relatively greater number differentially expressed genes and proteins between the two groups, suggesting a significant disturbance in the 'omics' profile of CHF patients in general.

While I have highlighted the potential pathways and mechanisms suggested by the differentially expressed biomarker identified, more work is required in the future studies to further examine how the different pieces of the 'omic' puzzle fit together. One of the many challenges associated with 'omics' based work is the integration of data when they are generated from different platforms. These challenges are sometimes made even more difficult given that low to moderate correlation between genomic and proteomic changes is not surprising and has been reported in previous studies,[249-252] even in experiments where single sample source is used, e.g., breast cancer[251] and yeast cell lines,[250] as well as human liver.[249]

Beyond genomics and proteomics, emerging approaches in the sphere of cardiovascular research such as metabolomics may serve as great complementary technology to existing 'omics' platforms.[253,254] Currently, metabolomics is a minimally explored area, and the interpretation and integration of metabolites, in the context of molecular biology dogma (i.e., relationship between DNA, RNA and protein), is made even more complex by the involvement of peptidic and nonpeptidic metabolites. However, with the recent initiation of the human serum metabolome study,[255] metabolomics holds immense possibilities as a powerful tool that is certainly worth exploring in future studies.

In the next chapter of my dissertation, I transitioned my focus to the post-transplantation phase, and first focused on the discovery and examination of whole-blood based biomarkers in the context of acute cardiac allograft rejection. In recent years, the use of microarrays to discover differentially expressed genomic biomarkers to develop molecular signature assays for clinical use has been gaining increasing amount of attention. In the field of cardiac transplantation, the potential use of peripheral blood gene expression profiles to diagnose acute cardiac rejection has been suggested through studies such as the CARGO trial, one of the most recognized studies in this regard to date.[115]

One factor which contributed to the success of projects such as CARGO may be in part due to the fact that acute rejection episodes is a an immune process that involves a number of critical leukocyte-mediated events (i.e., recognition of alloantigen on the allograft, release of

effector molecules, initiation of the inflammatory response, and activation/recruitment of circulating immune cells). As such, peripheral blood (mononuclear cells) [PBMC] has been considered a logical and biologically plausible surrogate tissue source to detect potential biomarkers of interest that can help indicate the underlying immune or inflammatory response.[137,158-164,256] Further, there is also growing evidence that gene expression profiles can be closely correlated with biopsy-proven acute cardiac allograft rejection.[115,137,139] The results presented in chapter 4 of this dissertation were consistent with these views. When the whole blood samples from AR and NR patients were compared, a large number of genes were significantly differentially expressed between the two groups. Many of the genes that were upregulated in the AR population are linked to both innate/humoral immune responses, where as others indicate response to wounding and acute inflammatory process. Of the potential candidates, 12 genes were further identified together as the best combination of biomarkers (i.e., biomarker panel/classifier) that was able to effectively discriminate between the AR and NR samples on the basis of whole-blood gene expression profiles. As noted earlier in the chapter 4 discussion, a number of these biomarkers (e.g., TFR2, KLF4 and BID) have been specifically tied to the regulation (i.e., activation, differentiation, proliferation) of cells such as T cells, B cells, and macrophages, based on results from *in vitro* studies published in the literature. These immune cell types are known contributors in the acute rejection process and are typically observed as part of the inflammatory infiltrate population in EMBs of ISHLT grade 2R or above rejection episodes. [47,51]

While the acute rejection biomarker data observed is contextually consistent with the current understanding of acute (cellular) rejection, and the characteristics of the biomarkers identified appear to be biologically plausible in an immune-driven pathological setting, the potential clinical utility of the biomarker panel/classifier itself remains primarily with its performance. Based on internal validation, the sensitivity and specificity of the biomarker panel were estimated to be approximately 83% and 100%, respectively. However, there are several factors to consider when discussing the potential application of such findings.

First, the sensitivity and specificity of EMB (the current gold standard for the diagnosis of acute cardiac allograft rejection) has been reported to vary between approximately 75-90% and 80-90+%, respectively.[219-221] In order to 'replace' the current gold standard, one would

expect the biomarker panel to perform better, if not at least just as well, relative to EMB. That said, a biomarker panel doesn't necessarily need to *outperform* the current gold standard for it to be useful. Beyond the economic considerations, a biomarker panel that is not inferior compared to the existing diagnostic (in terms of sensitivity or specificity) but has the advantage of being less-invasive can also help improve patients' quality of life.[257]

Second, the AR biomarker panel identified in this dissertation may also be applied as a complementary, pre-screening tool, similar to the *Allomap* test,[257,258] to help determine which patients really need the EMB. Finally, an important consideration in assessing the application of the biomarker panel described in this dissertation is the technology used. In chapter 4, the expression levels of the AR classifier genes were also assessed using an alternative technological platform, i.e., qPCR. This in turn, helped test the potential utility and transition to using qPCR as a possible clinical assay, given that qPCR is currently more readily available in clinical laboratories; in some cases they may already be validated for clinical diagnostic tests.[178,216] While most of the genes were consistent in the direction of regulation of AR relative to NR between the microarray and the qPCR platforms, it is important to acknowledge that differences were also observed. These differences, beyond the potential contributing factors discussed earlier in chapter 4, warrant additional work in future studies to bring the results to existing platforms used in the clinic. The change between discovery and clinically used platforms is one of the many known challenges associated with the translation of biomarker findings from bench to bedside.[259-262] One strategy that has been proposed in the literature to help expedite this transition is the use of custom arrays – ideally ones that carries the identical gene-detecting probes as the microarray used for biomarker discovery.[260] However, this approach is still limited by the fact that microarray is not yet widely implemented in clinical diagnostic laboratories.

Nevertheless, the initial AR biomarker results presented in this dissertation suggest that peripheral blood-derived genomic biomarkers hold considerable potential in distinguishing acute rejection from non-rejection in heart transplant recipients.

In the last portion of my thesis, I focused on another major hurdle faced by cardiac allograft recipients during the post-transplantation phase – CAV as an expression of chronic

rejection. Although the pathobiology behind CAV is an area of great interest for many research groups and its complexity is increasingly recognized, the use of proteomics technology to study CAV in a holistic approach remains a minimally explored territory. Specifically, proteomic biomarkers have yet to be examined in the context of detecting allograft coronary artery stenosis as a strong indicator of CAV development and expression of chronic rejection.

The prevailing view in the literature is that both immunologic and non-immunologic contributing factors and mechanisms are involved in the pathogenesis of CAV.[40,45,67,68] In chapter 5, the protein biomarker that were differentially expressed between the patients with and without significant CAV were in line with the current concepts. Intriguingly, the potential involvement of the complement system was highlighted by the biomarkers that were part of the CAV panel/classifier. While the activation and deposition of complement factors have been suggested to participate in the development of CAV through their interaction with cells within in the allograft (e.g., vascular endothelial and smooth muscles, as well as fibroblasts),[45,230] the results from chapter 5 represent the first time this perspective was supported using evidence derived from a blood-based proteomics approach. Another interesting finding arising from results in chapter 5 is that the differentially expressed biomarker identified also suggested the significance of non-immunologic components and metabolic variables, such as lipids and hormones, in the context of CAV. Collectively, these results further highlight the complexity underlying the CAV pathogenesis.

Another significance of the work in chapter 5 is testing the potential utility of blood-based molecular signature in diagnosis of CAV. In the context of acute rejection, which is considered primarily an intense, cell-mediated process, peripheral blood (mononuclear cells) [PBMC] is a logical surrogate tissue source to detect biomarkers with diagnostic values. In contrast, it is not clear whether such biomarkers exit and whether they can also be identified in the peripheral blood of patients with CAV, which is often described as a low grade chronic inflammatory response and is characterized by occlusion of the allograft coronary arteries. The initial results in this work however, suggest that biomarker panel/classifier identified on the basis of blood-derived proteomics data may be useful in classifying and differentiating patients with significant CAV (i.e., angiographically significant coronary stenosis in the cardiac allograft)

from those without; the CAV biomarker panel/classifier demonstrated a sensitivity and specificity of approximately 80% and 89%, respectively.

The potential clinical application of the CAV biomarker panel/classifier is similar to that of the AR rejection panel, in the sense that it may also serve as a complementary tool to the existing gold standards (coronary angiography in the case of CAV monitoring) that are unfortunately more invasive. It is important to recognize however, that this work has initially, as a proof-of-concept, focused on the coronary stenosis in the LAD vessel as it is the most severely stenosed vessel in majority of the patients. While previous studies in the literature have used comparable criteria for defining CAV, it will be of interest in the future to examine a wider spectrum of CAV patients, including those who have relatively clear LAD vessels but stenosed RCA and/or LCX.

Other limitations shared between the studies presented throughout this dissertation include the size of the patient population, as well as the selection of specific phenotypes for comparison. Ideally, larger patient cohorts would be desirable. Further, in the current work, patients of 'extreme' phenotypes were compared in part to elucidate the most dramatic molecular profiles differences and differentially expressed genes and/or proteins that are potentially reflecting the underlying mechanisms. For this reason, another limitation of the biomarker panels discussed in the work described is that they are initially designed to discriminate the AR (ISHLT grade≥2R) from the NR (ISHLT grade=0R), and differentiate significant CAV (Max %DS≥ 40%) from the non-significant CAV (Max %DS ≤20%) patients. As indicated in chapter 5, the PCA plot result highlights the difficulty both from a clinical and research perspective in dealing with the intermediate/'possible' patients. While currently not the focus of this thesis, it will be of great interest in future studies to include these intermediate patients to further examine the nuances of the AR and CAV molecular profiles described.

6.2 Future opportunities

Taken together, the results shown in this dissertation also open a number of possibilities for future investigations. For instance, drawing parallel to the ESHF study results, is there a 'point no return' or final common pathway for the development of CAV? The current

understanding is that CAV is largely an irreversible process.[263] Moreover, it has also been suggested that the occurrence of acute rejection episodes may predispose patients to develop CAV, possibly through initial damage and dysfunction of the endothelium.[264,265] While this correlation was not clear in the cohorts examined in this dissertation – in part due to the relatively small samples size – future studies may warrant larger patient cohort, with longer follow-up time. Importantly, serial samples from the same patients will need to be analyzed to determine how and if each occurrence of acute cardiac rejection can affect the peripheral blood expression profile, and whether the post-acute rejection expression profile eventually become more similar to those patients who have developed significant CAV.

Another interesting area worth investigating is the effects of immunosuppressive therapies on the AR and CAV biomarkers/molecular expression profiles. As an example, in the case of CAV, currently the only definitive treatment for patients is retransplantation,[45,55] but recent preliminary studies have suggested that drugs such as sirolimus (rapamycin) may have suppressive effects on the development of CAV.[266,267] However, it is not clear whether these observations are solely due to the decreased incidence of acute rejection episodes (and thus decreased damage to the endothelium), or due to direct anti-proliferative effects of the immunosuppressant on allograft vascular endothelial cells, smooth muscle cells, and/or fibroblasts. Through serial sample studies such as the one proposed earlier and taking specific medications into consideration, it may be possible to detect significant changes in either genomic or proteomic profiles in cardiac transplant patients post-treatment. Depending on the changes observed and the characteristics of the biomarker impacted, this may provide clues as to why certain medications are more successful than others in suppressing CAV development. This in turn, can also help shed light on the pathological mechanisms of CAV.

In summary, the contents in this dissertation provided data to suggest that peripheral blood-derived biomarkers can help provide the means for sensitive and specific diagnosis of acute and chronic cardiac allograft rejection/CAV (summarized in Figure 16). In particular, the evidence presented here also demonstrate, for the first time, the potential utility of blood-based protein biomarkers in diagnosis of CAV, i.e., presence of angiographically significant coronary stenosis in the cardiac allograft. Importantly, through comparison of molecular profiles between patients with extreme phenotypes, i.e., AR *versus* NR and significant CAV

versus non-significant CAV, a number of previously unrecognized gene and protein biomarkers in the context of AR and CAV were also identified. These biomarkers, based on preliminary analysis and put in context of existing literature, not only reinforce our current understanding of the pathological features and mechanisms involved, but also provide foundational framework for future studies to further examine their precise biological involvement in acute and chronic rejection.

Ultimately, these biomarkers represent another step forward in 'omics' research that may one day lead to identification of novel therapeutic targets but perhaps more importantly, development of clinical tools to help better manage cardiac transplant patients.

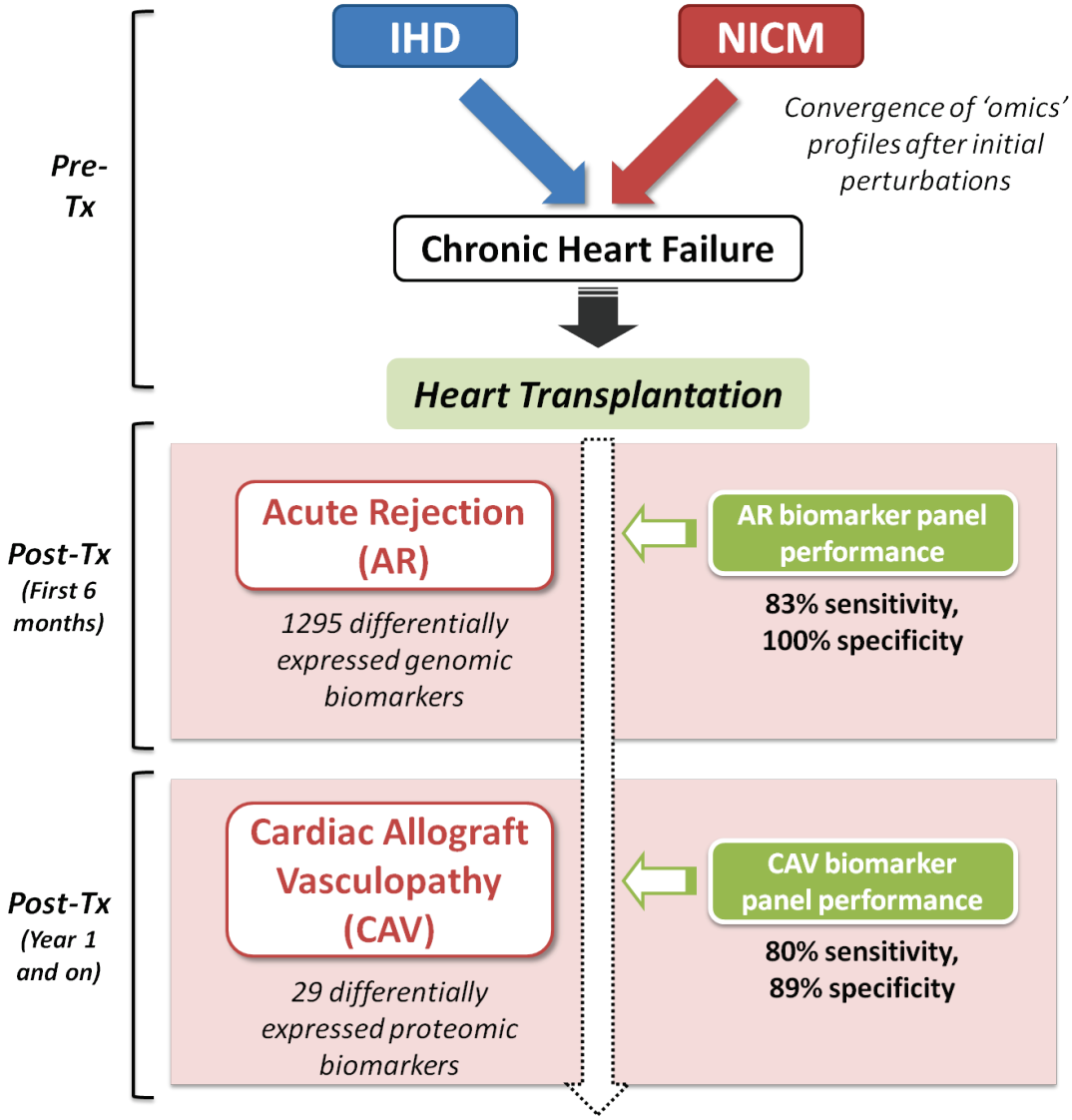

Figure 16. Summary of potential utility of AR and CAV biomarkers identified

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Appendices – Assay and 'omics' methodologies

Appendix A - Genomics technology – Affymetrix GeneChip® microarray

Microarray is a technology which allows the detection and measurement of multiple genes and their expression levels simultaneously. One example of the microarray platform is the Affymetrix GeneChip® Human Genome (HG) U133 Plus 2.0 array (Agilent, CA, USA). Each U133 Plus 2.0 array contains multiple oligonucleotide probes, which are 25-mer nucleotides that hybridize to specific RNA target with the correct complementary sequence.[268-271]

A collection of probes, e.g., 11-20, make up what is called a 'probe set'; probe sets are used to interrogate and measure the expression level of different individual genes.[268-271] On the Affymetrix GeneChip® Human Genome U133 Plus 2.0 microarrays, there are more 54,000 probe sets which corresponds to over 30,000 genes.[272]

The RNA targets used in a Affymetrix GeneChip® array analysis are those generated from the sample of interest. More specifically, total RNA from the sample is processed via reverse-transcription-*in vitro* transcription (RT-IVT) to synthesize amplified antisense cRNA, which is biotin labeled and then fragmented.[268-271] The biotinylated cRNA fragments are loaded onto the array (one sample per array) and analyzed.[268-271] Hybridization occurs between specific oligonucleotide probes on the array and biotinylated cRNA fragment targets with the correct complementary sequence.[268-271] The array is then washed and stained with streptavidin-phycoerythrin (SAPE), i.e., via addition of biotinylated anti-streptavidin antibody (which binds to the hybridized biotinylated cRNA fragments) and more SAPE (which binds to biotin).[268-271]

A confocal laser scanner is used to excite the fluorochrome, i.e., phycoerythrin, and capture the fluorescent emission signals, including their intensities and positions.[268-271] These signals, which are the basis of a microarray results, are summarized by software into values at a probe set level, allowing for further statistical analysis.[268-271]

Appendix B - Proteomics technology – Mass spectrometry and iTRAQ proteomics

Mass spectrometry (MS)-based proteomics, such as those involving isobaric tags for absolute and relative quantitation (iTRAQ^{TM}) of proteins, has been gaining increasing amount of attention in biomarker research. In principle, mass spectrometry relies on the measurement of mass-to-charge ratio (m/z) of ionized analyte molecules.[273-275] Results from a MS analysis typically include a mass spectrum (a plot with signal intensity of ion fragments on y-axis and m/z on x-axis), which is then computationally assessed to help identify and quantify the analytes present in the sample analyzed.[273-275]

In MS, first, the molecules within the biological sample of interest (e.g., human plasma) undergo volatilization and ionization.[273-275] An example of ionization method, i.e., converting molecules and atoms into ions, is called matrix-assisted laser desorption/ionization (MALDI). The ionization source is coupled with a mass analyzer, e.g. MALDI-MS, where the ions are sorted and detected.[273-275] One example of this is the time-of-flight (TOF) mass analyzer, which relies on the principle that for ions with the same charge and same amount of kinetic energy, the heavier ones travel slower.[273-275] Sometimes two mass analyzers are placed in tandem, e.g., TOF/TOF. In this setup, the first TOF is used to select (precursor) ions of a particular m/z ratio, which then enters and becomes fragmented in a collision cells that connects the two mass analyzers.[273-275] The fragmented ions are analyzed by the second TOF. Tandem mass spectrometry such as (MALDI)-TOF/TOF offers high selectivity, sensitivity and resolution, characteristics which are useful in analyzing biological samples.[273-275] It is also possible to use liquid chromatography (LC) to separate a given biological sample into different fractions (e.g., based on charge via ion-exchange chromatography or based on total hydrophobicity via reverse-phase chromatography), prior to analysis with tandem mass spectrometry, e.g., MALDI-TOF/TOF.[273-275]

The use of iTRAQ^{TM} labeling has allowed higher analytical throughput and multiplexing of biological samples in quantitative, MS-based proteomics. The principle behind iTRAQ^{TM} involves the use of isobaric tags, which consist of unique combination of report and balancer groups. The reporter group, which can have a mass of 114, 115, 116, or 117 Da (in a 4-plex

setup), is coupled with a balancer group with a mass of 31, 30, 29, or 28 Da, respectively. This ensures the tags are of the same total molecular weight (isobaric) at 145 Da.[276-278]

As an example, in a multiplex experiment involving four plasma samples, each sample is processed and the peptides within are labeled with one of the four unique iTRAQ™ tags, i.e., tags containing reporter 114, 115, 116 or 117. The labeled samples are combined into one sample mixture and analyzed. During the tandem mass (MS/MS) spectrometry analysis, the fragmentation process gives rises to unique reporter ions, with m/z of 114, 115, 116 or 117. By examining the intensity of these reporter ions as part of the MS/MS fragmentation spectrum, one can simultaneously identify and quantify the relative abundance of specific peptides present in the biological samples.[276-278] Software programs are also available, e.g., ProteinPilot™, which help summarize and interpret the peptide data, and provide the results at a protein level.[276-278] Additionally, given the nature of iTRAQ multiplex experiments, statistical tools, such as the Protein Group Code Algorithm (developed in-house by Dr. Gabriela Cohen Freue from the Biomarker in Transplantation initiative), have also been developed to help link proteins identified across different iTRAQ experiments as protein 'groups' (PG) for comparison and analysis purposes.