Chronic Heart Failure: The Roles of the Tumor Necrosis Factor-Alpha Axis and Left-Atrial Structural and Functional Remodeling

by

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ABSTRACT

Heart failure (HF) is a global epidemic, with a substantial burden on healthcare systems. Effective therapies have emerged for patients suffering from HF with reduced ejection fraction (HFREF), but not for those with preserved ejection fraction (HFPEF). Recently, systemic inflammation, driven by comorbid conditions, was attributed a larger role in the pathogenesis of HFPEF than HFREF. On the other hand, the role of the left atrium (LA) in HF remains unclear. Herein, the roles of inflammatory dysregulation and cardiac remodeling are explored in HF. The results presented suggest that dysregulation of the tumor necrosis factor-alpha axis may be a primary mediator of disease manifestations in HFPEF. The results show the LA plays variable role in in HF, whereby some patients rely on LA contraction for sufficient cardiac output. Altogether these findings contribute to a growing body of knowledge about the roles of inflammation and the LA in HF.

PREFACE

This thesis is an original work by Brendan Nicholas Bridge Putko. The Alberta Heart Failure Etiology and Analysis Research Team (HEART) project, to which the research presented in this thesis contributes, was a case-control study on chronic heart failure that followed human research subjects. The Alberta HEART project received Health Research Ethics Board approval at the University of Alberta (Pro00007105; October 9, 2009) and at the University of Calgary (22657; October 1, 2009), where data were collected. The collaborators for this project were the Alberta HEART Investigators led by Drs. Jason R.B. Dyck and Todd Anderson.

The research presented in Chapter 3 is a version of a publication on behalf of the Alberta HEART team of investigators: B.N. Putko, Z. Wang, J. Lo, T. Anderson, H. Becher, J.R. Dyck, Z. Kassiri and G.Y. Oudit "Circulating levels of tumor necrosis factor-alpha receptor 2 are increased in heart failure with preserved ejection fraction relative to heart failure with reduced ejection fraction: evidence for a divergence in pathophysiology," *PLoS One* vol. 9, issue 6, e99495. I was responsible for data collection, biochemical assays, statistical analysis, manuscript preparation and figure preparation as the primary author.

The technique employed for analyses of left-atrial volumes in Chapter 4 made use of a customized program for MATLAB software that was developed by colleagues: Mr. Kelvin Chow and Drs. Joseph Pagano, Richard Thompson and D. Ian Paterson from the University of Alberta. Drs. Thompson and Paterson are members of the Alberta HEART team of investigators.

DEDICATION

I dedicate this thesis to my mother, father and brother for always supporting me unconditionally and reminding me that I can be great, without ever making me feel I needed to be.

I am immensely grateful for the fortune of having them standing behind me for every step of my journey.

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LIST OF ABBREVIATIONS

ACCF – American College of Cardiology Foundation

ACEi – angiotensin-converting enzyme inhibitor

ACE2 – angiotensin-converting enzyme 2

AEVI – active emptying volume indexed to BSA

AF-CHF – Atrial Fibrillation in Congestive Heart Failure

AFib – atrial fibrillation

AHA – American Heart Association

ANP – atrial or A-type natriuretic peptide

ARB – angiotensin II receptor antagonist/blocker

ATTACH – Anti-TNF Therapy Against Congestive Heart Failure

BNP – brain or B-type natriuretic peptide

BSA – body surface area

CMR – cardiac magnetic resonance imaging

CVD – cardiovascular disease

CVS – cardiovascular system

DiVI – diastasis volume index to BSA

ECG – electrocardiogram

ECM – extracellular matrix

EDD – end-diastolic dimension

EDVI – end-diastolic volume indexed to BSA

ESD – end-systolic dimension

ESVI – end-systolic volume indexed to BSA

ECM – extracellular matrix

eGFR – estimated glomerular filtration rate

HF – heart failure

HFPEF – HF with preserved LVEF

HFREF – HF with reduced LVEF

IL-6 – interleukin-6

LA – left atrium; may form composite abbreviations, e.g. LA EDVI

LV – left ventricle; may form composite abbreviations, e.g. LV EDVI

LVDD – left-ventricular diastolic dysfunction

LVEF – left-ventricular ejection fraction

LVH – left-ventricular hypertrophy

LVMI – left-ventricular mass indexed to BSA

LVPW – LV posterior wall thickness

LVSD – left-ventricular systolic dysfunction

MAGGIC – Meta-analysis Global Group in Chronic Heart Failure

MI – myocardial infarction

MR-proANP – mid-regional pro-ANP

MRA – mineralocorticoid receptor antagonist

NPPA – Natriuretic Peptide Precursor A

NPPB – Natriuretic Peptide Precursor B

NSAID – Non-steroidal anti-inflammatory drug

NT-proBNP – N-terminal pro-BNP

NYHA – New York Heart Association

PEVI – passive emptying volume indexed to BSA

PVD – peripheral vascular disease

RA – right atrium

RENEWAL – Randomized Etanercept Worldwide Evaluation

ROS – reactive oxygen species

RV – right ventricle

SR – sinus rhythm

SV – stroke volume

SVI – stroke volume indexed to BSA

TACE/ADAM17 – TNFα converting enzyme

TEVI – total emptying volume indexed to BSA

TNFα – tumor necrosis factor alpha

TNFR1 – TNFα receptor 1

TNFR2 – TNFα receptor 2

TTE – transthoracic echocardiogram

CHAPTER 1
INTRODUCTION

1. Introduction

1.1. The Syndrome of Heart Failure

Heart failure (HF) is a clinical syndrome that has reached epidemic levels, particularly in Western nations [1]. The cardinal manifestations of HF are fluid retention, fatigability and dyspnea, which can lead to any or all of the following signs and symptoms: pulmonary or splanchnic congestion; peripheral edema; and exercise intolerance [2,3]. The cardinal signs and symptoms of HF are non-specific, while the more specific signs, such as elevated jugular venous pressure or a third heart sound appear to varying degrees [2]. The diagnosis of HF is therefore made by an astute clinician based on the analysis of signs and symptoms and physical examination findings in the context of a clinical history suggestive of HF [3,4].

In general terms, HF may either refer to an acutely decompensated syndrome that requires hospitalization and urgent care, or a chronic condition that consistently affects the lives of afflicted individuals. This thesis focuses exclusively on HF as a chronic disease. Within chronic HF, the most common classification system is based on left-ventricular ejection fraction (LVEF), as this scheme is frequently employed as a major enrolment criterion for clinical trials [3]. The American Heart Association (AHA) and American College of Cardiology Foundation (ACCF) define three categories of HF patient based on LVEF: those with preserved LVEF (HFPEF; LVEF≥50%), those with borderline HF (40%<LVEF<50%), and those with reduced LVEF (HFREF; LVEF≤40%). For practical purposes, a binary classification of HFPEF or HFREF is typically used, wherein the LVEF cutoff has varied between studies and clinical trials [5]. Presently, the consensus binary LVEF cutoff is LVEF≥50% for HFPEF and LVEF<50% for HFREF [3,6].

HF can also be broadly classified according to disease progression indices, of which the two most common are New York Heart Association (NYHA) functional classification and AHA HF staging [7,8]. NYHA class is based on increasing lifestyle limitation, from asymptomatic in class I to severe limitations present even at rest in class IV [7]. AHA HF stage meanwhile is based on increasing severity of structural disease

with concomitant symptoms, from at-risk without structural disease in stage A to severe structural disease with refractory HF symptoms in stage D [8]. Interestingly, a large HF staging study based on community-dwelling patients identified more than 50% of individuals over 45 years of age as either being at-risk (stage A) or having asymptomatic cardiac abnormalities (stage B), which illustrates that a significant proportion of the population have a latent risk of developing HF [8].

1.2. Incidence and Prevalence

The rate of HF incidence increases with age in the population; beyond 65 years of age, the incidence exceeds 1% [1,9,10]. Interestingly, large epidemiological studies have not shown a significant reduction in five-year HF mortality despite the emergence of several effective therapies for HFREF—it appears only that people are surviving longer in the short term after the initial diagnosis [10]. The incidence of HF has also remained stable over time, despite substantial improvements in the management for predisposing disorders of the cardiovascular system (CVS), such as acute coronary syndromes, hypertension, dysrhythmias, valvular lesions and congenital heart disease [11]. Because HF represents the end pathway for many of the myriad conditions that affect the CVS, therapies that treat but do not cure the precipitating condition present the possibility of developing future HF instead of dying, which may have historically been the outcome [10].

Alarmingly, the overall prevalence of HF is believed to be rising, apparently as a result of stable incidence coupled with longer survival after diagnosis [2,11]. Heart failure prevalence varies by race, sex and geographical location; however, combined estimates of prevalence based on North American and European figures suggest that it is between 1% and 12%, depending on the stringency of diagnostic criteria [1]. The number of patients discharged from hospital with a primary diagnosis of HF has increased to a stable point in North America, and may be declining, but total annual HF hospitalizations still exceed 1 in 400 in the general population [11]. The epidemic of HF presents a massive burden on the healthcare system, as the estimated lifetime cost per HF patient exceeds $100,000 per year in North America [11]. The aforementioned cost estimate is

largely generated by in-hospital costs, typically due to acute decompensation or incident severe HF. Nonetheless, those living with chronic HF are at a constant risk for acute decompensation due to various triggers, such as infection, anemia or poor adherence to medications [11].

1.3. Etiology and Outcomes

Underlying the clinical manifestations of HF are a constellation of cardiac structural or functional abnormalities—typically, although not necessarily, these first appear in the left ventricle (LV); however, non-myocardial causes, such as disorders of the pericardium or heart valves can also cause HF [3]. As mentioned above, the treatment of a CVS disorder to alleviate associated symptoms might not eliminate the risk of future HF. Among those comorbidities commonly associated with the development of HF are diabetes, hypertension, dyslipidemia, overweight/obesity, smoking, atrial fibrillation (AFib), prior myocardial infarction (MI), peripheral vascular disease (PVD) and renal dysfunction [12]. Presently, a relative risk for HF can be assigned to the aforementioned comorbidities, but more work is still necessary to identify how each of these individually plays into the biochemical changes that lead to the pathogenesis of HF [1].

Comorbid conditions, while implicated in both HFPEF and HFREF, may contribute differentially to the development of each syndrome. Predisposing causes for HFREF are generally believed to originate in the heart due to myocardial injuries, such as MI, which then trigger myocardial oxidative stress [2,13,14]. Myocardial oxidative stress thereafter causes imbalances in various systems, such as neurohormones, which then lead to adverse cardiac remodeling and yet more oxidative stress [13,14]. Atherosclerosis is thus a major predisposing condition in HFREF, as it can lead to MI and subsequent pathogenesis of HF. In HFPEF, on the other hand, numerous comorbidities, including diabetes, hypertension and overweight/obesity, may drive dysregulation of the systemic inflammasome, which leads to primary oxidative stress at the coronary microvascular endothelium as opposed to the myocardium [14]. Cardiac remodeling thereafter occurs as a consequence disrupted nitric oxide-dependent protein kinase G activation [14].

All-cause mortality between HFPEF and HFREF are high, although mortality is slightly lower in HFPEF [15], and differences exist in causes of death: non-cardiovascular causes account for the largest proportion of deaths in HFPEF, while coronary heart disease is the predominant cause of death in HFREF [5,16-18]. Comorbidities appear to play a greater role in affecting outcomes in HFPEF as compared to HFREF [17,18], but comorbidities are certainly an important precipitating factor for hospitalization in all HF phenotypes [1]. Particularly in HFPEF, the paucity of effective therapies, and the neutral or negative results of promising trials in the last decade [19-25] suggest that the collective understanding of the molecular pathology in HFPEF remains incomplete. Chapter 3 of this document explores some of the biochemical differences that underlie HFPEF versus HFREF.

1.4. Biochemical Alterations in Heart Failure

1.4.1. Biomarkers

Biomarkers offer an output that reflects tissue- or organ-specific or whole body consequences of a disease process, which will be helpful in bridging the gap that exists between effective therapies for HFPEF versus HFREF [26]. Biomarkers are any metric that reflects a biological process, but the markers described herein are circulating biochemical markers that can be measured in blood, plasma or serum; are obtained through relatively non-invasive means; and reflect systemic biochemical changes [11]. Biomarkers can increase diagnostic accuracy and provide prognostic information, but no single test can replace the diagnostic algorithm for HF due to the diversity of presentations and multiple etiologies that underlie the syndrome [3]. Therefore, the search for a panel of biochemical markers to improve the ability of clinicians to identify risk of HF; or diagnose, prognosticate and describe the pathophysiology of this syndrome more effectively is an area of considerable research. Through biomarker studies, several pathways have been shown to act in HF, including fibrosis and extracellular matrix remodeling, oxidative and cardiomyocyte stress, and inflammation [26-31].

1.4.2. TNFα Axis Inflammation

The tumor necrosis factor-alpha (TNFα) axis is one component of the inflammasome that is consistently associated with HF [32-38]. The fundamental components of the TNFα axis are TNFα, the ligand, and its two receptors, TNFR1 and TNFR2 [39]. Tumor necrosis factor-alpha is synthesized as a transmembrane protein that associates into homotrimers [40,41], after which, proteolytic cleavage by TNFα converting enzyme (TACE/ADAM17) generates a soluble pyrogenic cytokine with various effects, including inflammation, cachexia and anorexia [32,42-44].

Tumor necrosis factor-alpha, TNFR1 and TNFR2 are each expressed by all of the nucleated cell types of the heart [44]; however, TNFα expression is silenced under conditions of normal cardiac physiology, as is the case in most other cell types in which TNFα is expressed [45]. Although macrophages are the canonical cellular producer of TNFα, cardiomyocytes may produce substantial amounts per unit mass under conditions of stress or injury, so endogenous cardiac TNFα may play a significant role in cardiac inflammatory signaling [45]. Initial conceptions of the cardiovascular response to TNFα signaling were of uniformly deleterious effects; however, further evidence has elucidated a more complex interplay between TNFα and its two receptors, whereby TNFα can mediate seemingly contradictory effects on cardiomyocytes [46].

The paradoxical effects attributed to TNFα in the heart can be traced to the differences in the respective functions of TNFR1 and TNFR2. Indeed, in a study of rat cardiomyocytes, TNFR1 mediated increased reactive oxygen species production, reduced Ca^{2+} transience amplitude, reduced fractional shortening, and stimulated apoptosis, while TNFR2, mediated the opposite effects [46]. The combined effect *in vivo* is typically TNFR1 dominance over TNFR2, which results in the ostensibly deleterious effects of TNFα in the heart [45], such as the downregulation of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2) [47]. The divergent effects of TNFR1 and TNFR2 were also apparent in a small animal model of ischemic HF: TNFR1-knockout mice demonstrated improved remodeling and less apoptosis in response to MI, while TNFR2-knockout mice showed worse remodeling and more apoptosis [48]. Additionally, these two receptors have

divergent effects in non-cardiac tissues: TNFR1 mediates cytokine production in airway cells [49] and high fat diet-induced obesity in adipocytes [50], while TNFR2 mediates angiogenesis [51].

1.4.3. Natriuretic Peptides

There are four types of natriuretic peptide that have been characterized in scientific literature: atrial or A-type (ANP), brain or B-type (BNP), C-type (CNP) and dendroaspis (DNP) [52]. ANP and BNP are known as cardiac natriuretic hormones: they are peptide hormones secreted by the heart to effectuate its role as an endocrine organ. The main effects of ANP and BNP are to lower systemic vascular resistance and to stimulate natriuresis, culminating in blood pressure reduction [53]. Basic science investigations have shown that expression of the genes that encode ANP and BNP, *Natriuretic Precursor Peptide A* (*NPPA*) and *B* (*NPPB*), respectively, is ubiquitous in the heart during prenatal development, but is primarily confined to the left atrium (LA) in normal adult cardiac physiology [54]. The syntheses of ANP and BNP follow a similar cascade that involves intracellular processing. ANP or BNP pre-propeptide that contains the active, cysteine-bridged loop structure in its C-terminus is proteolytically processed at its N-terminus to generate a propeptide, which is then secreted and subsequently cleaved in the plasma to generate two portions: the active C-terminal loop fragment, and the inactive linear N-terminal fragment [52]. In response to pathological stimuli that stress the heart, such as increased afterload due to uncontrolled hypertension, marked increases in *NPPA* and *NPPB* expression are observed in the LA and LV [54].

Presently BNP and its N-terminal pro-peptide (NT-proBNP) are the circulating markers most routinely used in clinical practice, as they respond to advancing disease [55-57]. NT-proBNP may have a longer half-life than BNP, and so may be a better reflector of *NPPB* gene expression [57,58]. A benefit of measuring circulating levels of BNP is that they increase exponentially in response to increasing severity of HF, while levels of ANP increase in a linear manner [52]. However, measuring NT-proBNP and BNP is challenged by paradoxical reductions in these molecules in the presence of obesity, and by the observation of elevated NT-proBNP and BNP in other cardiac or

renal conditions beyond HF [59-61]. MR-proANP, which has a significantly longer half-life and is a more reliable marker of *NPPA* expression than active ANP [62], may therefore still be a useful addition to biomarker panels for HF; it has been useful for diagnosing acute HF [63,64], but its potential has not been explored in chronic HF. Chapter 4 of this thesis explores plasma levels of NT-proBNP and MR-proANP as biomarkers for LA remodeling in chronic HF.

1.5. Left Heart Structure and Function in Heart Failure

1.5.1. Left Ventricle

The general classification scheme of HFPEF or HFREF is accompanied by a conception of classical types of remodeling for each phenotype. A concentrically remodeled heart with marked left-ventricular hypertrophy (LVH) is characteristic of HFPEF [10]. An eccentrically remodeled and hypertrophic heart, on the other hand, characterizes HFREF [65]. Surveys of patients from clinical trials have largely confirmed this conception; however, it is worth noting that HF patients with clinical diagnoses of HF may fall within the established cut-off values for structurally normal hearts [66], while asymptomatic individuals may exhibit LVH or other structural abnormalities [8].

During normal pump function, layers of longitudinally and circumferentially arranged myocardium allow the heart to contract in the most mechanically efficient manner possible [67]. Proper LV function is reliant on the coordination of longitudinal and circumferential contraction during systole, but is also reliant on efficient relaxation to allow filling through the LA during diastole. The various contributors to HF—systemic perturbation and myocardial injury—typically beget different types of remodeling, such as MI leading to cardiomyocyte death and replacement fibrosis in the infarcted region, or hypertension leading to cardiomyocyte hypertrophy [68]. Importantly, these different remodeling processes, which are initially compensatory, typically converge in a deleterious cascade that leads to some degree of LV systolic and diastolic dysfunction (LVSD and LVDD, respectively) [3,68].

In terms of existing HF classification schemes LVEF is regarded as the most important measure of LV systolic function, and it is also the most widely reported [69].

More than 40% of patients present with an LVEF above the normal cutoff of 50% [3], which has led to controversy with respect to describing patients as HFPEF or HF with preserved/normal systolic function. HFPEF is the preferred terminology: just as with structural abnormalities, systolic dysfunction may be subclinical or may not be reflected in resting LVEF [3]. Indeed, echocardiographic strain and strain rate imaging studies have revealed the presence of subclinical resting LVSD in a substantial portion of HFPEF patients [69,70]. Similarly, individuals may have no overt HF symptoms and yet have subclinical LVDD [8,9], and while most HFPEF patients meet criteria for LVDD, some do not [66]. Therefore, the difference between preclinical LVDD and symptomatic HFPEF is likely more complex than a comorbidity-induced evolution of LVDD into HFPEF, although this scenario certainly appears to represent one possibility [71]. Nonetheless, LVDD is an important metric in HFPEF, as advancing LVDD increases the risk of all-cause mortality in this group [72]. Interestingly, marked LVDD is also a common finding in HFREF in addition to HFPEF [2,3]; therefore, it is the type of LV dysfunction of focus in this thesis: in Chapter 3, biochemical changes underlying LVDD in HFPEF and HFREF are explored. Left-ventricular diastolic dysfunction manifests both in early, passive filling at the start of diastole and late, active filling at the end [71]; the relative balance between early and late filling may shift in cardiac abnormalities. Furthermore, LVDD is not an entirely intrinsic process: it is also dependent, particularly in the late phase, on the chamber to which the LV is apposed, the LA.

1.5.2. Left Atrium

Limited LA structural information in the form of volume assessment is often reported as a metric in HF populations. There is evidence that enlarged LA represent a risk factor for incident HF in populations with predisposing cardiovascular disease (CVD) [73] or in the general community [74]. Indeed, LA enlargement is also a characteristic finding in symptomatic HF [8,75], and it is an indicator of worse outcomes in patients with established HF [76,77].

The functional role of the LA has also been characterized to a lesser extent than for the LV; however, some foundational studies have shown that in both asymptomatic

LVDD and HF, there may be changes in the reservoir (elastic recoil) and booster pump (active contraction) contributions of the LA to LV stroke volume (LV SV). Seminal work by T Kono *et al.* showed progressive LA dilation and loss of contractility simultaneous to progressive LVSD in a dog model of ischemic HF [78]. Translating this work into humans, A Prioli *et al.* found an initial compensatory increase in LA function from normal diastolic function to moderate LVDD, but progressive LA dysfunction in conjunction with progression from moderate to severe LVDD [79]. More recently, M Kurt *et al.* reported analyses of LA strain in early and late diastole, and found that the average E/e' to early strain ratio, a dimensionless surrogate for LA stiffness, is effective at identifying HFPEF from asymptomatic LVDD [80]. Similarly, D Morris *et al.* showed that reduced early and late LA strain are characteristic of HFPEF over asymptomatic LVDD, and also of increasing NYHA class within HFPEF [81]. Likewise, H Motoki *et al.* found that reduced LA strain, particularly during atrial contraction, is a strong predictor of grade III diastolic dysfunction in patients with HFREF [82]. In Chapter 4 of this thesis, the role of the LA in LV filling is explored.

1.5.3. Cardiac Imaging Modalities

Many modalities exist to image the heart and associated vasculature; however, the two techniques used for the analyses herein were echocardiography and cardiac magnetic resonance imaging (CMR). Both of these modalities are used extensively in clinical practice and research due to their non-invasive nature, and use of non-ionizing radiation. Transthoracic echocardiogram (TTE) is the most widely available modality for cardiac imaging; it is advantageous due to the low risk posed and minimal discomfort caused to the patient, and the ability to image the heart in real time [83]. Furthermore, several practice guidelines, including Canadian guidelines, exist for TTE-based evaluation of cardiac structure and function, which allows relatively reliable diagnostic and prognostic insights [84]. The accurate assessment of diastolic function, which is the most challenging determination in cardiac imaging, is also the most important in the HFPEF population, but can be made by combining measurements in motion mode (M-mode) and tissue Doppler mode in TTE [85,86]. In Chapter 3, diastolic dysfunction investigations made use of established echocardiography techniques for diastolic function assessment.

With respect to diastolic function analyses, CMR is less developed, and practice guidelines do not presently recognize it as a standard technique. Furthermore, CMR is not recommended for patients with renal failure, as gadolinium contrast agents used for imaging tissue lesions are potentially harmful in this group [84]. The benefit of this modality, however, is the substantially improved resolution over echocardiography, which allows for determination systolic function parameters to at least the same accuracy as echocardiogram, but with better anatomical insight [83]. The enhanced resolution in CMR allows for sophisticated analyses of LV and LA function, including detection of subclinical LV systolic dysfunction using similar techniques to TTE [86]. In Chapter 4, CMR is used in novel ways to add to the small body of knowledge on the role of LA biomechanical function in LV hemodynamics in patients with HF.

CHAPTER 2
MATERIALS AND METHODS

2. Materials and Methods

General ethics principles and methods that apply to both Chapters 3 and 4 are detailed herein. Chapter-specific techniques are appropriately elaborated therein.

2.1. Ethics and Confidentiality for Human Research Subjects

All research detailed in this document was conducted on human subjects who were recruited from across the province of Alberta, Canada at two city sites, Edmonton and Calgary, to participate in the Alberta Heart Failure Etiology and Analysis Research Team study (HEART) [87]. Human subjects were evaluated according to the tenets set forth in the Declaration of Helsinki [88], whereby subjects were given sufficient protocol information to give informed consent, and were free from coercion to participate in the study. The Alberta HEART study received Health Research Ethics Board approval at the University of Alberta (Pro00007105; October 9, 2009) and at the University of Calgary (22657; October 1, 2009).

Individuals of both sexes who were at least 18 years of age were eligible to participate in the study, with the exception of those who had a known malignancy, with expected survival time less than one year; a pregnancy within the previous six months; a recent cardiac event, including acute MI and decompensated HF; history of moderate or severe pulmonary hypertension; or previously known severe mitral or aortic valvular stenosis. Study enrollees were assigned an alphanumeric identifier code to protect their identities. These codes reflected only the relative order of study enrolment and the site of enrolment and, importantly, did not provide any information as to the clinical status of the enrollee. In this way, analyses were carried out in a blinded fashion, after which other information was used to stratify subjects into pertinent study categories.

All data collected, particularly sensitive personal information, is being stored as paper or digital files with restricted access, backup in case of loss, and internal oversight to ensure proper usage. No part of the data collected for these studies will be sold or given to third parties, and enrollees may access summary information for the study, or withdraw at any time.

2.2. Study Enrolment and Baseline Analysis

Study subjects were consecutively enrolled for the analyses described herein. Community-dwelling ambulatory patients with clinical diagnoses of chronic HF were studied in comparison to a healthy volunteer reference group. Heart failure patients were referred to study coordinators in either Calgary or Edmonton, and were enrolled contingent on not fulfilling any of the aforementioned exclusion criteria. Community outreach strategies were employed to recruit healthy volunteers.

During one-day enrolment events, study subjects were processed through physical examination, including blood pressure recording; blood sampling; electrocardiogram (ECG); TTE; and CMR. Of the enrollees, healthy volunteers were only selected for the analyses detailed in this thesis if they had yielded sufficient plasma during blood sampling for biochemical marker analysis and fulfilled the following additional criteria: no history of cardiovascular or renal disease, hypertension, diabetes, or AFib; and no prescriptions for antiarrhythmics; ACEi; ARB; beta-blockers; digoxin; loop or thiazide diuretics; or MRA. Likewise, HF patients were selected who had also yielded sufficient plasma for biochemical marker analysis. Altogether 250 individuals comprised the full study group: 50 healthy individuals, 100 individuals with HF and an LVEF<50%, and 100 individuals with HF and an LVEF≥50%, based on enrollment TTE-derived LVEF, which was calculated using Simpson's biplane method of disks. The 250 total study subjects were included in the analyses in Chapter 3, while only those subjects from the Edmonton study site who underwent CMR were studied in Chapter 4. The initial study group was closed at 250 for data evaluation purposes; the demographic and clinical history data was extensively cross-checked for accuracy.

2.3. Enrollee Data and Study Categorization

Anthropometric, demographic and physical measurements were taken at the time of enrolment, including height, weight, age and sex. Obesity was then defined as BMI≥30kg/m^2 based on World Health Organization criteria [89]. Subjects' clinical history, particularly for relevant cardiovascular risk factors, such as obesity, hypertension, diabetes and AFib, as well as medication history were pulled from Netcare

and subsequently cross-checked with a history taken at study enrolment, a province of Alberta-wide healthcare database, by authorized members of the study team. New York Heart Association (NYHA) functional class was adjudicated by teams of board-certified cardiologists in Edmonton or Calgary after reviewing HF patient charts.

2.4. Blood Sampling and Laboratory Values

Subjects were rested and sitting while blood was collected into cooled tubes, with lithium-heparin or EDTA as anticoagulant, which were immediately placed on ice prior to plasma fractionation and deep freezing at -80°C. Plasma was stored at the Canadian Biosample Repository (Edmonton, AB, CAN) for retrieval and subsequent biochemical testing as needed.

2.5. Electrocardiogram and Cardiac Imaging

Subjects were studied by full 12-lead ECG and fitted with a heart rate (HR) monitor on the day of enrollment. Heart rate was recorded and rhythm was determined based on ECG tracing. Conduction abnormalities were determined based on QRS axis determination and ECG intervals. Cardiac images, meanwhile, were obtained using two modalities: TTE and CMR. Details on TTE and CMR analyses are provided in Chapters 3 and 4, respectively.

2.6. Data Analysis

Box-and-whisker plots, scatterplots, forest plots and histograms are displayed herein; they were generated from raw data using OriginLab software version 9.1 (OriginLab, Northampton, MA, USA). Box-and-whisker plots of biochemical marker levels are displayed on a base-10 logarithmic scale to account for skewed distributions, where the box represents the interquartile range, the bisecting line represents the median, and the whiskers extend to maximum and minimum values. Scatter plots that include biochemical marker levels also display those values on a base-10 logarithmic scale. Forest plots display odds ratio (OR) and 95% confidence interval (95%CI) on a base-10 logarithmic scale. Graphics generated in OriginLab were imported into Adobe Illustrator CS5 (Adobe Systems Canada, Ottawa, ON, CAN) for visual editing to produce publication-quality figures.

Categorical data are expressed in tables as percentages, while tabulated continuous data are expressed as median with interquartile range in parentheses. All statistical analyses were performed using SPSS software version 20 (IBM Corporation, Armonk, NY, USA) with a p-value<0.05 taken as significant. Associations between categorical variables were compared using the Pearson Chi-square Test. Associations between continuous variables and binary categorical, ordinal or continuous variables were explored using binary logistic regression, ordinal logistic regression or linear regression analyses, respectively. Biochemical marker levels were base-10 log-transformed for regression analyses to account for non-normal distribution patterns. Continuous variables were compared across categories using the Mann-Whitney U Test for binary categories, or the Kruskal-Wallis Test with Mann-Whitney U Test for multiple comparisons for multiple categories. Non-parametric tests were performed on raw biochemical marker levels, although graphical displays show base-10 logarithmic scales.

CHAPTER 3
THE ROLE OF THE TUMOR NECROSIS FACTOR-ALPHA AXIS

3. The Role of the Tumor Necrosis Factor-Alpha Axis

3.1. Introduction and Rationale

HFPEF accounts for approximately 40% of HF diagnoses, with a rising incidence relative to HFREF, and has mortality and morbidity comparable to HFREF [15,90,91]. In general, the healthcare burden attributed to HFPEF is expected to grow as the general population is living longer than ever before, and HF risk factors, such hypertension and overweight/obesity, have reached stable highpoints in terms of prevalence [92-94]. Despite epidemiological similarities, in terms of pathophysiology, HFPEF and HFREF may represent distinct groups of patients along the continuum of the HF syndrome [14]. This was suggested by findings of striking dissimilarities in responses to therapeutic interventions between these two groups. Indeed, clinical trials have validated angiotensin-converting enzyme inhibitors (ACEi), angiotensin II-receptor blockers (ARB), beta-adrenergic receptor antagonists (beta-blockers) and mineralocorticoid receptor antagonists (MRA) as therapeutics in HFREF [3]. Conversely, trials of ACEi [20], ARB [19], beta-blockers [22], MRA [24,25] and phosphodiesterase-5 inhibitor [23], a proposed unique therapy for HFPEF, were neutral or negative, with non-significant improvements in quality of life or patient outcomes.

Biomarker studies have shown that several pathways are dysregulated in HFPEF and HFREF, including fibrosis and ECM remodeling, oxidative and cardiomyocyte stress, and inflammation [26-31]. A recent model for HFPEF proposed by WJ Paulus and C Tschöpe (2013) suggests that comorbidity-induced inflammation is a primary mediator in HFPEF [14]. In this scheme, an increase in systemic inflammation due to comorbid conditions leads to oxidative stress at the coronary microvascular endothelium, which triggers pathological changes that lead to cardiac remodeling and hypertrophy. By contrast, in HFREF, a trigger, such as myocardial infarction or infection, leads to myocardial oxidative stress, which begets a secondary, cyclical dysregulation in inflammatory cascades and neurohormonal signaling that further damages the myocardium [2,13,14]. In both cases, circulating levels of various cytokines from different cascades are increased, including interleukins and TNFα [13,14].

Multiple lines of evidence support a role of the TNFα axis in HF, and circulating TNFα, TNFR1 and TNFR2 are elevated in patients with HF relative to control subjects [37]. A recent report showed that circulating TNFR1 levels are significant predictors of incident HF in a population of at-risk individuals, and that TNFR1 predicts the incidence of HFPEF over HFREF [95]. The work presented in the following chapter expands on previous work by exploring associations between indices of diastolic function or disease progression, and levels of circulating TNFα, TNFR1 and TNFR2, as well as a non-TNFα family cytokine, interleukin-6 (IL-6). Our findings that plasma levels of TNFR2 are elevated in HFPEF relative to HFREF and associated with elements of the disease phenotype suggest this could be a point of pathophysiological difference between HFPEF and HFREF.

3.2. Materials and Methods

3.2.1. Laboratory Values

Plasma was sent for analysis of serum creatinine, lipid profiles and BNP levels at Alberta Health Services laboratories. BNP was measured in EDTA-anti-coagulated plasma using an Alere Triage Reagent pack (Alere Inc., Ottawa, ON, CAN) on a UniCel DxI 800 Immunoassay System (Beckman Coulter, Missisauga, ON, CAN) [96]. Testing was performed off site at an Alberta Health Services laboratory. Estimated glomerular filtration rate (eGFR) was calculated using the revised Modification of Diet in Renal Disease equation based on serum creatinine measurement [97].

3.2.2. Plasma Inflammatory Marker Level Assays

Plasma levels of TNFα, TNFR1, TNFR2 and IL-6 were assessed using commercially available enzyme-linked immunosorbent assay (ELISA) kits (catalogue no.'s STA00C, SRT100, SRT200 and S6050, respectively, R&D Systems, Minneapolis, MN, USA). Optimized versions of previously described protocols were used with lithium-heparin anti-coagulated plasma as the assay substrate in all cases [36,38,98]. No modifications were made to the assay protocols for TNFR1 and TNFR2: 20µL of plasma was diluted ten-fold in preserved animal serum buffer (supplied with assay kit) to produce 200µL solution, which was then incubated for 2 hours at 22°C on a

corresponding plate coated with inflammatory marker antibodies. This incubation step was modified in the TNFα assay: plasma was incubated in the anti-TNFα coated plate for 24 hours, eight of which were at 22°C and 16 of which were at 4°C. Likewise, the IL-6 protocol was modified in the following ways: 200µL of plasma was used as opposed to 100µL, and the plasma was incubated in the anti-IL-6 coated plate for three, instead of two hours at 22°C. For the TNFα and IL-6 assays, no preserved animal serum buffers were used to dilute plasma.

After the incubations described above, plasma samples were aspirated from the plates, and the wells were subsequently washed using buffered surfactant wash buffer (supplied with assay kit) diluted to the recommended 1:25 stock buffer:H_2O ratio. 200µL of buffered anti-TNFα, -TNFR1, -TNFR2 or -IL-6 horseradish peroxidase-linked conjugate was then added to the appropriate assay plate and incubated at 22°C for six hours for TNFα and two hours for TNFR1, TNFR2 and IL-6. Following incubation with the second antibody, the wells were once again aspirated and washed with diluted wash buffer before 200µL of hydrogen peroxide-activated chromogenic solution (supplied with assay kit), which contained tetramethylbenzidine, was added to the wells. Colour was allowed to develop in the wells protected from light at 22°C for one hour for TNFα and for 20 minutes for TNFR1, TNFR2 and IL-6, after which the chromogenic reaction was arrested by addition of 1M sulfuric acid acid solution.

Absorbance was measured at 450nm with the wavelength correction set to 540nm for all assays using a SpectraMax M5 Plate Reader linked to SoftMax Pro version 5.4.1 software (both from Molecular Devices, Sunnyvale, CA, USA). Detection rates for ELISA assays were 72.4%, 100%, 100% and 81.2% for TNFα, TNFR1, TNFR2 and IL6, respectively. The inter- and intra-assay coefficients of variation were 13.1% and 9.5% (n=4); 7.0% and 5.2% (n=4); 5.3% and 3.5% (n=4); and 8.0% and 6.3% (n=4), respectively. The sensitivities of the assays were 0.04pg/mL, <7.8pg/mL, <7.8pg/mL and 0.12pg/mL, respectively.

3.2.3. Plasma ACE2 Activity Assay

Plasma ACE2 activity was assayed using an adapted version of a previously established protocol [99]. Lithium heparin anti-coagulated platelet-free plasma samples were diluted to a final ratio of 30:70 in plasma assay buffer: 1M NaCl (Sigma Chem. Co., St. Louis, MO, USA), 75mM Tris-HCl (Invitrogen, Carlsbad, CA, USA) and 5mM $ZnCl_2$ (Sigma Chem. Co., St. Louis, MO, USA) at pH 6.5. Assay buffer was made to contain various protease inhibitors: 10µM captopril (ACE inhibitor; Sigma Chem. Co., St. Louis, MO, USA); 5µM amastatin (aminopeptidase inhibitor; Sigma Chem. Co., St. Louis MO, USA); and Protease Inhibitor Cocktail (Sigma Chem. Co., St. Louis, MO, USA) dissolved to achieve a concentration of 10µM bestatin (aminopeptidase inhibitor), 1µM E-64 [N-(trans-Epoxysuccinyl)-L-leucine-4-guanidinobutylamide; cysteine protease inhibitor], 770nM pepstatin A (aspartyl protease inhibitor), 154µM AEBSF [4-(20aminoethyl)benzenesulfonyl fluoride hydrochloride; serine protease inhibitor], 75 nM phosphoramidon (neprilysin inhibitor), 15nM aprotinin (serine protease inhibitor) and 75 nM leupeptin (cysteine, serine and threonine proteases inhibitor). The use of protease inhibitors in the buffer solution was meant to ensure that the fluorogenic substrate and specific ACE2 inhibitor (both described below), which are integral to the assay, were not cleaved by other proteases naturally present in human plasma.

Samples were incubated with dinitrophenol-quenched methoxycoumarin-containing fluorogenic substrate (catalogue no. ES007, R&D Systems, Minneapolis, MN, USA) at a final concentration of 10µM at 37°C. To find the fluorescence increase due to ACE2 activity, plasma samples were assayed both in the presence and absence of the specific, linear ACE2 inhibitor, DX600 (catalogue no. 002-26, Phoenix Pharmaceuticals, Burlingame, CA, USA). Fluorescence was measured with excitation and emission settings at 320nm and 405nm, respectively, using SpectraMax M5 plate reader linked to SoftMax Pro version 5.4.1 software (both from Molecular Devices, Sunnyvale, CA, USA). The maximal fluorescence increase due to ACE2 activity was determined from the maximum fluorescence difference between inhibited and uninhibited aliquots; this was normalized to a standard curve for methoxycoumarin-containing fluorescent peptide (catalogue no. M-1975, Bachem, Torrance, CA, USA) and scaled for time of

measurement and plasma volume over 24 hours, with 1 hour as the baseline. All ACE2 enzymatic activity values herein are expressed in pmol/hr/mL, which describes the amount of substrate turned over per unit time per unit volume of plasma. The detection limit was 2.3pmol/hr/mL, and the inter- and intra-assay coefficients of variation were 10.2% and 7.5% (n=10), respectively.

3.2.4. Echocardiographic Analyses

Echocardiograms were performed using the Phillips IE33 ultrasound platform, and were interpreted by cardiologists with specialized echocardiography training who were blinded to both the clinical classification and biomarker analyses. LVEF was assessed using Simpson's biplane method of disks. HF patients were adjudicated as HFREF or HFPEF using an LVEF cutoff of 50%, according to clinical practice guidelines [3,6]. Adjudication of New York Heart Association (NYHA) functional class and primary etiology of HF were determined by cardiologists blinded to biomarker analyses. Grading of diastolic dysfunction was performed by blinded members of the authorship team based on previously published guidelines [85]. LA volume index, lateral e' and medial e' were used to make a binary decision for diastolic dysfunction, after which E/A ratio, or average E/e' ratio for patients in AFib, was used to ascertain grade in those determined to have diastolic dysfunction. Diastolic dysfunction analyses could not be performed due to poor echocardiographic visualization in nine HFPEF patients and 14 HFREF patients, and a further one HFPEF patient and seven HFREF patients were excluded from diastolic function analyses due to the presence of severe mitral regurgitation (MR).

3.3. Results

3.3.1. Baseline Clinical Profile and Cardiac Assessments

Demographic and clinical information for the control, HFPEF and HFREF groups is displayed in Table 3.1. In this study, the community-dwelling HF patient populations recapitulated observations from previous landmark studies [12,15,90,91]. HFPEF patients were older (p=0.003), and significantly more were obese (p=0.016) and hypertensive than HFREF patients (Table 3.1). Medical histories for important HF risk factors, such as

current or former smoking habit (p=0.199), diabetes, PVD (p=0.234) and AFib were not different between HFPEF and HFREF patients (Table 3.1). Interestingly, total cholesterol (p=0.774), triglycerides (p=0.656) and total cholesterol:high-density lipoprotein (HDL) ratio (p=0.187) were not significantly different between HF phenotypes, despite differences in obesity prevalence. This also recapitulates observations that dyslipidemia is equally prevalent in HFPEF and HFREF [12,91]. In line with results from the Meta-analysis Global Group in Chronic Heart Failure (MAGGIC) study [15], ischemia as the primary etiology of HF was more prevalent in HFREF than HFPEF (Table 3.1). Despite these differences, both groups were on evidence-based therapeutic regimens and both were well covered with standardized front-line HF therapeutics in ACEi/ARB, beta-blockers and MRAs (Table 3.1). Importantly, the two HF groups comparably used drugs that can affect inflammatory signaling: NSAID use was equivalent, while statin use was not significantly different (p=0.438; Table 3.1). NYHA functional class distribution was also not significantly different between the HFPEF and HFREF groups (Table 3.1).

Electrophysiological changes were not apparent in heart rates, as these did not differ significantly between study groups; however, more HFPEF patients were in AFib on the day of study than HFREF patients (p=0.018; Table 3.2). Despite a binary cutoff for LVEF at 50%, the HFREF group had significantly lower LVEF compared to control and HFPEF groups, with notably different median values (Table 3.2). Both HFPEF and HFREF had greater LV posterior wall thickness (LVPW; p<0.001 for both) and mass index (LVMI; p<0.001 for both); LA volume index (p<0.001 for both); and average E/e' ratio (p<0.001 for both) compared to control (Table 3.2). Left ventricle posterior wall thickness (p<0.05), and LV end-diastolic and -systolic dimensions (LV EDD and LV ESD, respectively; p<0.001 for both) were significantly different between the two HF groups as well (Table 3.2). Significantly more HFREF subjects had left-ventricular hypertrophy (LVH) than HFPEF (p<0.001; Table 3.2). Interestingly, the HFPEF group exhibited a milder distribution of diastolic dysfunction than the HFREF group (p=0.043), and while these findings are congruent with previous reports of LVDD in HFPEF [66], AFib may also be acting as a confounder in this analysis (Table 3.2). Nonetheless

moderate (pseudonormal filling pattern; grade II) and severe (restrictive filling pattern; grade III) diastolic dysfunction were present in both HF groups in marked proportions.

3.3.2. Circulating Inflammatory Marker Levels

Relative to control subjects, plasma levels of TNFα were only significantly elevated in HPFEF, while plasma levels of TNFR1 and TNFR2 were significantly elevated in both HFPEF and HFREF (Figure 3.1 A-C). Meanwhile, plasma IL-6 was not significantly different between control and either HF group, or between HF groups (Figure 3.1 D). Notably, the only significant difference in TNFα axis markers between HFPEF and HFREF was observed in TNFR2, while TNFα itself and TNFR1 were non-significantly different (Figure 3.1 A-C).

A central element in the HFPEF paradigm proposed by WJ Paulus and C Tschöpe is the influence of HF risk factors on systemic inflammation [14]. In order to establish whether this mechanism might be at work in our study subjects, we explored the relationship between age, eGFR, sex, obesity, LVH, hypertension, diabetes, current or former smoking habit, PVD or AFib with circulating levels of TNFα, TNFR1, TNFR2 and IL-6 in the combined HF group (Table 3.3). Advanced age was significantly associated with elevated circulating TNFR1 and TNFR2, while eGFR was significantly associated with elevated TNFα in addition to its two receptors. Tumor necrosis factor-alpha, TNFR1 and TNFR2 were increased differentially in concert with common HF risk factors, but were always associated with at least two risk factors, while IL-6 was not associated with any risk factors. Elevated circulating TNFR1 was significantly associated with the greatest number of risk factors: increasing age; decreased eGFR; and history of hypertension, diabetes, PVD and AFib. Sex, obesity and LVH were not significantly associated with any of the inflammatory markers studied.

3.3.3. Modulators of Inflammatory Markers

We investigated how circulating inflammatory markers were related to ordinal classifiers of disease severity, grade of diastolic dysfunction and NYHA functional class. Using ordinal logistic regression, we found that elevated TNFR2 was significantly associated with increasing grade of diastolic dysfunction in HFPEF, but not in HFREF,

while TNFR1 was not associated with diastolic dysfunction in either HF group (Figure 4.2 A). Interestingly, elevated TNFR1 and TNFR2 levels were significantly associated with increasing NYHA functional class in both HPFEF and HFREF (Figure 4.2 A). Neither TNFα, nor IL-6 was significantly associated with diastolic dysfunction or symptom severity in either HFPEF or HFREF (data not shown). Given that AFib can confound diastolic dysfunction analyses, we investigated associations between TNFR1 or TNFR2 and LA volume index or average E/e' ratio as alternative measures of diastolic function that can be used for patients in AFib [100]. Left-atrial volume index was not associated with plasma TNFR1 or TNFR2 levels in HFPEF ($r=0.110$, $p=0.298$; and $r=0.170$, $p=0.105$, respectively) or HFREF ($r=0.083$, $p=0.443$; and $r=0.056$, $p=0.607$, respectively). Plasma levels of TNFR1 and TNFR2 were weakly, but significantly, associated with average E/e' ratio in HFPEF (Figure 4.2 B & C), but not HFREF ($r=0.215$, $p=0.053$; and $r=0.112$, $p=0.318$, respectively).

Increased activity of TNFα converting enzyme (TACE), a sheddase involved in proteolytic processing of TNFα, TNFRs, and angiotensin-converting enzyme 2 (ACE2) might represent a mechanism, along with increased expression as the other, of increased circulating TNFα and TNFRs in HF [101,102]. ACE2 is a counter-regulatory homologue of angiotensin-converting enzyme (ACE) and a major regulator of endothelial function and myocardial fibrosis [103-105]. Since the membrane-bound localization of TACE poses a limitation in assessing its levels or activity in the plasma, we measured plasma ACE2 activity as a surrogate, as this biomarker increases with increased TACE activity [106]. Previous reports of plasma ACE2 activity showed an association with clinically diagnosed HFREF, symptom severity and worsening clinical outcomes [99,107]. In our HFPEF cohort, plasma ACE2 activity was significantly elevated relative to control (Figure 4.3 A); however, it was not associated with increasing grade of diastolic dysfunction or NYHA class (Figure 4.3 B). We did not evaluate the potential role of increased expression, as we did not have access to tissue samples from our study subjects.

3.4. Discussion

In this comparative analysis of healthy controls, and HF patients with preserved or reduced EF, we found that our cohorts recapitulated previous characterizations, whereby HFPEF subjects were more likely to be older, hypertensive and obese. Despite differences in comorbidities, age and HF etiology the NYHA class distribution was similar between HFREF and HFPEF, which indicates that the two patient populations experienced significant burdens of disease. Additionally, both groups had cardiac hypertrophy and exhibited marked diastolic dysfunction. In patients whose LV dysfunction is primarily diastolic as opposed to both systolic and diastolic, a greater prevalence of comorbidities might account for similar overall symptom severity.

Improvement in exercise capacity in a cohort of HFPEF patients after an exercise training regimen was largely mediated by peripheral improvements, which suggests a systemic component to HFPEF, including skeletal muscle dysregulation [108]. Systemic inflammation, induced by a confluence of comorbid conditions, such as diabetes, and behaviours, such as smoking, may be the primary driver of HFPEF [14]. We investigated dysregulation of the TNFα-axis in this context, and included IL-6 as non-TNF-family cytokine for comparison. We expand on previous work in HF: our data showed that levels of TNFα and TNFR1 were significantly increased in HFPEF relative control but not HFREF, while TNFR2 was significantly increased relative to both control and HFREF, which follows from previous reports [37]. Plasma IL-6 levels were very comparable between HFPEF and HFREF, which suggests that TNFα-mediated inflammation might be a point of pathophysiological difference between HF phenotypes.

We found that low eGFR, hypertension, current or former smoking habit and history of AFib were significantly associated with elevated plasma TNFα levels, while elevated plasma TNFR1 levels were associated with aging, low eGFR, hypertension, diabetes and PVD, and elevated TNFR2 levels were associated with aging, low eGFR, diabetes and AFib. The advanced age and greater prevalence of comorbidities in the HFPEF population could drive the observed elevation in circulating inflammatory markers relative to HFREF. Indeed, non-cardiac comorbidities are more prevalent in

HFPEF, with a larger fraction of adverse clinical outcomes attributable to non-cardiac events compared to HFREF [5,15-18]. Likewise, increased TNFα-axis inflammation has been linked to an increased risk of adverse cardiovascular and all-cause outcomes in HF [33,38,109]. Altogether, these data are congruent with the paradigm for HFPEF that includes comorbidities-driven dysregulation of TNFα-mediated signaling [14,18].

Our finding that circulating TNFR2 levels are significantly associated with increasing average E/e' ratio and grade of diastolic dysfunction in HFPEF, but not in HFREF, suggests a greater role for TNFα-mediated inflammation in this cohort. However, we found that elevation of both TNFRs was associated increasing symptom severity in both HF groups, so the role of the TNFα-axis cannot be discounted in HFREF. The association of diastolic dysfunction and symptoms with TNFR2 in HFPEF is consistent with findings in experimental models, as the TNFR1/TNFR2 axis is involved in mediating divergent effects: TNFR1 has been implicated in adverse cardiac remodeling and adipogenesis, while TNFR2 antagonizes the pathological effects of TNFR1, and also stimulates angiogenesis [46,48,50,51]. TNFR1 and TNFR2 also mediate opposite effects on phospholamban and SERCA2, key Ca^{2+} handling proteins involved in myocardial contraction and relaxation [46,47,110]. An increase in circulating TNFR2 levels might reflect a loss of protective signaling mechanisms due to tissue shedding of TNFR2, thereby leading to the correlation between circulating TNFR2 levels and diastolic dysfunction and symptoms in HFPEF.

The differential roles of TNFRs might explain why TNFα is not associated with elements of the HF syndrome: the variable response to TNFα is mediated at the receptor level. Furthermore, high levels of circulating TNFRs may bind circulating TNFα, which would diminish any apparent increase in circulating levels of the cytokine [39]. Our finding that TNFα is not differentially associated with HFPEF or HFREF is consistent with findings by C Marti *et al.* [95], but each study's conclusions differ in that C Marti *et al.* implicated TNFR1 in HFPEF, while our study implicates TNFR2. The key difference between the two studies is that C Marti *et al.* explored risk of incident HF, while we examined existing HF populations. Indeed, we found TNFR1 to be associated with the

greatest number of HF risk factors, which is congruent with a role in HFPEF incidence. Meanwhile, our data show that TNFR2 might then be a better biomarker for gauging severity of established HFPEF.

Circulating fragments of TNFα, TNFR1 and TNFR2 are generated from full, transmembrane proteins through the activity of TACE [101,102]. We hypothesized that increased circulating levels of TNFR1 and TNFR2 might reflect increased activity of TACE. ACE2 is also a substrate of TACE, and plasma ACE2 activity increases in conjunction with increased TACE activity [101,106]. Since TACE is membrane-bound, as a surrogate of TACE-activity, we measured plasma ACE2 activity in HFPEF patients relative to healthy controls. Previous work showed a significant association between ACE2 and symptom severity and adverse clinical outcomes in patients with HFREF [99,107]. We did not find this relationship in HFPEF, but found plasma ACE2 activity to be elevated to relative controls. This is consistent with the idea that elevated ACE2 activity reflects secondary neurohormonal dysregulation in HFPEF, as proposed by WJ Paulus and C Tschöpe [14]. In contrast, dysregulated ACE2 may be a component of the neurohormonal dysregulation that is a primary driver of HFREF [99,107].

3.4.1. Conclusion

Taking our data together with previous reports indicates that the TNFα-axis is a component of the dysregulated inflammatory signaling that characterizes HFPEF [27-29,31,46,48,50,51]. In the context of the failures of the Anti-TNF Therapy Against Congestive Heart Failure (ATTACH) [111] and Randomized Etanercept Worldwide Evaluation (RENEWAL) [112] trials, our data suggest that a downstream approach involving TNFR1 inhibition or TNFR2 potentiation might represent a more effective therapeutic approach for patients with HFPEF.

3.4.2. Limitations

This study is relatively small compared to other recent biomarker studies in HF. Indeed, TNFα might also be informative with respect to differentiating HFPEF from HFREF, but our study cannot resolve whether this is the case. Also, our results only provide correlative evidence of whether these biochemical markers are primary mediators

of the HF syndrome, or end-effectors of pathogenic processes. While there is likely an interplay between plasma and tissue levels for TNFR1 and TNFR2—shedding increases plasma levels at the expense of tissue levels—direct tissue and plasma comparisons from the same subjects will be necessary to determine the relative effects of proteolytic processing and changes in gene expression.

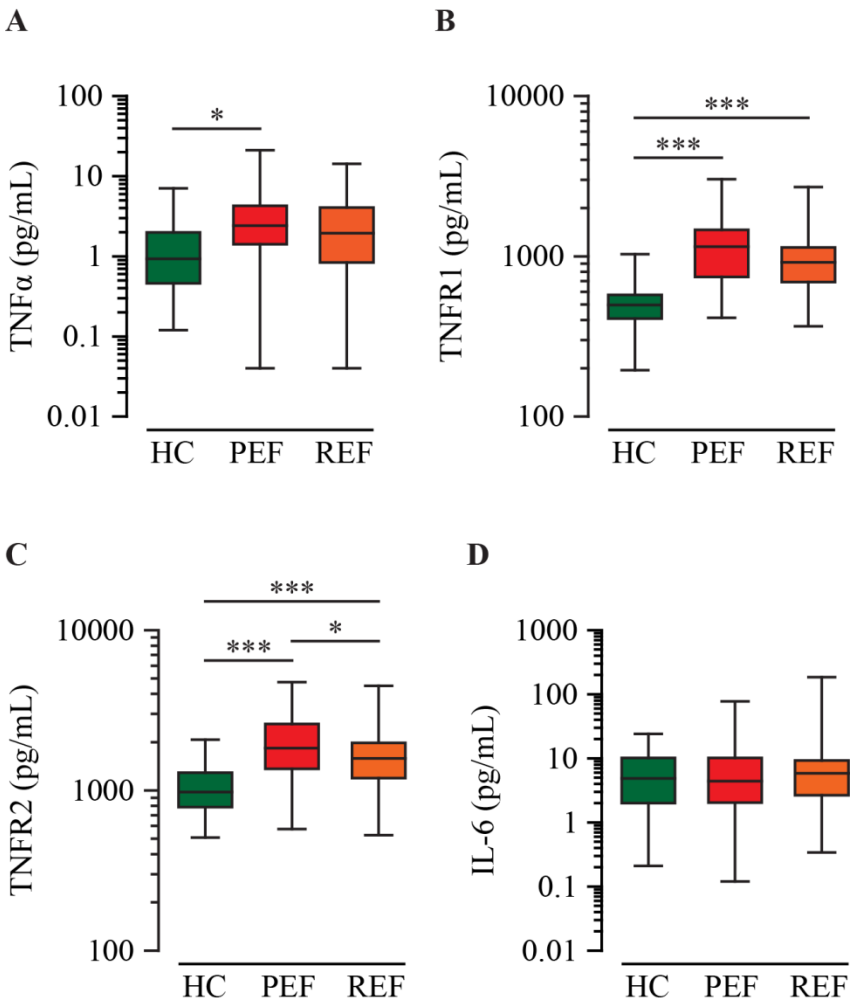

Figure 3.1. Plasma Levels of Inflammatory Markers between Study Groups

Circulating TNF-α (A), TNFR1 (B), TNFR2 (C) and IL-6 (D) levels in healthy control (HC), HFPEF (PEF) and HFREF (REF). * P<0.05, *** P<0.001 for Kruskal-Wallis Test with pairwise comparisons.

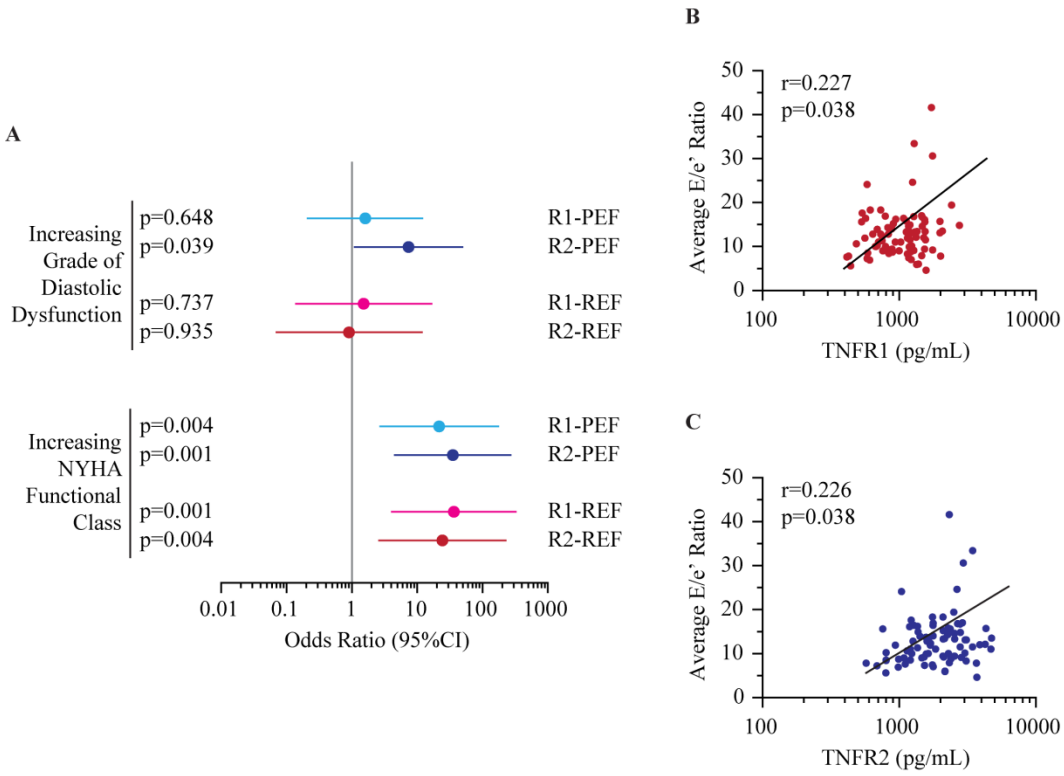

Figure 3.2. Associations of TNFR1 and TNFR2 with Diastolic Dysfunction and Disease Severity

Odds ratios and 95% confidence intervals (95%CI) for ordinal logistic regression analyses to evaluate TNFR1 (R1) or TNFR2 (R2) as predictors of increasing grade of diastolic dysfunction or NYHA class in HFPEF (PEF) or HFREF (REF) (A). Average E/e' ratio as a function of TNFR1 (B) and TNFR2 (C) in HFPEF.

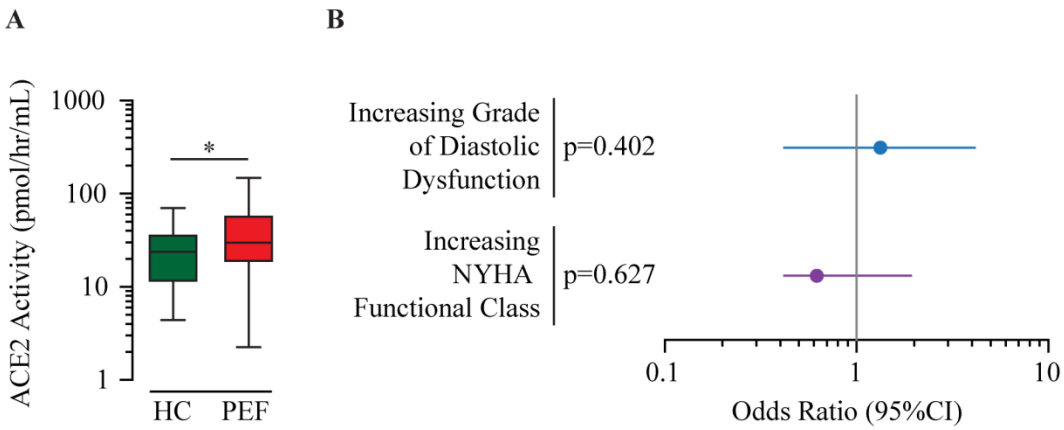

Figure 3.3. Angiotensin-Converting Enzyme 2 (ACE2) in HFPEF

Plasma ACE2 activity in healthy control (HC) and HFPEF (PEF) (A). Odds ratios and 95% confidence intervals (95%CI) for ordinal logistic regression analyses to evaluate ACE2 as predictor of increasing grade of diastolic dysfunction or NYHA class in HFPEF (B). * P<0.05 for Mann Whitney U test.

Table 3.1. Clinical Profiles of Study Subjects

Demographics	**HC**	**HFPEF**	**HFREF**	**p-value**
Number	50	100	100	---
Age, years	54 (52-62)	72 (63-79)	65 (59-73)	<0.001
Sex: male, %	48	62	71	0.029
Physical Characteristics				
Obese, %	22	59	42	<0.001
Systolic BP, mmHg	122 (115-136)	128 (118-141)	119 (104-132)	0.005
Diastolic BP, mmHg	74 (67-78)	71 (63-79)	72 (64-80)	0.449
Medical History				
Smoker, %	18	61	52	<0.001
HTN, %	N/A	78	60	0.006
DM, %	N/A	46	36	0.134
PVD, %	0	10	5	0.071
AFib, %	N/A	52	40	0.089
NYHA Class, %				0.072
I	N/A	12	22	
II	N/A	56	47	
III	N/A	32	28	
IV	N/A	0	3	
Primary Etiology of HF, %				<0.001
Ischemic	N/A	14	37	
Non-ischemic	N/A	86	63	
Laboratory Values				
BNP, pg/mL	16 (11-28)	76 (44-236)	162 (79-398)	<0.001
SrCR, µM	72 (59-85)	99 (79-138)	96 (82-116)	<0.001
Total Cholesterol, mM	5.4 (4.8-5.8)	3.8 (3.2-4.4)	3.6 (3.0-4.4)	<0.001
Triglycerides, mM	1.3 (0.8-1.6)	1.2 (0.9-1.8)	1.3 (0.9-2.4)	0.662
Cholesterol: HDL Ratio	3.9 (3.1-4.5)	3.3 (2.8-4.3)	3.6 (2.9-4.5)	0.276
eGFR, mL/min/1.73m²	76 (62-85)	57 (41-78)	59 (48-72)	<0.001

Medication				
Antiarrhythmic, %	N/A	11	7	0.323
ACEi or ARB, %	N/A	86	89	0.521
Beta-blocker, %	N/A	30	36	0.367
Digoxin, %	N/A	11	16	0.301
Loop diuretic, %	N/A	78	68	0.111
MRA, %	N/A	19	38	0.003
NSAIDs, %	0	8	8	0.118
Thiazide diuretic, %	N/A	12	7	0.228
Statin, %	2	73	68	<0.001

Abbreviations: HC, healthy control; HFPEF, heart failure (HF) with preserved ejection fraction; HFREF, HF with reduced left-ventricular ejection fraction; BP, blood pressure; HTN, hypertension; DM, diabetes; PVD, peripheral vascular disease; AFib, atrial fibrillation; NYHA, New York Heart Association; BNP, B-type natriuretic peptide; SrCr, serum creatinine; HDL, high-density lipoprotein; eGFR, estimated glomerular filtration rate; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor antagonist/blocker; MRA, mineralocorticoid receptor antagonist; and NSAIDs, non-steroidal anti-inflammatory drugs. P-value represents Mann-Whitney U Test, Kruskal-Wallis Test or Chi-square Test where appropriate. Number was not tested, as the sample sizes were selected *a priori*.

Table 3.2. Electrocardiogram and Echocardiogram

	HC	HFPEF	HFREF	p-value
HR, bpm	65 (60-76)	65 (60-78)	65 (60-76)	0.757
AFib, %	0	43	23	<0.001
LVEF, %	63 (60-67)	59 (54-63)	35 (27-41)	<0.001
LV EDD, cm	4.4 (4.1-4.6)	4.8 (4.3-5.2)	5.9 (5.4-6.4)	<0.001
LV ESD, cm	2.8 (2.5-3.2)	3.1 (2.8-3.6)	4.7 (3.8-5.6)	<0.001
LVPW, cm	0.9 (0.8-1.0)	1.1 (1.0-1.2)	1.0 (0.9-1.1)	<0.001
LVMI, g/m²				
Female	62 (54-69)	89 (78-111)	103 (80-119)	<0.001
Male	74 (56-87)	105 (83-119)	129 (104-152)	<0.001
LVH, %	6	34	60	<0.001
LA index, mL/m²	23 (19-27)	34 (28-43)	37 (30-50)	<0.001
MR, %				<0.001
None	76	45	32	
Trace/mild	24	43	41	
Moderate	0	11	20	
Severe	0	1	7	
E-wave velocity, cm/s	74 (67-82)	87 (73-107)	76 (60-97)	<0.001
Medial E/e' ratio	9 (8-11)	14 (10-17)	15 (11-20)	<0.001
Lateral E/e' ratio	7 (6-8)	10 (8-13)	11 (8-16)	<0.001
Average E/e' ratio	8 (7-10)	12 (9-15)	13 (10-18)	<0.001

A-wave velocity, cm/s	68 (61-78)	79 (63-96)	74 (55-89)	0.052	
E/A ratio	1.0 (0.8-1.2)	0.9 (0.8-1.3)	1.0 (0.6-1.6)	0.745	
Grade: diastolic dysfunction*, %				<0.001	
0 (Normal)	94	34	14		
I (Impaired relaxation)	4	25	31		
II (Pseudonormal filling)	2	24	34		
III (Restrictive filling)	0	17	21		

Abbreviations: HC, healthy control; HFPEF, heart failure (HF) with preserved ejection fraction; HFREF, HF with reduced left-ventricular ejection fraction; LVEF, left-ventricular ejection fraction; LV EDD, left ventricle (LV) end diastolic dimension; LV ESD, LV end systolic dimension; LVPW, LV posterior wall thickness; LVMI, LV mass indexed to body surface area (BSA); LVH, left-ventricular hypertrophy; LA index, left-atrial volume indexed to BSA; MR, mitral regurgitation; E-wave, early diastolic wave velocity; e', mitral valve annular velocity as measured medially or laterally by way of tissue Doppler imaging; and A-wave, late diastolic velocity due to atrial systole. P-value represents Kruskal-Wallis Test or Chi-square Test where appropriate. *Grade of diastolic dysfunction excluding those patients with severe MR or poor visualization on echocardiography.

Table 3.3. Associations of Risk Factors and Inflammatory Markers

	TNFα		**TNFR1**		**TNFR2**		**IL-6**	
Scale	*R*	*p-value*	*R*	*p-value*	*R*	*p-value*	*R*	*p-value*
Age	0.077	0.331	0.204	0.004	0.161	0.023	0.038	0.617
eGFR	0.187	0.02	0.534	<0.001	0.433	<0.001	0.082	0.28
Binary	*OR (95%CI)*	*p-value*	*OR (95%CI)*	*p-value*	*OR (95%CI)*	*p-value*	*OR (95%CI)*	*p-value*
Sex	1.68 (0.87-3.23)	0.121	0.60 (0.13-2.76)	0.513	0.50 (0.11-2.26)	0.368	1.42(0.78-2.57)	0.251
Obese	1.40 (0.75-2.62)	0.294	2.27 (0.54-9.60)	0.266	1.84 (0.45-7.60)	0.397	1.17 (0.67-2.05)	0.578
LVH	1.68 (0.84-3.36)	0.142	0.69 (0.16-2.99)	0.622	0.46 (0.11-1.95)	0.29	1.13 (0.64-1.99)	0.677
HTN	2.04 (1.04-4.00)	0.038	11.59 (2.21-60.75)	0.004	3.24 (0.69-15.18)	0.136	0.64 (0.34-1.19)	0.162
DM	1.58 (0.82-3.04)	0.172	20.21 (4.05-100.96)	<0.001	11.29 (2.42-52.75)	0.002	0.97 (0.55-1.71)	0.918
Smoker	2.00 (1.04-3.82)	0.037	1.68 (0.39-7.13)	0.485	3.57 (0.84-15.24)	0.085	0.95 (0.54-1.67)	0.857
PVD	1.04 (0.35-3.12)	0.944	23.63 (1.30-430.17)	0.037	11.78 (0.68-204.67)	0.09	1.04 (0.37-2.92)	0.942
AFib	1.97 (1.01-3.81)	0.045	4.09 (0.94-17.77)	0.06	4.81 (1.12-20.74)	0.035	0.90 (0.52-1.58)	0.716

Abbreviations: TNFα, tumor necrosis factor-alpha; TNFR1, TNFα receptor 1; TNFα receptor 2; eGFR, estimated glomerular filtration rate; LVH, left-ventricular hypertrophy; HTN, hypertension; DM, diabetes mellitus; PVD, peripheral vascular disease; and AFib, atrial fibrillation.

CHAPTER 4
THE ROLE OF LEFT-ATRIAL STRUCTURAL AND FUNCTIONAL REMODELING

4. The Role of Left-Atrial Structural and Functional Remodeling

4.1. Introduction and Rationale

Patients with chronic HF are routinely characterized according to LV structure or function, such as classification based on LVEF [3]. In contrast, the informative value of the structure or function of the LA is generally confined to maximum volume assessment. Complementary ECG is then typically performed to assess rhythm, whereupon AFib, the electrophysiological abnormality where LA contractility is lost, is a frequent finding for patients in HF. Extensive evidence exists that AFib is a significant contributor to HF morbidity. Indeed, reports showed that patients with HF that have concurrent AFib or experience incident AFib have reduced exercise capacity and a greater risk for adverse outcomes than their counterparts in sinus rhythm (SR) [113-116]. While part of the morbidity associated with AFib is due to its predisposing sufferers to lethal ventricular dysrhythmias, the hemodynamic consequences of AFib, particularly with respect to LV SV may also be impactful.

To this end, there is evidence that LA biomechanical dysfunction is also indicative of reduced exercise capacity in patients with HFREF [117], and is a characteristic finding in both HFPEF and HFREF [80,118]. Nonetheless, the collective understanding of the biomechanical role of the LA during early- and late-filling phases in diastole remains incomplete. Sophisticated analyses of LA structural and functional remodeling, beyond rhythm assessment by ECG or maximal volume determination by imaging, allow the determination of reservoir (elastic recoil) versus booster pump (active contraction) contribution to LV hemodynamics [119]. The study reported in this chapter explored the variation in LA structure and function within the syndrome of HF, and, to our knowledge, is the first MRI-based report of LA function in the context of LV function for patients with chronic HF. We tested the hypothesis that, similar to LV structure and function, a continuum of LA structural and functional remodeling exists in HF, which may partially be independent of LV remodeling. Cardiac remodeling was assessed using biochemical markers and CMR. MR-proANP and NT-proBNP were used as the biochemical reflectors of cardiac remodeling processes, as expression of these natriuretic peptides increases markedly in conditions of cardiomyocyte stress or disease

[53,54]. MR-proANP and NT-proBNP were measured in place of ANP and BNP, respectively, as regional propeptides are likely better reflectors of *NPPA* and *NPPB* expression [57,58,62].

4.2. Materials and Methods

4.2.1. Study Participants

A subset of the 250 study subjects recruited for the analyses in Chapter 3 were selected for this study. Only study subjects from the Edmonton study site who underwent MRI were available. After excluding those individuals with moderate or severe valvular lesions as assessed by CMR, 27 healthy community-based individuals and 68 ambulatory patients with clinical diagnoses of chronic HF, for a total 95 subjects, constituted the study group. In order to capture a range of patients along the HF spectrum from HFREF to HFPEF, LVEF was not used as a selection criterion for this study—it was simply included as a study parameter during data analysis.

4.2.2. Biochemical Markers

Blood for plasma analysis was collected on the same day that CMR assessments were performed. Plasma collected into EDTA anticoagulant tubes was used for the analysis of MR-proANP and NT-proBNP levels. MR-proANP was measured using the BRHAMS KRYPTOR Immunoassay platform (Thermo Fisher Scientific, Portage, MI, USA) as previously described [62]. Testing was performed off-site at the Phadia Immunology Research Lab in Portage, MI, USA. NT-proBNP was measured using the Roche Elecsys 20.10 Immunoanalyzer platform (Roche Diagnostics, Laval, QC, CAN) as previously described [96]. Testing was performed at an Alberta Health Services laboratory.

4.2.3. Cardiac MRI

Cardiac MRI was performed on a 1.5T Sonata MRI platform (Siemens Healthcare, Erlangen, DEU) at the Mazankowski Alberta Heart Institute Elko MRI Centre under the direction of cardiologists and technicians with specialized CMR training. Left-atrial tracings were performed using three long-axis view of the heart: two-chamber (LA and LV), three-chamber (LA; LV outflow tract; LV; and partial right

ventricle, RV) and four-chamber (LA; LV; right atrium, RA; and RV). Using Matrix Laboratory (MATLAB) software version R2012a (MathWorks, Natick, MA, USA), LA areas and lengths were traced in order to compute a weighted average volume using a triplane area-length method. The weighted volume equation for LA volume was built into a customized MATLAB program that had been cross-checked by contributing developers: Mr. Kelvin Chow and Drs. Joseph Pagano, Richard Thompson and D. Ian Paterson, all from the University of Alberta. Length tracings were from the mitral annular plane to the LA base. Area tracings were made along the inner border of the LA, excluding wall tissue, pulmonary vein inlets and LA appendage, and ending at the plane of the mitral annulus. Using 2-, 3- and 4-chamber views, LA volumes were calculated at three phases of the cardiac cycle: end diastole, diastasis and end systole. For the measurement in end systole, the area from the plane of the mitral annulus to the point of leaflet coaptation was also excluded, as diastasis and end diastole measurements were made with an open mitral valve, which necessitated defining the border of the LA to be the mitral annulus. Left atrium volumes were indexed to body surface area (BSA), which was derived from ideal bodyweight (IBW), as described below. This was done to account for the significant prevalence of obesity in the HF cohort. Left atrium or ventricle volumes at end diastole, diastasis and end systole indexed to BSA are denoted as EDVI, DiVI and ESVI, respectively. Left ventricle volumes were available in the Mazankowski Alberta Heart institute database, and were analyzed by Dr. D. Ian Paterson.

4.2.4. Cardiac Function Analyses

From measured cardiac chamber volumes, we determined basic functional volumes indexed to BSA. For the LA, we calculated the elastic emptying volume index (LA EEVI; LA ESVI – LA DiVI), active emptying volume index (LA AEVI; LA DiVI – LA EDVI), and total emptying volume index (LA TEVI; LA ESVI – LA EDVI). LA EEVI is a reflection of the reservoir capacity of the LA, while LA AEVI is a reflection of the active contractility of the LA. For the LV, we calculated stroke volume index (SVI; LV EDVI – LV ESVI) and also measured LVEF. From LA EEVI, LA AEVI and LA TEVI, we calculated the reservoir (elastic), booster pump (active) and total LA contributions to LV SV, expressed as percentage.

4.2.5. Data Analyses

Ideal bodyweight was calculated using the Robinson equation [120]. Body surface area was calculated based on IBW using the Du Bois equation to control for the significant adiposity and prevalence of overweight/obesity in HF patients [121].

4.3. Results

4.3.1. Baseline Clinical Characteristics

Heart failure subjects were significantly older and more likely to be male than their healthy counterparts (Table 4.1). Despite similar population heights, HF subjects were significantly heavier and substantially more were obese (Table 4.1). Histories of hypertension, diabetes, and AFib were much more prevalent in the HF group by design, as control subjects were necessarily not afflicted by these conditions as an enrollment criterion (Table 4.1). However, HF subjects were also significantly more likely to be current or former smokers, an uncontrolled risk factor (Table 4.1). In general, the HF group was well-treated with evidence-based therapeutics, while no healthy control was on medications for cardiovascular disease or risk factors, such as hypercholesterolemia (Table 4.1). The generally poorer cardiovascular health of the HF group was also reflected in the 26% of these subjects that were in AFib at the time of study, but was not reflected in HR, which did not differ significantly between the two groups (Table 4.2). Of the 26 HF subjects (38%) with a history of AFib, nine were in SR on their study day, while one study subject without a prior history did have AFib on their study day according to ECG.

4.3.2. Cardiac Biomarkers and Structure

Plasma levels of MR-proANP and NT-proBNP were significantly elevated in the HF group (Table 4.2). This reflected significantly larger indexed LA volumes, at end diastole (LA EDVI), diastasis (LA DiVI) and end systole (LA ESVI), in the HF group relative to controls (Table 4.2). Indeed, this was also true for controls: we found significant relationships between MR-proANP and LA EDVI, LADiVI and LA ESVI in the total study subject cohort (Figure 4.1 A-C). Significant relationships were found between NT-proBNP and LA EDVI ($r=0.675$, $p<0.001$), LA DiVI ($r=0.629$, $p<0.001$)

and LA ESVI (r=0.542, p<0.001), as well. This relationship was further highlighted by the strong correlation between NT-proBNP and MR-proANP levels (Figure 4.1 D).

To account for significant differences in age and sex ratios between the control and HF groups, we included these as covariates in a limited logistic regression model to test the relationship between indexed LA chamber volumes and presence of HF. We found that increased LA EDVI (OR, 95%CI: 1.08, 1.03-1.14; p=0.004) and LA DiVI (OR, 95%CI: 1.07, 1.02-1.11; p=0.003) remained independently associated with the presence of HF. LA ESVI was slightly non-significant after including the aforementioned covariates (OR, 95%CI: 1.03, 1.00-1.06; p=0.052).

Similar to LA volumes, indexed LV volumes at end diastole (maximum volume; LV EDVI) and end systole (minimum volume; LV ESVI) were significantly larger in the HF group, although the ranges overlapped substantially, which shows the wide variation in phenotypes captured in our study, from concentric to eccentric remodeling (Table 4.2). Indexed LV masses (LVMI) were also significantly higher in the HF group, indicating generally prevalent LV hypertrophy, regardless of remodeling type, in this group (Table 4.2).

4.3.3. Relationships in Cardiac Structure and Function

Left-atrial elastic and total emptying volume indices were significantly lower in the HF group, while AEVI was not; however, the range was substantially larger in the HF group due to the many subjects with no detectable active LA contribution (Table 4.2). Stroke volume indexed to BSA, meanwhile, was not significantly different between the two groups, while LVEF was, although the ranges overlapped due to the range in HF phenotypes studied from HFPEF to HFREF (Table 4.2). Despite strong relationships with LA chamber volumes at three phases of the cardiac cycle, neither MR-proANP nor NT-proBNP showed any relationship with LA AEVI in the combined (r=0.134, p=0.276 and r=0.210, p=0.107, respectively) or HF cohorts (r=0.026, p=0.866 and r=0.098, p=0.546, respectively).

For control subjects, the median elastic contribution to LV SV was 24% (IQR: 17-30%; Figure 4.2 A), the median active contribution was 24% (IQR: 21-31%; Figure 4.2 A) and the median total contribution was 50% (IQR: 41-55%). The elastic and active contributions were approximately normally distributed for control subjects (Figure 4.2 A). In HF subjects, elastic LA contribution to LV SV followed a generally unimodal distribution that was smaller in magnitude than that of controls (median, IQR: 14%, 8-23%; p=0.001; Figure 4.2 B); however, there was a bimodal distribution pattern in active LA contribution to LV SV in HF (median, IQR: 23%, 0-34%) that was not significantly different than control (p=0.163, Figure 4.2 B). The bimodal distribution for active LA contribution in HF subjects showed two distinct groups: one where the LA no longer makes an active contribution to LV SV and another where the LA, on average, makes a marked contribution that is comparable to healthy individuals. The majority of HF subjects with no active contribution were in AFib on the study day, although seven of 43 HF patients in SR had less than 10% active contributions, with five of these exhibiting less than 2.5% contribution, which were thus defined as zero contribution for the purpose of categorizing subjects.

Heart failure subjects that had a non-zero active LA contribution to LV SV had a slightly, but significantly, larger contribution than controls (Figure 4.2 C). Altogether, total LA contribution to LV SV was significantly lower in HF (median, IQR: 36%, 26-43%; $p<0.001$). Interestingly, elastic and active SV contributions did not correlate in HF or control, which shows the variety in LA involvement from an elastic and active perspective (Figure 4.2 D). Furthermore, active LA contribution to SV was not associated with LA volume after contraction, LA EDVI, in HF patients (r=0.033, p=0.836), which indicates that those individuals with large LA, who are not in AFib, could have substantial active LA contributions to LV SV. Finally, we evaluated whether LVEF was associated with active LA contribution to LV SV, but found no relationship in the HF group when considering those individuals with non-zero active contributions (r=0.145, p=0.353).

4.3.4. Impact of Rate and Rhythm on Function

Heart rate was not a determinant of active LA contribution to LV SV (r=0.187, p=0.140). In the HF cohort, both present AFib as assessed by ECG and a history of AFib were significantly associated with increased log-transformed MR-proANP and NT-proBNP levels, and with increased LA EDVI, LA DiVI and LA ESVI on logistic regression analyses (Table 4.3).

AFib on ECG and history of AFib were both significantly associated with larger elastic contributions to SV (Table 4.3). Since individuals in AFib on the day of study necessarily did not have active LA contributions to LV SV, we did not evaluate this association (Table 4.3). When zero-value contributions were included, history of AFib was significantly associated with decreased active LA contribution to LV SV; however, this relationship was non-significant when considering only those individuals with a non-zero active LA contribution to LV SV (Table 4.3). Indeed, of nine HF subjects with a history of AFib, but in SR on the day of examination, seven had greater than 10% active LA contributions to LV SV, which ranged from 17 to 61% contribution.

4.4. Discussion

In this comparative analysis of healthy control subjects and an HF population, comprised of a range of subjects encompassing HFPEF and HFREF, we evaluated LA structural and functional remodeling. In terms of prevalence of comorbid conditions and general demographics, the HF population studied was characteristic of community-dwelling populations living with chronic HF [12,15,90,91]. The HF patients in our study were also characteristic of a community-based HF group with respect to disease severity: the majority of patients were in NYHA classes II and III HF, while none were in class IV [122].

In keeping with previous findings, LA volumes were strongly correlated with increased plasma MR-proANP and NT-proBNP levels, which are significantly elevated in HF relative to control [123]. We found that indexed LA volumes at end diastole and diastasis were significantly elevated in HF, even after controlling for the significantly advanced age and larger proportion of males in the HF group relative to controls. Left-

trial enlargement has previously been identified as both an indicator for incident HF risk [73,76] and a characteristic finding in symptomatic HF [8,75]. Interestingly, increased LA volume at end systole was not significantly associated with presence of HF after controlling for age and sex. This is may be due to elasticity healthy LA myocardium that allows stretching to maximal volumes that are similar to those seen in the HF group. The similarity between age- and sex-corrected maximal LA volumes may prove to be a challenge to effectively using indexed LA volume at end systole as a risk stratification criterion, as has been proposed [124]. While small, our study provides evidence that indexed LA volume at end diastole may be a better reflector of true LA enlargement.

Stiff LA myocardium will not store as much elastic potential energy if filling pressures do not increase, and thus may not have as much elastic recoil. In this case, the volume change from end systole to diastasis would be reduced, which we found to be true of HF patients: elastic change in LA volume and elastic contribution to LV SV were both significantly reduced relative to healthy controls. On the other hand, and of great interest, was the finding that active change in LA volume and active LA contribution to LV SV were not significantly reduced in the HF group. Indeed, we showed a substantial range for active contribution, where those individuals in the HF group with a non-zero active contribution actually had a slightly, but significantly higher median active contribution than healthy controls, and this was independent of LVEF, and elastic contribution. This adds to the small body of work that has examined LA reservoir and booster pump functions in LV hemodynamics [79,117-119], but is, to our knowledge, the first report of LA function in the context of the LV for HF patients using powerful CMR techniques. The remaining proportion of LV SV flows through the LA as fluid through a conduit; those individuals with minimal elastic or active contributions are thus less reliant on LA elasticity or contractility, respectively, to achieve a sufficient LV SV. Indeed, we observed seven individuals in SR who had less than 10% active contribution, of which five had a near-zero contribution. In contrast, it is those individuals with large active LA contributions to LV SV who would be most affected by changes in LA biomechanical function, such as in episodes of paroxysmal AFib.

Interestingly, we found that patients with a history of paroxysmal AFib may have significant active LA volume changes when in SR. We further found that active contribution to LV SV and LA EDVI did not correlate, which suggests that individuals may have large LA while still relying on substantial booster pump capacity. This confluence is alarming, because enlarged LA are good substrates for the onset of AFib [125], and LA enlargement follows with the progression of LVDD [71], a common finding in HF [3].

Coupling the above information with the finding that both MR-proANP and NT-proBNP are strongly associated with the presence and history of AFib presents the salient potential for using these biochemical markers as risk stratification tools. However, we found that the two aforementioned biomarkers are significantly increased in HF relative to healthy controls, so resolving cut-off values for risk stratification will require large data samples.

Those HF patients whose LA make a large active contribution to LV SV may be those who benefit most from restoration of the biomechanical function of the LA through a rhythm control strategy. This is a point that should be investigated further, as the evidence is presently conflicted. Indeed, the Atrial Fibrillation in Congestive Heart Failurem (AF-CHF) trial found that a rhythm control strategy was no more effective in HFREF patients with AFib in reducing cardiovascular or all-cause mortality, or secondary outcomes, such as strokes; however, these results cannot be generalized to HFPEF patients [126]. Furthermore, our data show that active LA contribution is independent of LVEF, which should prompt the re-evaluation of HF management strategies for some HFREF patients. Given findings that reduced LA strain during LV systole is a risk factor for returning to AFib after cardioversion [127], natriuretic peptides, clinical history and measurements of relative LA reservoir and booster pump contributions may identify those HF patients in whom an actively contracting LA is most critical, but who are at a greatest risk of an episode of AFib.

4.4.1. Conclusion

These data indicate that the LA may play a significant role in maintaining cardiac output and effective hemodynamics, even in HF patients with enlarged LA and a history of paroxysmal AFib. This warrants further study to explore if this information can add value to risk-stratification schemes in HF. Combined with MR-proANP, NT-proBNP or both to quantify risk of AFib, LA size and function measurements could help tailor management strategies for some HF patients. Our data indicate that it is possible that a small subset of the patients in the AF-CHF trial [126] had a significant active LA contribution to LV SV and had a history of paroxysmal AFib, and thus may have benefited more from rhythm control, but that this effect was not substantial enough to show in the entire cohort studied.

4.4.2. Limitations

The sample size of the study is small from the standpoint of making generalizations about the entire HF population. Furthermore, patients with electrophysiological abnormalities severe enough to warrant implantable device therapy could not be studied due to the nature of CMR.

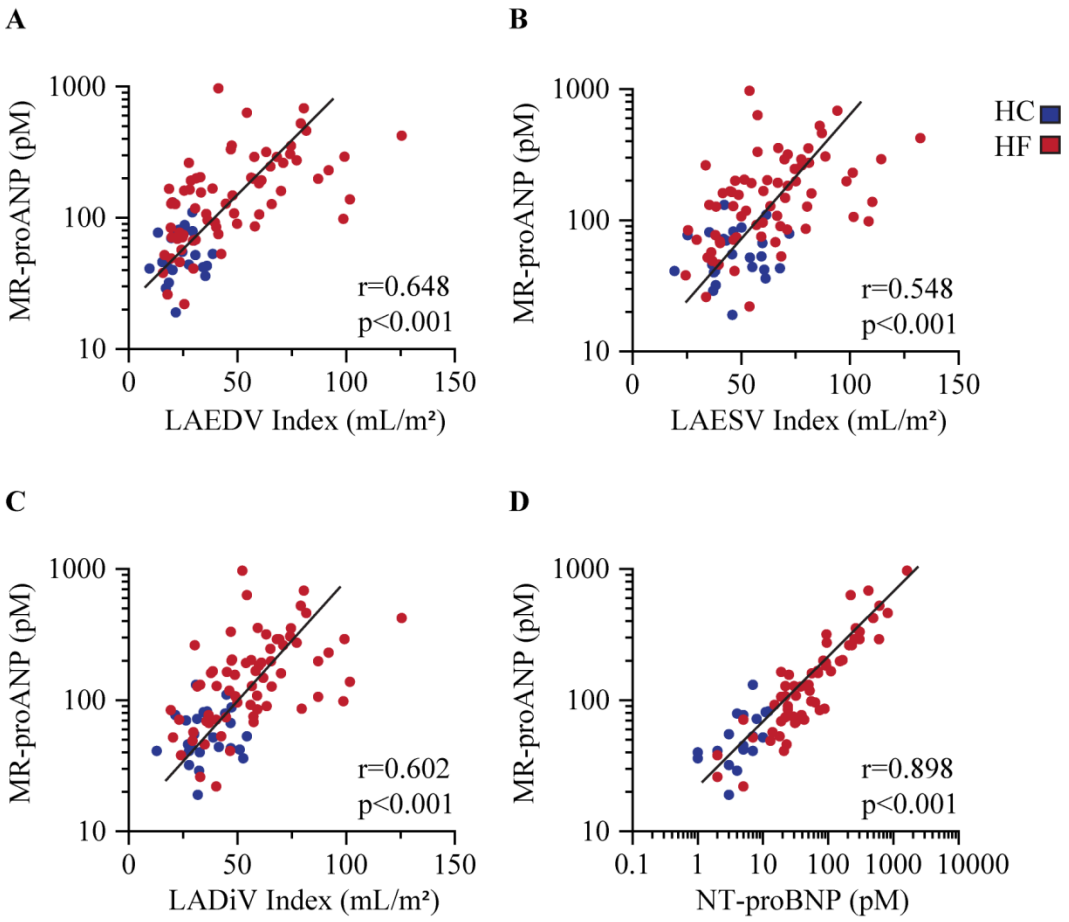

Figure 4.1. Correlates of Natriuretic Peptides

MR-proANP levels as a function of LA EDVI (A), LA DiVI (B) or LA ESVI (C). Association between MR-proANP and NT-proBNP levels (D). HC in dark; HF in light.

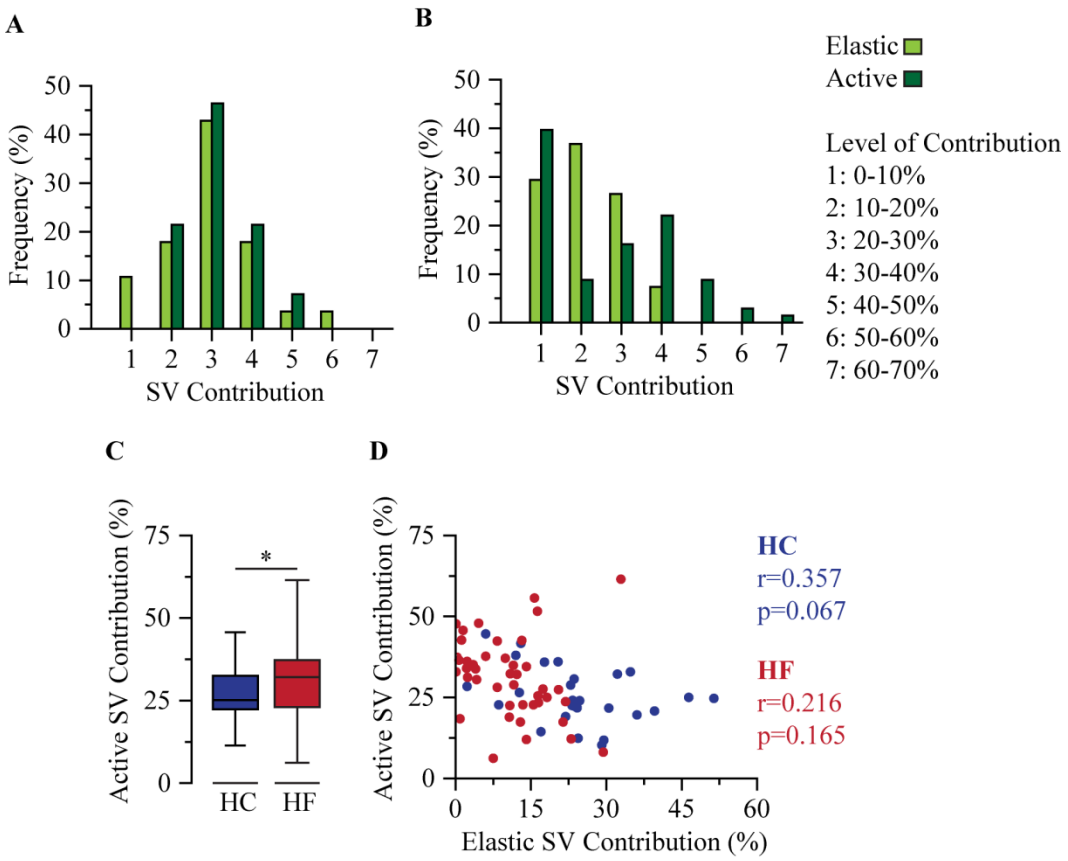

Figure 4.2. Elastic and Active LA contributions to Stroke Volume

Elastic (light) and active (dark) LA contributions to LV SV in healthy controls (HC) (A) and HF patients (B). Active LA contribution to LV SV in healthy controls (dark) and HF patients with non-zero contributions (light) (C). Active as a function of elastic LA contribution to LV SV in healthy (dark) and HF (light) subjects. * $p<0.05$ for Mann-Whitney U Test.

Table 4.1. Clinical Profiles of Study Subjects

Demographics	**HC**	**HF**	**p-value**
Number	27	68	---
Age, years	54 (52-68)	69 (60-75)	<0.001
Sex, % male	37	60	0.033
Patient Characteristics			
Height, cm	170 (160-173.5)	173 (165-178)	0.239
Weight, kg	75 (67-84)	89.5 (79.8-101.3)	<0.001
BMI, kg/m^2	25.5 (22.9-28.0)	30 (28.1-33.4)	<0.001
Obese, %	15	50	0.002
SBP, mmHg	116 (104-133)	126 (114-139)	0.327
DBP, mmHg	69 (62-80)	76 (65-84)	0.383
NYHA Class			---
I	0	18	
II	0	57	
III	0	25	
Medical History			
HTN, %	0	65	---
DM, %	0	35	---
Smoker, %	19	51	0.003
AFib, %	0	38	---
Medications			
Antiarrhythmic, %	0	7	0.148
ACEi/ARB, %	0	88	<0.001
ASA, %	0	60	<0.001
Antithrombotic, %	0	44	<0.001
Betablocker, %	0	0	---
CCB, %	0	29	0.002

Digoxin, %	0	7	0.148
Loop diuretic, %	0	75	<0.001
MRA, %	0	18	0.020
Thiazide diuretic, %	0	6	0.198
Statin, %	0	71	<0.001

Abbreviations: HC, healthy control; HF, heart failure; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; NYHA, New York Heart Association; HTN, hypertension; DM, diabetes mellitus; AFib, atrial fibrillation; ACEi/ARB, angiotensin-converting enzyme inhibitor/angiotensin II receptor blocker; ASA, acetylsalicylic acid; CCB, calcium channel blocker; and MRA, mineralocorticoid receptor antagonist. P-value represents Mann Whitney U Test or Fisher's Exact Test where appropriate.

Table 4.2. Cardiac Biomarkers, Structure and Function

Cardiac Biomarkers	**HC**	**HF**	**p-value**
MR-proANP, pM	52 (41-77)	143 (82-250)	<0.001
NT-proBNP, pM	5 (3-7)	54 (22-151)	<0.001
Heart Rate, bpm	63 (58-72)	67 (60-80)	0.148
Rhythm			<0.001
NSR, %	100	74	
AFib, %	0	26	
Cardiac Structure			
LA EDV Index, mL/m^2	23.0 (18.5-29.6)	42.5 (27.6-65.1)	<0.001
LA DiV Index, mL/m^2	32.6 (28.3-44.6)	56.2 (40.2-68.4)	<0.001
LA ESV Index, mL/m^2	45.9 (38.2-57.2)	60.1 (46.1-77.7)	<0.001
LV EDV Index, mL/m^2	75.6 (69.4-83.2)	93.6 (76.6-125.9)	0.001
LV ESV Index, mL/m^2	30 (22.9-33.5)	46.9 (31.3-74.6)	<0.001
LV Mass Index, g/m^2	52.7 (49.7-60.5)	82.4 (69.2-96)	<0.001
Cardiac Function			
Elastic EVI, mL/m^2	10.5 (7.4-14.7)	6.3 (3.2-9.4)	<0.001
Active EVI, mL/m^2	11.1 (9.3-15.2)	10.4 (0-15.1)	0.154
Total EVI, mL/m^2	21.8 (20.0-26.4)	15.8 (9.7-19.0)	<0.001
Stroke Volume Index, mL/m^2	46.7 (42.2-48.7)	44.1 (37.4-52.0)	0.253
LVEF, %	61 (59-64)	50 (38-60)	<0.001

Abbreviations: MR-proANP, mid-regional pro-A-type natriuretic peptide; NT-proBNP, N-terminal pro-B-type natriuretic peptide; NSR, normal sinus rhythm; AFib, atrial fibrillation; LA EDV, left atrium (LA) end diastolic volume; LA DiV, LA diastasis volume; LA ESV, LA end systolic volume; LV EDV, left ventricle (LV) end diastolic volume; LV ESV, LV end systolic volume; EVI, emptying volume index; and LVEF, LV ejection fraction.

Table 4.3. Parameters Associated with Atrial Fibrillation

	AFib on ECG		**History of AFib**	
Cardiac Biomarkers	*OR (95%CI)*	*p-value*	*OR (95%CI)*	*p-value*
MR-proANP	76.84 (6.54-902.48)	0.001	33.19 (4.36-252.81)	0.001
NT-proBNP	11.98 (2.73-52.62)	0.001	4.74 (1.57-14.34)	0.006
Left-Atrial Volumes				
LA EDVI	1.10 (1.05-1.15)	<0.001	1.09(1.05-1.13)	<0.001
LA DiVI	1.08 (1.04-1.12)	<0.001	1.09 (1.05-1.13)	<0.001
LA ESVI	1.07 (1.03-1.11)	<0.001	1.08 (1.04-1.13)	<0.001
Left-Atrial Function				
Elastic Contribution to LV SV	1.10 (1.03-1.17)	0.004	1.07 (1.01-1.13)	0.016
Active Contribution to LV SV (Zero Values Included)	Not applicable		0.93 (0.90-0.97)	<0.001
Active Contribution to LV SV (Zero Values Excluded)	Not applicable		1.00 (0.94-1.07)	0.972

Abbreviations: MR-proANP, mid-regional pro-A-type natriuretic peptide; NT-proBNP, N-terminal pro-B-type natriuretic peptide; AFib, atrial fibrillation; LA EDVI, left atrium (LA) end diastolic volume index; LA DiVI, LA diastasis volume index; LA ESVI, LA end systolic volume index; LV SV, left-ventricular stroke volume.

CHAPTER 5
DISCUSSION, LIMITATIONS, FUTURE DIRECTIONS AND CONCLUSIONS

5. Discussion, Limitations, Future Directions and Conclusion

5.1. Discussion

The syndrome of HF has reached epidemic proportions, and is a significant health burden on a global scale [1,9]. Its diverse signs and symptoms are the result of concomitant biochemical changes and abnormalities in cardiac structure and/or function [3]. In this thesis, investigations into both the aforementioned elements of HF were explored. Detailed discussions follow Chapters 3 and 4, while a summary discussion follows in this chapter, including general conclusions in the context of all of the presented findings, limitations, future directions and a general conclusion.

5.1.1. Summary of Main Findings and Conclusions: Chapter 3

- Circulating levels of TNFR1 and TNFR2 are elevated in both HFPEF and HFREF patients in comparison to healthy individuals, and TNFR2 is significantly elevated in HFPEF in comparison to HFREF patients.
- Elevated TNFα and its two receptors are differentially associated with the presence of HF risk factors, such as hypertension and habitual smoking.
- Elevated levels of TNFR2 are associated with worsening indices of diastolic function and disease severity in HFPEF.

Our results are congruent with a recent report [95], as we show that TNFα axis dysregulation is a general hallmark of HF, but that this phenomenon is more substantial in HFPEF than in HFREF. However, it is difficult to compare our results directly to the results reported by C Marti *et al.*, because we examined established HF populations, while their study tracked incident HF in community-dwelling individuals [95]. We indirectly investigated one potential mechanism for increased circulating TNFRs: activation of TACE/ADAM17. The increase in plasma ACE2 activity that we observed may reflect increased TACE activity, which would shed tissue TNFR2 into the plasma, thereby diminishing protective signaling cascades mediated by TNFR2 in the myocardium among other tissues [46].

From the perspective of understanding the molecular pathology of HF, further study will be needed to resolve why TNFR1 appears to better reflect the onset of HFPEF,

while TNFR2 appears to better reflect established HFPEF. Because TACE processes both TNFR1 and TNFR2 [102], a loss of cardio- and vasculoprotective TNFR2 signaling would also seem to precipitate HF development in addition to progression. On the other hand, from a clinical standpoint, the aforementioned resolution is not critical: if TNFR1 can be validated as a marker for HF incidence and TNFR2 can be validated as a marker of HF progression, then the two could be combined in a biomarker panel to cover the HF syndrome over a large portion of its natural history. Biomarker panels have the potential to offer greater insight than single biomarkers on their own [26,28].

Our results contribute to a growing body of knowledge that provides important retrospective context when analyzing the failure of the ATTACH and RENEWAL trials, which looked at the effectiveness of infliximab and etanercept, TNFα antagonists, in patients with moderate to severe chronic HF [111,112]. The complex syndrome of chronic HF, with its myriad causes and variability in presentation may require more specific interventions, as the syndrome might partially hang in the balance of the multiple effects of TNFα, which are mediated through TNFR1 and TNFR2.

5.1.2. Summary of Main Findings and Conclusions: Chapter 4

- Increased LA size is associated with presence of HF in comparison to healthy individuals, independent of age or sex.
- Among individuals with HF, there is a range in the active contribution of the LA to LV SV that over ranges from negligible to greater than that of healthy individuals, and which is independent of LVEF or LA volume.
- Individuals with a history of paroxysmal AFib may also have marked active LA contributions to LV SV when they are in SR.

Few studies have reported LA function in the context of LV hemodynamics such that functional profiles are compared between HF and healthy subjects. Seminal work has, however, shown changes in reservoir and booster pump capacity in patients with HF without LV context [80,118]. Our results indicate that, at least in patients with moderate HF, the LA booster pump may be very important in achieving a sufficient LV SV. The LA may be more impactful on some HF patients' prognosis than others: some study

subjects with histories of paroxysmal AFib had substantial active LA volume changes, and LA enlargement, a risk factor for AFib [125], is characteristic of HF [3].

Similar to the potential of combining TNFR1 and TNFR2 into a biomarker panel, as described above, LA volume, clinical history and natriuretic peptides could be used to development a risk stratification panel based around the LA. Indeed, those individuals with extensive LA booster pump function and a significant risk of AFib in AF-CHF, where rhythm control was not superior to rate control with respect to outcomes [126], might have been the best candidates for rhythm control. A future re-development of the AF-CHF trial with more specifically-selected patients could thus have more success.

5.1.3. Implications of the Presented Research

Taking the findings presented in chapters 3 and 4 together, it is clear that within the syndrome of HF there are clusters of patients with distinct disease phenotypes; however, between different categories of HF patient there are also common characteristics, thereby linking them within the greater spectrum of HF. Indeed, we found in Chapter 3 that increased circulating levels of TNFRs are associated with increasing NYHA class in both HFPEF and HFREF. This observed effect was strong in both HF phenotypes, which suggests that dysregulation of the TNFα-axis may, to some extent, be characteristic of a cluster of patients that is not defined by LVEF. Biomarker investigations of several inflammatory systems have also found a more general effect with regards to HF prognosis in community-dwelling individuals [33,36,38]. Likewise, in chapter 4, our investigations of LA function across the whole spectrum of HF showed that the extent of LA reservoir and booster pump contribution to LV stroke volume is variable in subjects with moderate severity HF. Previous reports in HFREF and HFPEF patients have indeed shown a reduction in reservoir capacity across LVEF [80,118]. That we also found LVEF-independent effects underscores the existence of various clusters within the syndrome of HF with some overlapping characteristics.

Improvements in HF care, particularly for HFPEF, will require creativity and a potential re-imagining of the syndrome using current knowledge, such as that by WJ Paulus and C Tschöpe [14]. Critical to optimized care will be novel methods of

stratifying patients within the HF population using a collection of classification schemes, such as adding information from biomarker panels or other metrics of cardiac function to LVEF classification [26]. Indeed, as the HF syndrome is characterized to greater extents, various inputs may be combined to generate individualized patient profiles, such that therapeutic regimens can be most effectively tailored, while avoiding inefficient use of resources.

5.2. Limitations

The most significant limitation of both the studies presented in Chapters 3 and 4 is their single point-of-study, cross-sectional nature. Follow-up measurements are, therefore, not included, nor are outcomes data. Additionally, the overall study cohort used for the analyses in both Chapters 3 and 4 may have been subject to some selection bias, because enrollment in the study was voluntary. It is possible that those individuals willing to participate in such a study would be those individuals who might be more judicious with respect to medications and more proactive in terms of lifestyle modification. While this scenario would make resolving trends between HF patients and healthy individuals more rather than less difficult, it nonetheless may be a source of deviation between our study cohorts and the true community-dwelling population.

5.3. Future Directions

As mentioned above, longitudinal studies are the necessary next step to fully implicate measured biomarkers or metrics of cardiac remodeling in the syndrome of HF. One- and three- or five-year follow-up studies are indeed planned as part of the Alberta HEART project. Smaller interval longitudinal studies may also be valuable, such as before and after application of a physiologic stressor. Indeed, baseline measures of cardiac function may not always reflect reduced cardiac reserve, which becomes apparent in exercise stress situations [71], so stress testing to see how biomarkers and metrics of cardiac function relate to validated measures of exercise capacity may be valuable. With respect to inflammatory markers, it may be worthwhile to examine how unstressed levels relate to stress test performance, but also to evaluate how circulating inflammatory markers change in exercise stress situations. Similarly, evaluating how LA contributions

to LV SV change in response to exercise stress, if at all, will be important for making statements about their relative importance.

On the other hand, more basic knowledge, generated using non-human models of CVD, will be necessary to make concrete statements about the pathogenesis of HF. Genetic knockout models coupled with pharmacologic inhibition, such as TNFR1-knockout and TNFR2-knockout mice, might provide compelling evidence of the role of various inflammatory markers in the pathogenesis of HF [128]. With respect to cardiac function, large animal models, such as dogs, may be necessary in order to best approximate human hemodynamics [128]. A translational approach in which clinical findings, such as those presented in this thesis, are used to inform new directions for basic investigations, and in which a confluence of basic and clinical findings is used to inform new interventions will be the most effective way to finding a lasting solution to the HF epidemic.

5.4. Conclusions

The research presented in this thesis provides support for some previously established concepts about the biochemical and cardiac structural and functional characteristics of patients with HF. Using well-defined study cohorts, the potential role of the TNFα axis as a key mediator, particularly in HFPEF, was elaborated. On the other hand, the variability in LA remodeling was assessed from the perspective of a pan-HF approach. Altogether, the findings presented herein may lead to improvements in personalizing HF care such that the present situation, in which overall HF mortality is not reduced, but, rather, delayed [11], can be modified such that improvements in survival are absolute.

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