Fabry Cardiomyopathy and Heart Failure: The Roles of Inflammation and Valvular Heart Disease

by

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Abstract

Fabry disease (FD) is an X-linked recessive multisystem disorder and an underrecognized secondary cause of hypertrophic cardiomyopathy. A gene variant encoding the enzyme α-galactosidase A results in deficient or absent hydrolase activity, resulting in the accumulation of glycosphingolipids in various organs including the nervous system, kidneys, skin, eyes, and heart. Cardiac involvement of FD includes biventricular hypertrophy, conduction disease, and coronary microvascular dysfunction leading to heart failure with preserved ejection fraction. Indeed, cardiovascular disease is now a leading cause of morbidity and mortality in FD patients. Our research explores the role of inflammation and valvular heart disease in FD and their contributions to HF. Our data suggests that FD patients have increased inflammatory biomarkers, which correlate with end-organ dysfunction. In addition, our data demonstrates the increased burden of valvular disease in this population. Our findings contribute to a growing understanding of Fabry disease and its cardiovascular consequences.

Preface

This thesis is an original work by Haran Yogasundaram. All included research received research ethics approval from the University of Alberta (Pro00058233) and collaborator institutions.

A portion of the research included in this thesis was conducted as part of an international research collaboration led by Dr. Gavin Oudit at the University of Alberta. A portion of the data acquisition, as well as the data analysis, interpretation, and writing of the manuscript, in chapters 3 and 4 were my original work.

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Dedication

This thesis is dedicated to my family, friends, and mentors for their unwavering support.

I offer my deepest gratitude to my parents, Yoga and Jayanthi Yogasundaram, for their unconditional love and encouragement. I would also like to thank my brother and sister-in-law, Dilan and Eesha Yogasundaram, for their support.

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CHAPTER 1

INTRODUCTION

Portions of this chapter have been published:
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1. Introduction

1.1 Fabry Disease

The differential diagnosis of hypertrophic cardiomyopathy includes sarcomeric hypertrophic cardiomyopathy, mitochondrial syndromes,[1] and three storage disorders: Fabry disease, PRKAG2 deficiency, and Danon's disease.[2, 3] Fabry disease (FD, OMIM 300644) is an X-linked recessive lysosomal storage disorder caused by genetic deficiency or absence of alpha-galactosidase A (α-Gal A, EC 3.2.1.22) activity due to a loss-of-function mutation in the galactosidase alpha (*GLA*) gene.[4] This inborn defect of catabolism results in intracellular accumulation of glycosphingolipids, particularly globotriaosylceramide (Gb3), causing neurological, cardiac, ocular, gastrointestinal, dermatological, and renal manifestations.[5-7] Fabry disease represents an important cause of infiltrative cardiomyopathy. Classical and variant FD comprise the two major clinical phenotypes and the clinical course is variable.[8, 9] Missed or delayed diagnoses are common as initial clinical manifestations such as intermittent pain, hypohidrosis and gastrointestinal problems can be subtle and nonspecific.[4, 10] Cardiovascular disease has overtaken renal disease as the leading cause of mortality in FD patients.[5] Enzyme-replacement therapy represents the mainstay of disease-modifying treatment in FD and halts or reverses the pathogenesis of FD, as well as improve outcomes.[11, 12]

1.2 Epidemiology

The prevalence of Fabry disease is historically estimated between 1 in 40,000 to 1 in 117,000 individuals, which represents a significant underestimation given the propensity of this disease to be underdiagnosed.[4, 13] Indeed, the pooled prevalence of alpha-galactosidase A deficiency in dried bloodspot screening of over 200,000 neonates was found to be 1/1781

(0.056%), the majority of which had subsequent positive genetic testing.[14-17] A systematic review found the prevalence of GLA variants to be similar at 0.039%.[18] This discrepancy is primarily due to variant disease phenotypes obfuscating the clinical picture, as these patients may not manifest disease until later in life, if at all; penetrance is highly variable, largely due to differing vascular risk factors including hypertension, diabetes, and smoking history. Many patients with unexplained sequelae of FD, including cerebrovascular and renal disease, may represent undiagnosed FD. In these high-risk populations, the prevalence of GLA variants was 0.62% (1/161), with definitive FD diagnoses established in 0.12% (1/833).[18] Specifically, the prevalence of decreased alpha-galactosidase A activity in cohorts of patients with cryptogenic stroke (1.2%)[19] or end-stage renal disease necessitating hemodialysis (1.2%)[20] is significant. These cohort studies have been reinforced by findings from a multidisciplinary clinic screening study in which 1.8% of probands with clinically suspected FD were carriers of *GLA* mutations.[21] The diagnosis of these patients, especially in conjunction with subsequent identification of affected relatives, highlights the potential benefit of screening for FD in selected cohorts.

Likewise, cohorts of unexplained hypertrophy have a relatively high prevalence of FD. The prevalence of decreased alpha-galactosidase A activity in cohorts of males with unexplained LVH was 6.3%[22] and 3.0%.[23] In one study of females with late-onset hypertrophic cardiomyopathy, this prevalence was a staggering 11.8%.[24] These findings were confirmed via genetic analysis; alpha-galactosidase A mutations were found in 0.5% and 1.0% of large cohorts of patients with unexplained LVH.[25] [26] In addition, as with any heritable disease, certain regions may have increased prevalence of FD due to a founder effect. From these data, it is clear that cardiologists need to be cognizant of the under-recognition of FD, particularly in select cohorts of patients.

1.3 Pathogenesis and Clinical Manifestations

Fabry disease is caused by the deficiency or absence of alpha-galactosidase A activity secondary to loss-of-function mutation in the galactosidase alpha (*GLA*) gene located on Xq22.1 chromosome locus, leading to the accumulation of glycosphingolipids, particularly globotriaosylceramide (Gb3) (Figure 1.1 A). Despite heterozygous females possessing a normal copy of the *GLA* gene, random X-inactivation can lead to clinical manifestations of FD. However, it is also important to recognize that the pathogenesis of FD is not simply through the physical accumulation of glycosphingolipids in tissues alone. In fact, less than 5% of the LV mass can be accounted for by glycosphingolipid deposition alone suggesting secondary metabolic effects and activation of hypertrophic signaling pathways (Figure 1.1 B).[27] The clinical manifestations of FD can be broken down into two major phenotypes: classic FD and variant FD. Classic FD patients are males with severely diminished or absent alpha-galactosidase A activity and typically have multiorgan involvement, and are often identified from nephrology, cardiology, or neurology clinics (Figure 1.2 A). These products accumulate in various tissues, particularly the heart (68%), peripheral nerves (45%), kidney (45%), eye (38%), brain (34%), skin (34%), gastrointestinal tract (31%), and auditory system (19%) leading to a diverse end-organ involvement (Figure 1.2 B).[21] These patients generally present in childhood or adolescence with acroparesthesias, diffuse angiokeratomas, cornea verticillata, heat intolerance, and abdominal pain crises.[21] Endothelial dysfunction is a feature of FD and progressive cardiac, neurological, and renal disease feature prominently.[10, 28] Concentric ventricular hypertrophy, microvascular dysfunction, and arrhythmia are important cardiac manifestations of FD.[29, 30] Renal FD causes proteinuria and chronic renal failure[31], while neurological manifestations include cryptogenic stroke, transient ischemic attack, sensorineural hearing loss, and migraines.[32] Of patients diagnosed with FD, 60% have a history of

abnormal cardiovascular signs and symptoms.[33] Cardiac symptoms first manifest in females and males at approximately 40 and 33 years of age, respectively.[33] Patients may present with signs and symptoms corresponding to heart failure with preserved ejection fraction (HFpEF), valvular dysfunction including mitral and aortic insufficiency, angina, and conduction abnormalities. Accordingly, hypertension, peripheral edema, and murmur are the most common signs, while dyspnea, palpitations, and angina are frequent symptoms.[33]

Variant FD affects patients with less severe mutations and residual alpha-galactosidase A activity is preserved. Variant FD typically involves either the heart or kidneys alone,[34, 35] and the clinical progression of disease is usually slower.[8, 36] Nevertheless, disease manifestations in the variant phenotype can cause significant morbidity and mortality. Progressive renal disease has been overtaken by cardiac disease as the most significant cause of morbidity and mortality in patients with FD. However, it remains a significant management concern in these patients.[37, 38] Classic FD, cardiac, and renal variants FD can all lead to cardiorenal syndromes.[39] Proteinuria and male gender are important predictors for progression of renal disease in FD.

In confirmed FD, the well-known, validated Mainz Severity Score Index (MSSI) incorporates many of the aforementioned disease manifestations and can be used to stratify patients according to severity of disease.[40] A newer system, the Fabry International Prognostic Index is based upon clinical data and has been shown to correlate with prognosis in FD.[41] These indices have been evaluated in following clinical response to treatment via enzyme replacement therapy, making it a useful tool for serial evaluation of patients.[40, 41]

1.4 Cardiomyopathy Associated with FD

Gb3-mediated infiltration and microvascular damage in FD lead cardiomyopathy characterized by restriction and concentric hypertrophy, which has emerged as the leading cause of mortality in patients with FD.[5] Given the severe diastolic dysfunction and restrictive filling pattern in advanced FD, it can be considered in the differential diagnosis of restrictive cardiomyopathy.[42] Diastolic dysfunction and progressive LVH comprise the major features of cardiac involvement in FD.[9, 27, 33] Accordingly, heart failure with preserved ejection fraction and valvular disease are major cardiac complications of FD.[9, 43]

1.4.1 Structural and Functional Changes

Progressive LVH and impaired diastolic dysfunction leading to heart failure with preserved ejection fraction are major features of both classic and cardiac variant FD.[29, 44] In contrast to many other etiologies of LVH, concentric, symmetrical hypertrophy is typically seen in FD. LVH was reported in 46-61% of males and 18-28% of females with FD and is associated with increasing disease severity.[7, 29, 45] Accumulation of Gb3 in the heart induces inflammation and oxidative stress, along with corresponding concentric hypertrophy and extracellular matrix remodeling.[46] Interestingly, this responsive hypertrophy that leads to the vast majority of increased myocardial mass in FD patients, rather than the infiltration of Gb3 alone. However, even in the absence of overt LVH, significant irreversible cardiac disease can be present, in both male and female patients.[24, 47, 48] Alarmingly, 38.1% of men and 16.7% of women older than 40 years from a Taiwanese mutation, IVS4+919G>A, had MRI findings suggestive of irreversible myocardial fibrosis prior to the development of LVH or major cardiac manifestations.[47] While LVH is a hallmark of FD, right ventricular (RV) involvement is also commonly seen, including ventricular hypertrophy and dysfunction,[49-52] which is consistent with sphingolipid deposition occurring in

both ventricles.[53] Right ventricular hypertrophy and dysfunction likely contributes to the presence of heart failure symptoms in those with preserved LV ejection fraction.

Minor valvular disease in FD is common; mitral leaflet and aortic valve thickening, leading to regurgitation, were found in 57% and 47% of patients, respectively.[44] Despite accumulation of Gb3 in both sides of the heart in FD, left-sided valves are much more often involved, implying a pressure-mediated mechanism of valvular degeneration. As with LVH, valvular disease correlate with worsening disease severity.[29] Other structures are often involved in FD; prominent papillary muscles appear to be a relatively specific marker of FD[54], while aortic root dilatation occurs in severe disease.[29] Aortic dilatation at the sinus of Valsalva is common in FD, present in 32.7% and 5.6% of males and females, respectively.[55] Aneurysmal disease in this location was present in 9.6% of males and 1.9% of females.[55]

1.4.2 Microvascular Disease and Angina

Angina pectoris in FD is present in about 25% of patients[33] and is related to microvascular disease[56] and mostly seen in the absence of significant epicardial coronary lesions.[30, 56, 57] Moreover, it can precede the appearance of other cardiac manifestations such as left ventricular hypertrophy.[30, 58, 59] Accordingly, early diagnosis of microvascular coronary disease is desirable, not only in symptomatic patients, but also as an early indicator of cardiac involvement. Myocardial perfusion imaging is a sensitive method to detect the degree of microvascular disease in patients with FD.[30]

1.4.3 Electrophysiological Remodeling

Common electrophysiological manifestations of FD include shortened PR interval (in early stages), atrial fibrillation (in advanced stages), and increased QRS duration.[68, 69] Electrocardiographic changes can precede the development of LVH or symptoms of cardiovascular disease.[27] Atrial fibrillation has a prevalence in FD that is four times greater than the general population, and twelve times greater specifically in those over the age of 50.[70] Late manifestations include high-grade atrioventricular block, supraventricular and ventricular dysrhythmias, and sudden cardiac death. [70, 71] Age, left atrial size, LVH and myocardial fibrosis (as determined by cardiac MRI) are correlated with the arrhythmia burden in FD.[70, 72] In this population at high-risk for arrhythmia, implantable loop recorders are superior at detecting clinically significant arrhythmias, leading to placement of a pacemaker or cardioverter-defibrillator, relative to Holter monitoring.[73]

1.5 Diagnosis of FD: The Emerging Role of Biomarkers

The diagnosis of FD can be challenging, particularly when a variant phenotype is present.[74] The mean duration from onset of symptoms to the diagnosis of FD is 13.7 and 16.3 years in males and females, respectively.[7] Connective tissue disease (39%), arthritis (15%), neuropsychiatric diagnoses (13%), and fibromyalgia (7%), among others (49%), are often assigned as misdiagnoses.[7] Given the advent of enzyme-replacement therapy, early recognition and correct diagnosis of FD is crucial to prevent potentially irreversible progression of disease in both classic and cardiac-variant phenotypes.[75, 76] Consensus recommendations suggest a multipronged approach involving clinical features, biochemical findings, genetic testing, and pathological features.[77] Notably absent from these recommendations are promising imaging criteria, which confer benefits such as noninvasive testing and assessment of disease progression. An updated

approach to the diagnosis of FD should consider these features and involve expert opinion (Figure 1.3).[18, 78] As with any potentially systemic disease, detailed history and physical examination are critical. In particular, family history is important as FD exhibits an X-linked recessive pattern of inheritance and *de novo* mutations are rare. As described previously, signs and symptoms of FD may be nonspecific; however, findings such as cornea verticillata, acroparesthesias, and angiokeratomas tend to have much smaller differential diagnoses and high specificity.[18] Nevertheless, diagnosis should be not made on clinical findings alone. Importantly, drug-induced cardiomyopathy with medications such as hydroxychloroquine can mimic the clinical and pathological findings of FD and must be ruled out before a diagnosis of FD can be made.[79]

In suspected males, α-Gal A activity assays of plasma or leukocytes can be used to diagnose FD. Activity below 5% of mean in males is highly suggestive of classic FD.[80] Many patients, however, may have markedly reduced alpha-galactosidase A activity of uncertain clinical significance.[81] In females in particular, plasma α-Gal A activity is highly variable and can be normal even in the presence of clinical disease. [82] Indeed, at least one-third of female FD patients are missed via conventional alpha-galactosidase A testing, [83] which represents a significant shortcoming as 69.4% of female carriers have symptoms and signs of FD,[35] and clinically significant disease develops in the majority of these patients.[78, 84] Therefore, confirmatory genetic testing is necessary in many cases involving either gender.

Genetic testing is an important component in the diagnosis of FD. Hundreds of mutations of the *GLA* gene causing FD have been identified.[4] Once a mutation in the *GLA* gene of a patient is identified, it should be compared to known mutations in the literature to verify whether it is associated with the classic phenotype, variant phenotype, or if it is a genetic variant of unknown significance (GVUS). Ventricular hypertrophy, renal disease, or cerebrovascular disease, in the

setting of GVUS is not enough to establish the diagnosis of FD as other much more common disease processes can lead to a similar clinical picture. Some variants, such as p.Asp313Tyr, occur with relatively high frequencies in various populations and are not considered to be disease-causing.[85] On the contrary, large deletions over Xq22.1 predictably cause FD and are generally not identified via *GLA* mutation analysis.[86] Therefore, genetic screening alone is unreliable for the diagnosis of FD in the absence of clinical and biochemical evidence of disease. Pedigree analysis is an important adjunct to the work-up of an FD patient as relatives, including children, may need to be tested. Accordingly, genetic counseling is critical for patients with FD.[78] A recent screening study has demonstrated that irreversible disease can occur in the absence of clinical manifestations, suggesting a potential increased role for newborn screening.[47]

Given the highly variable phenotypic implications of differing alpha-galactosidase A levels and mutations, lyso-Gb3 has emerged as a powerful FD-specific biomarker with clinical relevance. [87] In patients with GVUS, lyso-Gb3 testing can be used to determine if the mutation is likely to be clinically significant, and, therefore, if the patient should be ascribed the diagnosis of FD. Furthermore, lyso-Gb3 can stratify patients according to classic versus variant phenotype, even before end-organ damage has manifested.[87] Gender-specific plasma protein biomarker panels are sensitive and specific for FD.[88] These panels identify abnormal levels of biomarkers other than Gb3 and its metabolites, such as apolipoprotein E and perioxiredoxin 2, highlighting contributors to the FD phenotype and providing insight into the pathophysiology of FD. Endomyocardial biopsy can confirm the diagnosis of FD, and may be useful in cases where the diagnosis is ambiguous, such as concomitant hydroxychloroquine use.[79] However, its role has diminished as the aforementioned noninvasive biochemical, imaging, and genetic testing is often sufficient.

Biomarker analysis has the potential to provide insight into pathophysiology of disease, expedite diagnosis, increase diagnostic efficiency, provide prognostic information, and monitor treatment effectiveness.[89, 90]

1.6 Role of Cardiac Imaging

1.6.1 Echocardiography

In FD, echocardiography is used in the assessment of diastolic filling, LVH, valvular regurgitation, and myocardial dysfunction. Patients with FD cardiomyopathy, as with other restrictive cardiomyopathies, may demonstrate echocardiographic findings including reduced mitral annular tissue Doppler imaging velocities, increase E/e' ratio, atrial enlargement, and restricted filling with tall E wave and small A wave. As 60% of patients do not have LVH at the time of diagnosis, conventional echocardiography had significant limitations in the assessment of FD in early stages.[44] However, newer techniques using tissue strain imaging are able to detect the subclinical presence of diastolic dysfunction and regional systolic dysfunction in patients with normal ejection fraction.[92, 93 94] Strain and strain rate analysis with two-dimensional speckle-tracking imaging can be used to identify patients with FD with reduced regional myocardial function, independent of LVH,[45] and those with subclinical diastolic dysfunction.[95] A cutoff strain rate during isovolumic relaxation of 0.235 sec^{-1}, even after correcting for LVH, distinguishes between FD and healthy controls with a sensitivity of 94% and specificity of 92%.[45] Indeed, diastolic dysfunction may precede LVH in FD and can be detected via echocardiography.[93] Lateral and septal velocity measurements Sa (<10 cm/s) and Ea (<10 cm/s) had sensitivities of 100% and specificities of 90-100% for identifying mutation-positive subjects without LVH.[93] In this context, reduced systolic strain is usually found in segments with replacement fibrosis,[96] while reduced

ejection fraction is typically seen only in advanced FD-related cardiomyopathy.[97] Speckle-tracking echocardiography is also useful for assessment of left atrial function; left atrial reservoir, conduit, and contractile function are affected in FD. [98] The Tei index can be used to detect LV dysfunction in FD, and, along with thinning of the basal inferolateral LV wall, are predictors of heart failure and mortality.[99, 100] Finally, echocardiography is useful in monitoring response to ERT as peak systolic strain, strain rate, and parameters of left atrial function improve, particularly in patients with no existing fibrosis.[75, 98, 101, 102]

1.6.2 Cardiac MRI

Cardiac MRI (CMRI) is the gold-standard imaging modality for assessment of structural disease and myocardial fibrosis in FD, and can be used to correlate morphological changes with functional changes.[92, 103] CMRI is especially useful in the evaluation of ventricular hypertrophy as asymmetrical LVH and FD-specific changes can be more easily appreciated.[104] Conventional imaging fails to distinguish hypertrophy associated with FD versus other etiologies. However, T1-mapping can discriminate between FD and other causes with a high degree of sensitivity and specificity.[91, 105] Delayed gadolinium enhancement is present in a consistent, specific pattern in patients with FD, allowing for reliable differentiation from symmetric hypertrophic cardiomyopathy.[106]

1.7 Medical Management of Fabry Disease

Given the plethora of disease manifestations and the numerous organ systems affected by FD, multidisciplinary teams potentially involving cardiologists, internists, medical geneticist,

nephrologists, neurologists, ophthalmologists, otolaryngologists, pulmonologists, and/or gastroenterologists are necessary to manage these complex patients. Recommended assessment parameters for non-cardiac organ systems are described elsewhere.[113]

1.7.1 Medical Management

Conventional management directed at prevention of end-organ damage is essential in FD and is typically undertaken irrespective of additional therapies such as ERT.[114]. As with all types of cardiovascular diseases, lifestyle modifications are an important first step in the management of FD patients. Optimal control of blood pressure in patients with FD, even in normotensive patients, prevents left atrial enlargement and progression of FD cardiomyopathy.[43, 116] In addition, adequate blood pressure control is suggested for a period of at least 12 months prior to evaluation for ERT as concurrent hypertension can obfuscate imaging findings.[117]

1.7.2 Enzyme Replacement Therapy

In 2001, enzyme replacement therapy (ERT) was introduced as a new biologic therapy for the treatment of FD.[12] Two ERT formulations of recombinant alpha-galactosidase A exist: agalsidase α (Replagal®; 0.2 mg/kg every 2 weeks) and agalsidase β (Fabrazyme®; 1 mg/kg every 2 weeks), the latter of which has sole approval in the United States and Canada. Several major clinical trials have demonstrated both the short- and long-term safety and efficacy of ERT.[11 120 12, 121-123] ERT results in significant reduction of glycosphingolipids accumulation in tissues,[124] specifically intracellular deposits in the coronary endothelium.[125] Unsurprisingly, ERT has been demonstrated to improve specific outcomes including pain scores and quality-of-life assessments,[126] cardiopulmonary exercise capacity,[127] cerebrovascular perfusion, [128] and

stabilization of renal function.[129] Cardiac-specific outcomes, including LV mass and myocardial function, in FD are improved with ERT [102, 130 101] FD cardiomyopathy may be halted or even partially reversed by ERT.[75, 76] Promisingly, ERT demonstrated a reduction in a composite endpoint of renal, cardiac, central nervous system events, and death.[122]

The 2016 Canadian FD guidelines for ERT are similar to other evidence-based guidelines across the globe and rely upon clinical manifestations of end-organ damage, regardless of gender. [117, 131] The Canadian Fabry disease initiative tracks outcomes of subjects with FD treated with ERT in subjects who meet the evidence-based treatment guidelines demonstrated that cardiovascular risk factor modification and targeted use of ERT reduce the risk of adverse outcomes related to FD.[114]

Given that the qualification criteria often necessitate advanced disease as a prerequisite for ERT, it is especially worrisome that ERT effectiveness is severely diminished in advance FD.[132] Specifically, FD cardiomyopathy in the advanced stages progresses despite ERT.[133] Indeed, there is significant evidence for improved outcomes with ERT in FD before irreversible myocardial fibrosis has occurred.[75] Accordingly, early diagnosis and treatment is paramount. Complicating the clinical picture is the advent of irreversible cardiac damage without LVH or significant cardiac manifestations,[47] which may lead to increased importance of newborn and/or pediatric screening in the future. It is important to note that the ERT qualification criteria unfortunately do not include advanced imaging modalities described previously; in particular, cardiac MRI with T1 mapping may improve detection of clinically significant disease progression and accordingly improve appropriate utilization of ERT.[91, 105]

1.7.3 Other Therapies

Due to failure of medical management or development of concomitant indications for therapy escalation, therapies such as implantable cardiac devices, cardiac transplantation, or novel therapies including gene therapy and substrate reduction therapy may need to be considered. High-grade atrioventricular block or cardiac bradyarrhythmias may necessitate the implantation of a permanent pacemaker or, in some patients with advanced cardiomyopathy and malignant ventricular arrhythmias, an implantable cardioverter-defibrillator.[113] Ultimately, 10-20% of FD patients require permanent cardiac pacing.[113] Cardiac transplantation remains a viable option for patients with severe disease using conventional indications for transplantation. Fabry disease does not appear to recur in the allograft, likely due to residual alpha-galactosidase activity in the donor heart.[135] Substrate reduction therapy (SRT) is an emerging potential therapy for FD, particularly in conjunction with ERT. SRT inhibits upstream biosynthesis of glycosphingolipids, thereby reducing Gb3 accumulation.[136] Gene therapy represents a novel treatment modality for FD with the potential for long-term cure. *In vivo* studies have demonstrated successful long-term correction of Gb3 levels in Fabry mice through the use of adeno-associated virus-mediated gene transfer,[138, 139] and corresponding improvement of cardiac hypertrophy.[140]

1.8 Rationale and Hypotheses

1.8.1. Chapter 3

Inflammation has been implicated in the pathogenesis of HFpEF. Accumulation of glycosphingolipids in the body is associated with increased inflammation. Accordingly, we sought to evaluate inflammatory biomarkers in FD. We hypothesized that inflammatory biomarkers are elevated in FD patients and are associated with end-organ dysfunction.

1.8.2. Chapter 4

The cardiovascular mortality of patients with FD, especially due to HF, is improving through the advent of ERT and ongoing risk-factor reduction. However, in addition to the vasculature and cardiac myocyte, glycosphingolipid also accumulates in valvular tissue. Accordingly, evaluated the prevalence of valvular heart disease in a FD cohort. We hypothesized that Fabry patients have a greater prevalence of valvular heart disease than the general population.

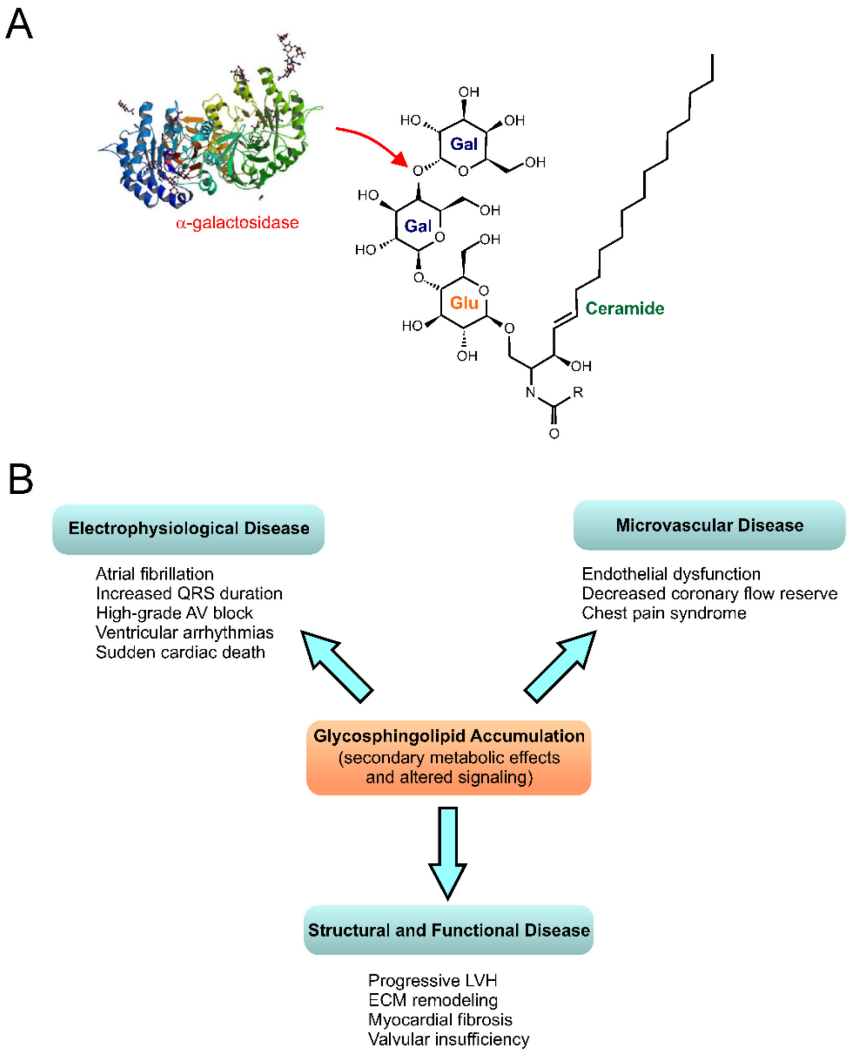

Figure 1.1. Pathophysiology of cardiac manifestations in Fabry disease (A). Accumulation of sphingolipid by deficient or absent alpha-galactosidase A activity leads to progressive ventricular hypertrophy, valvular thickening with insufficiency, conduction disturbances, dysrhythmias, and microvascular dysfunction (B).

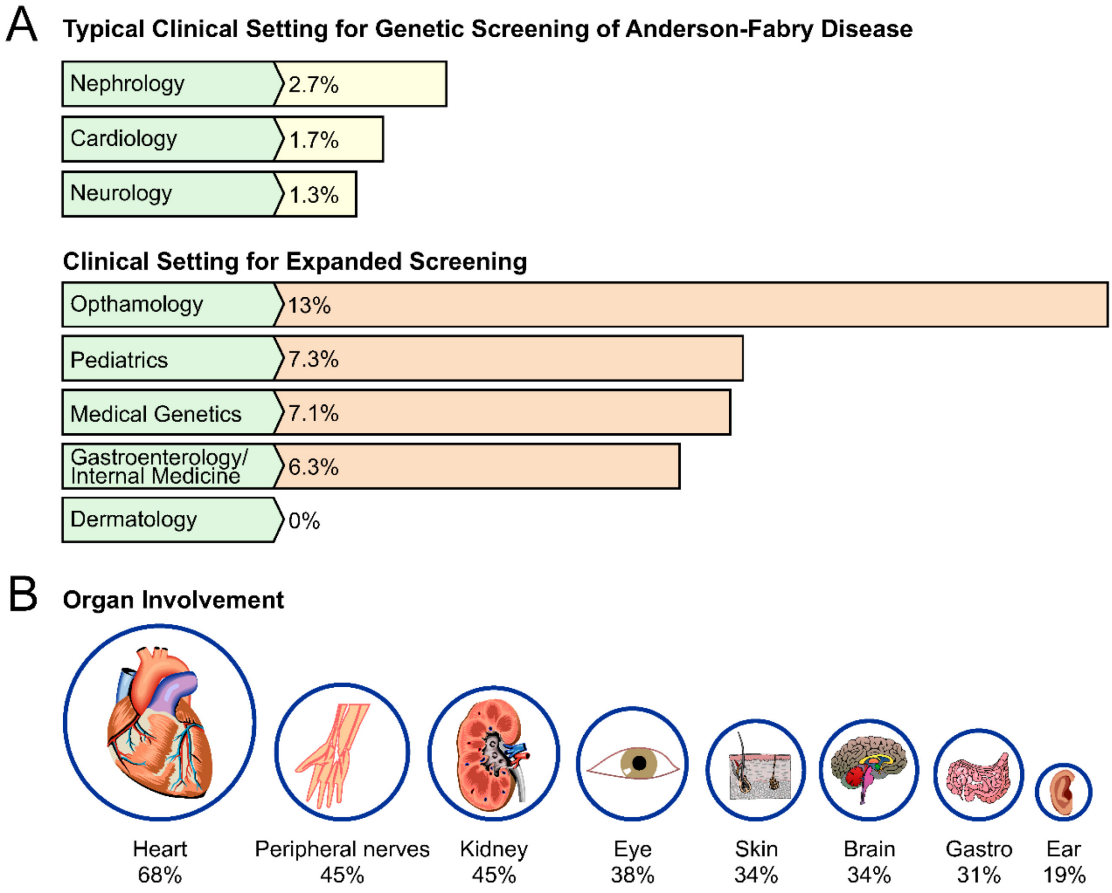

Figure 1.2. Percentages of screened patients in various clinical settings who were carriers of *GLA* mutations (A), along with frequency of organ involvement (B). The heart remains the most commonly involved organ in screened patients. This figure has been adapted from Favalli et al.

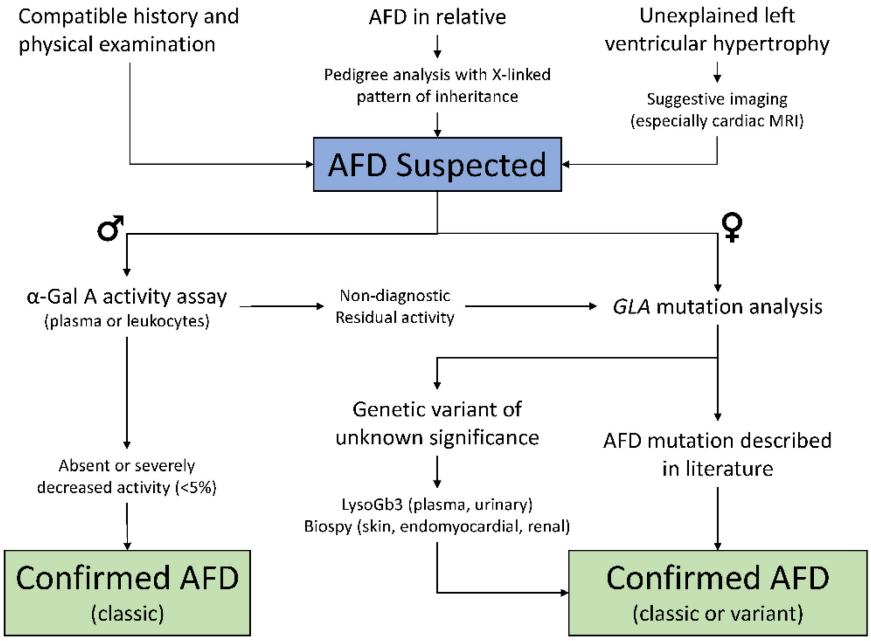

Figure 1.3. Proposed diagnostic algorithm for Fabry disease. When classic FD is suspected in males, the plasma alpha-galactosidase A activity assay can be used for diagnosis. In all other cases, genetic testing for a *GLA* mutation is warranted. This figure has been constructed using published recommendations by Laney DA et al. (2013), van der Tol L et al., Niemann M et al., and Putko B et al.

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CHAPTER 2

MATERIALS AND METHODS

2. Materials and Methods

General methodology applying to all chapters are included herein while methodology specific to Chapters 3 and 4 are detailed therein.

2.1 Ethics Approval

All subjects gave written informed consent and ethics approval was obtained from the University of Alberta (Edmonton, Alberta, Canada; Pro00058233). Similar approvals were obtained by collaborators at the University of Calgary (Calgary, Alberta, Canada) and QE II Health Sciences Centre (Halifax, Nova Scotia, Canada), where applicable.

2.2 Patient Recruitment and Data Handling

Patients with Fabry disease were recruited via metabolic and cardiac clinics in Edmonton, Calgary, and Halifax. Study patients were assigned an alphanumeric code reflecting order and site of enrollment. In order to protect patient identities, linking information was held by the supervisor, Dr. Gavin Oudit. All data was stored under restricted access with backups in case of loss. No data was made available to third parties outside of researchers involved in project as outlined in the author lists. Subjects were able to withdraw at any time.

2.3 Data Analysis

Data analysis was performed using OriginLab software version 9.1 (OriginLab, Northampton, MA, USA) and Microsoft Excel 2016 (Microsoft, Redmond, WA, USA). Statistical analyses were performed using SPSS software version 20 (IBM Corporation, Armonk, NY,

USA) with p values <0.05 considered significant in prespecified analyses. Adobe Illustrator CS5 (Adobe Systems Canada, Ottawa, ON, CAN) was used for the generation of figures.

CHAPTER 3

INFLAMMATION IN FABRY DISEASE AND HEART FAILURE WITH PRESERVED EJECTION FRACTION

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3. Inflammation in Fabry Disease and Heart Failure with Preserved Ejection Fraction

3.1 Introduction

Fabry disease (FD, OMIM 300644) is an X-linked lysosomal storage disorder characterized by diminished or absent alpha-galactosidase A (α-Gal A, EC 3.2.1.22) enzyme activity[1], leading to the accumulation of the glycosphingolipid globotriaosylceramide (Gb_3) in tissues.[2] Recent neonatal screening data suggests that the actual prevalence is close to 1:3,000.[3, 4] As Fabry disease is X-linked, hemizygous males typically have much lower α-Gal A activity than heterozygous females.[5] However, the majority of female heterozygotes develop clinically significant disease albeit with a milder disease course than hemizygous males, likely due to X-chromosome inactivation.[2, 6] Cardiac manifestations of FD include left ventricular hypertrophy (LVH), diastolic dysfunction, microvascular angina, valvular abnormalities, and conduction defects,[7, 8] while proteinuria and progression to end-stage renal disease (ESRD) are renal complications of FD.[9] Patients suffering from the classic phenotype of FD typically have early onset symptoms with noticeable cardiovascular effects between 30 and 40 years of age,[10] ultimately suffering from heart failure with preserved ejection fraction (HFpEF).[11, 12]

Cardiomyopathy with concentric hypertrophy and diastolic dysfunction is now the most common cause of death in patients with FD.[13] Fabry disease variants are characterized by the presence of certain *GLA* gene mutations and are known as cardiac or renal variant phenotypes.[2] Enzyme-replacement therapy (ERT) slows the progression of disease including the development of LVH.[7, 14] Plasma biomarkers are a rapidly growing area of research in FD and can provide prognostic value and insight into the pathophysiology of the disease.[15, 16] These cytokines have a

significant role in cardiac disease, particularly with respect to myocardial remodelling[17] and chronic heart failure.[18, 19] We report on the plasma levels of a variety of biomarkers in adults with FD compared with healthy controls to gain insight into the pathophysiology and burden of disease of this condition, as well as its link to the HFpEF phenotype.

3.2 Methods

3.2.1 Ethics and Transparency Statement

All subjects gave written informed consent and ethics approval was obtained from the University of Alberta (Edmonton, Alberta, Canada), University of Calgary (Calgary, Alberta, Canada), and QE II Health Sciences Centre (Halifax, Nova Scotia, Canada). Due to proprietary techniques used in certain portions of the data analysis section, analytic methods and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure. Clinicians and researchers are invited to contact the authors for the purposes of data replication or to share biomarkers samples as part of a collaborative effort.

3.2.2 Patient Population

Fabry disease patients (n=68) were recruited through Canadian metabolic clinics in Edmonton, Calgary, and Halifax. Healthy controls (n=40) with similar mean age and sex with no significant medical conditions were recruited through community outreach. Inclusion criteria for healthy controls included: no history of cardiovascular disease, hypertension, diabetes, or renal disease; and no prescriptions for angiotensin-converting-enzyme inhibitors, angiotensin-receptor blockers, beta-blockers, digoxin, mineralocorticoid-receptor antagonists, thiazide diuretics, or loop diuretics. Enzyme-replacement therapy with either agalsidase alfa (Replagal, Shire) or

agalsidase beta (Fabrazyme, Sanofi-Genzyme) in standard dose was given to patients who qualified under the Canadian Fabry Disease Initiative (CFDI) treatment guidelines.[20]

3.2.3 Baseline Analyses

Demographic information including date of birth, sex, height, and weight were collected. Clinical data including genetic mutation analysis, plasma leukocyte α-galactosidase activity, duration of treatment, serum creatinine, cardiac imaging, and Mainz Severity Score Index (MSSI)[21] data were also recorded for FD patients. Estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease equation.[22] A cut-off eGFR value of 60 mL/min/1.73m^2 was used for assigning kidney disease. LVH was defined using cardiac MRI, as described below.

3.2.4 Cardiac Imaging

Transthoracic echocardiography was performed for the Fabry disease cohort as described previously.[23, 24] Briefly, echocardiography was performed using standard techniques[25] on commercial ultrasound equipment (M3 S Probe, Vivid 7; GE Vingmed Ultrasound AS, Horten, Norway). Chamber quantification and ejection fraction was assessed using the modified Simpson's method.[26] Preserved ejection fraction (EF) was defined as EF ≥50% as assessed by echocardiography. The presence of diastolic dysfunction was graded using E/A ratio, peak E velocity, E/e' ratio, and LA maximum volume index according to current guidelines,[27] unless assessment was judged to be indeterminate or precluded by arrhythmia or patient factors.

Cardiac MRI was performed as previously described in a standardized fashion.[24, 28-30] Cardiac MRI was performed on 1.5T Siemens Sonata or Avanto scanners (Siemens Medical

Solutions, Erlangen, Germany). Typical imaging parameters using standard balanced steady-state-free precession short-axis and long-axis cines were: 1.24 ms echo time, 2.89 ms repetition time, 51° flip angle, 360x270 mm field of view, 8 mm slice thickness, 2 mm gap between short axis slices, 10-14 views per segment, reconstructed to 30 phases per cardiac cycle were used. Left-ventricular mass index was measured in end-diastole and was calculated using a modified method of disks[31] measured from steady-state free precession cines and analyzed using software (MATLAB 2010a; The MathWorks, Natick, MA) as previously described.[31, 32] Assessment of LVH was performed as previously defined using cut-offs of LVMI ≥ 85 g/m^2 in males and ≥ 81 g/m^2 in females to denote LVH.[33] Papillary muscles were included as part of the myocardium for LV mass calculations but excluded for volume assessment. Conventional late gadolinium enhancement (LGE) imaging was performed 7 minutes after contrast injection using a phase sensitive inversion recovery sequence in the short-axis, 2-, 3-, and 4-chamber views to match the cine slice locations. LGE imaging was not performed in 3 patients due to advanced renal disease precluding the administration of gadolinium contrast.

Due to the specialized nature of the data acquisition and analysis, T1 mapping and ECV calculations were only performed in the Alberta cohort. T1 mapping used the SAturation-recovery single-SHot Acquisition (SASHA) pulse sequence as previously described.[28, 30] T1 mapping was performed at baseline and 15 minutes after administration of 0.15 mmol/kg of gadopentetate dimeglumine (Magnevist; Bayer Inc, Toronto, Canada) as previously described.[28, 30] Endocardial and epicardial tracings were created for T1 analysis. Blood pool and myocardial T1analysis used based on a circular region of interest (ROI) drawn in the LV blood pool and a 2-mm width ROI drawn over the interventricular septum, respectively. Normal left-ventricular T1 values at our site in men are 1167±36 ms (baseline) and 600±38 ms (post-contrast) (n=30) and

1202±30 ms (baseline) and 539±46 (post-contrast) (n=30) in women.[28, 30] In each of the 18 segments, the extracellular volume (ECV) fraction, which is the volume in which gadolinium contrast agent is distributed, was estimated using the calculated concentrations of contrast agent in the blood and tissue.[28, 30]

3.2.5 Sample Collection and Processing

While patients were sitting and rested, whole blood for plasma analysis was collected into lithium-heparin and EDTA tubes and stored immediately on ice. Subsequently, plasma fractionation was completed and the samples were stored in liquid nitrogen at the Canadian BioSample Repository (Edmonton, Alberta Canada).

3.2.6 Classical Plasma Biomarker Quantification

Enzyme-linked immunosorbent assays (ELISA) were used to investigate plasma levels of TNF, TNFR1, TNFR2, IL-6, MMP-2, MMP-8, MMP-9, galectin-1, and galectin-3. Plasma BNP and MR-proANP levels were assessed as previously described using an Alere Triage reagent pack (Alere Inc., Ottawa, ON, CAN) and analyzed using an automated DxI 800 immunoanalyzer (Beckman-Coulter, Fullerton, CA, USA) at provincial heath laboratories in the province of Alberta, Canada.[34] Plasma α-Gal activity was assessed as previously described.[35] Plasma CRP levels were measured using high-sensitivity kits at provincial health laboratories in the province of Alberta, Canada. Commercial ELISA kits were used to assay plasma levels of TNF, TNFR1, TNFR2, and IL-6 (catalogue no.'s HSTA00D, SRT100, SRT200, and HS600B respectively, R&D Systems, MN, USA) as previously described.[36] The described kit protocol was used for ELISA assays for total MMP-2, MMP-8, MMP-9, galectin-1, and galectin-3 (catalogue no.'s MMP200, DMP800, DMP900, DGAL10, and DGAL30, respectively, R&D Systems, MN,

USA). Absorbance was measured using a SpectraMax M5 Plate Reader (Molecular Devices, CA, USA) at 450nm for all assays with the wavelength correction set to 540nm for TNFR1, TNFR2, MMP-2, MMP-8, MMP-9, galectin-1, and galectin-3 and to 650nm for TNF and IL-6. Detection rates for the ELISAs were 100% for all assays. The intra-assay coefficients of variation were 3.7% (n=8), 5.2% (n=8), 3.5% (n=8), 3.6% (n=8), 11.4% (n=8), 13.4% (n=8), 4.6% (n=8), 7.9% (n=8), and 2.1% (n=8) for TNF, TNFR1, TNFR2, IL-6, MMP-2, MMP-8, MMP-9, galectin-1, and galectin-3 assays, respectively.

3.2.7 Analysis of Plasma Lyso-Gb3 and Analogues

Plasma Lyso-Gb_3 and its six related analogues with modified sphingosine moieties (-C_2H_4; -H_2; +O; +H_2O; +H_2O_2, +H_2O_3) were analyzed in plasma of Fabry patients with a method previously published by Boutin and Auray-Blais[37] (Figure 3.1). Briefly, 100 µL of plasma was spiked with in-house synthesized N-glycinated Lyso-Gb_3 (Lyso-Gb_3-Gly) as the internal standard and purified by solid phase extraction using mixed-mode cation-exchange cartridges (Oasis MCX, 30 mg, 60 µm; Waters Corp., Milford, MA, USA). The samples were separated by ultra-performance liquid chromatography using an Acquity I-Class (Waters) system equipped with a BEH C18 column (2.1 x 50 mm, particles diameter 1.7 µm; Waters). The analysis of Lyso-Gb_3 and its six analogues was performed by tandem mass spectrometry using the multiple reaction monitoring mode on a Xevo TQ-S mass spectrometer (Waters). Positive electrospray was used for the ionization. Plasma total Lyso-Gb_3 was reported as the sum of Lyso-Gb_3 and its six analogues.

3.2.8 Statistical Analysis

Statistical analyses were carried out using IBM SPSS Statistics version 20 for Windows (SPSS Inc, Chicago, IL, USA). Discrete variables are presented as count and/or percent. Continuous variables with normal distributions are presented as mean±standard deviation, while continuous variables with skewed distributions, including all biomarkers, are presented as median (first quartile, third quartile), unless otherwise indicated. A *P*-value of <0.05 was considered statistically significant. Categorical data was compared using Pearson Chi-squared tests or Fisher's Exact Test, where appropriate. Pairwise comparisons were evaluated using Mann-Whitney U Tests or Kruskal-Wallis Test with Mann-Whitney U-Tests, where appropriate. Analyses of continuous covariates was performed using linear regression, i.e. Mainz Severity Score Index (MSSI) versus biomarker levels and left-atrial size versus MR-proANP. Outliers identified by visual analysis were tested for potential impacts on the regression analysis. Receiver-operator characteristic (ROC) curve analysis was performed using a diagnosis of FD via alpha-galactosidase levels and/or genetic testing as the gold-standard.

3.3 Results

3.3.1 Clinical Characteristics

The mean age (±standard deviation) of FD patients was 42±13 years (n=62) versus 46±12 for the healthy controls (n=40) and there were even numbers of females and males in both cohorts (Table 3.1). The mean body-mass index (BMI) in the healthy controls was 25.0±2.7 kg/m^2 versus 24.3±4.3 kg/m^2 for the FD cohort. Of the 68 FD patients, 41 patients (60%) had LVH by cardiac MRI criteria and 37 (54%) were receiving ERT. Among FD patients, median plasma α-Gal A activity was 1.9 (0.63, 3.6) μmol/hr/g protein. Using the MSSI[21], 16 (24%) FD patients were classified as mild disease (MSSI ≤20), 43 (63%) had moderate disease (MSSI 21-

40), and 9 (13%) had severe disease (MSSI ≥41). The mean score of the cardiac subset of MSSI (maximum of 20) was 5.5 ±4.9. Estimated GFR in FD patients was 83±33 mL/min/1.73 m^2. *GLA* mutation analysis, phenotype, medication use, and ERT status are reported in Table 3.2.

The cardiac phenotype of the Fabry cohort was characterized by hypertrophy, preserved ejection fraction, and diastolic dysfunction (Table 3.1).[23, 28] The high median LVMI is consistent with the relatively high prevalence of males in our FD cohort (50%). Increased post-contrast myocardial T1 values suggest increased myocardial fibrosis and low pre-contrast T1 values are consistent with a diagnosis of FD.[28] The mean ejection fraction (EF) via echocardiography was 63±8% and 2 male FD patients had reduced EF. Excluding patients who were unable to be assessed for diastolic dysfunction due to arrhythmia or judged to be indeterminate, 60% had none, 12% had Grade I (mild), 28% had Grade II (moderate), and no patients had Grade III (severe) diastolic dysfunction. The mean average E/e' ratio was 11.1±4.7, while the mean left atrial maximum volume index was 29.3±9.7 mL/m^2.

3.3.2 Differences in Plasma Biomarkers between FD and Healthy Cohorts

Plasma BNP and MR-proANP were elevated in FD relative to healthy controls (P =0.006 and P=0.013, respectively) (Figure 3.2, Table 3.3). While there was no statistically significant difference between plasma MMP-8 values in the FD and healthy control cohorts (P=0.079), there was a significant difference in MMP-2 and MMP-9 (P=0.017 and P<0.001, respectively) (Figure 3.3). Patients with FD had significantly elevated plasma levels of inflammatory markers TNF, TNFR1, and TNFR2 relative to healthy controls (P=0.008, P=0.003, and P<0.001, respectively) (Figure 3.3). There was no difference in CRP concentration between the FD and healthy control cohorts (P=0.839), but IL-6 was significantly elevated in FD patients (P=0.021). galectin-1 was

significantly elevated in the FD cohort when compared to healthy controls, while galectin-3 was not statistically different (P<0.001 and P=0.533, respectively) (Figure 3.2). TNFR2 and galectin-3 were found to have independent positive correlations with Lyso-Gb_3 (P=0.020 and P=0.024, respectively).

Receiver-operator characteristic curve analysis was performed for Lyso-Gb_3 which performed extremely well (area under curve, AUC=0.998) (Figure 3.4). As a biomarker, plasma total Lyso-Gb_3 and analogues performed flawlessly for classic mutations (AUC=1.0 for both) but performed poorly for cardiac/renal variant mutations (AUC=0.41) although our sample size was low (n=6). In our cohort, there was no additional utility to measuring Lyso-Gb_3 analogues (AUC=0.994) instead of Lyso-Gb_3 alone (Figure 3.4).

3.3.3 Cardiac and Renal Disease and the relationship with Plasma Biomarkers

In FD patients with LVH by cardiac MRI criteria (n=41, 60%), TNFR2, TNF, IL-6, MMP-2, and Lyso-Gb_3 were significantly elevated (P=0.045, P=0.025, P=0.001, P=0.046, and P=0.002, respectively) (Figure 3.5). Patients with late gadolinium enhancement on cardiac MRI had greater levels of BNP, MR-proANP, TNFR1, TNFR2, and MMP-2 (P=0.001, P<0.001, P=0.014, P=0.014, and P<0.001 respectively) (Figure 3.6). Patients with diastolic dysfunction had elevated BNP, MR-proANP, and MMP-2 levels (P=0.002, P=0.01, and P=0.003, respectively) (Figure 3.7 A). Maximal left-atrial size correlated with MR-proANP (P<0.0001) (Figure 3.7 B). Regression analysis revealed that disease burden assessed by the MSSI was positively associated with increased plasma MMP-9 (P=0.015), while the MSSI cardiac subset correlated with MMP-2 (P=0.003). Fabry patients with renal dysfunction (n=18, 26%) had higher levels of BNP, MR-proANP, TNF, TNFR1, TNFR2, MMP-2, MMP-8, galectin-1, and

galectin-3 (P=0.001, P<0.001, P<0.001, P<0.001, P<0.001, P<0.001, P=0.03, P<0.001, and P=0.004, respectively) (Figure 3.8).

3.3.4 Medical Therapy and Biomarkers

Fabry patients who qualify for and receive ERT (n=37, 54%) had greater plasma levels of TNF, TNFR1, TNFR2, MMP-2, and Lyso-Gb_3 (P=0.025, P=0.003, P<0.001, P<0.001, and P=0.001, respectively) (Figure 3.9). Among patients who were prescribed either an ACE-inhibitor or ARB (n=50, 74%), plasma MMP-2 and MMP-8 were elevated (P=0.027 and P=0.015, respectively). Patients prescribed a statin (n=39, 57%) had significantly increased plasma levels of BNP, MR-proANP, galectin-1, and galectin-3 (P=0.007, P=0.001, P=0.023, and P=0.001, respectively), while patients prescribed aspirin (n=46, 68%) had significantly elevated plasma levels of BNP, MR-proANP, and MMP-8 (P=0.008, P=0.001, and P<0.001, respectively).

3.3.5 Demographics and Biomarkers

Among patients with FD, TNF, TNFR1, TNFR2, MMP-2, and Lyso-Gb_3 were elevated in males (n=34, 50%) relative to females (P=0.003, P=0.002, P=0.035, P=0.012, and P<0.001, respectively) (Figure 3.10). Older FD patients (n=34, 50%) over the median age (45.5 years) had higher levels of BNP, MR-proANP, MMP-2, and galectin-3, relative to younger patients with FD (P=0.002, P=0.012, P=0.038, and P=0.011, respectively), while Lyso-Gb_3 levels were lower in these older patients (P=0.012). There were no significant differences in biomarkers between overweight or obese (n=36, 53%; BMI $\geq$ 25.0 kg/m^2) and normal BMI FD patients.

3.3.6 Genotype & Phenotype and Plasma Biomarkers

Galectin-3 concentrations differed significantly (P=0.03) between the type of mutation, missense (n=6, 9.1%) versus nonsense (n=60, 90.9%) while no other statistically significant differences were found for other biomarkers. Plasma concentrations of total Lyso-Gb_3, Lyso-Gb_3, and its analogues were significantly greater in patients with classic phenotypes (n=59, 90.8%; unclassified excluded) compared with those with cardiac/renal variant phenotypes (n=6, 9.2%), but no other significant differences were noted with other biomarkers (Table 3.4).

3.4 Discussion

Proteomic biomarker discovery platform have revealed several altered pathways in FD including vascular dysfunction, oxidative stress, and cytoskeletal remodeling.[16, 38] Our study used a directed approach whereby inflammatory and cardiac remodelling biomarkers were analyzed from plasma of healthy controls and FD patients. The elevation of inflammatory markers TNF, IL-6, TNFR1, and TNFR2 in FD patients strongly implicates chronic inflammation as a major driver in the pathogenesis of FD. Mechanistically, glycolipids, including Lyso-Gb_3, bind to toll-like receptor 4, activating nuclear factor kappa B and T lymphocytes, and subsequent production of proinflammatory cytokines, leading to a chronic inflammatory state and associated vasculopathy.[39-41] Both TNF and IL-6 plasma levels are elevated in chronic heart failure and correlate with decreasing functional status of these patients, as well as all-cause mortality.[42-44] Furthermore, the positive correlation of inflammatory biomarkers in FD patients with higher disease burden based on MSSI scores, cardiac-specific MSSI scores, LVH, LGE, and renal dysfunction suggests that systemic inflammation plays a central role in the morbidity and mortality associated with FD.[45] These findings are further

supported by the relatively high prevalence of diastolic dysfunction in our cohort. Vascular dysregulation in FD may also affect the coronary arteries,[46] leading to microvascular angina[7, 8] and subsequent HFpEF.[47, 48] These findings support the evolving paradigm of inflammation and vascular dysfunction as key pathogenic processes in HFpEF.[45, 48] Fabry patients develop significant renal dysfunction and the strong association of elevated inflammatory markers and worsened renal function observed in our cohort is especially relevant given the connection between HFpEF and renal disease.[49] Of particular interest to FD patients, biomarker panels may, in the future, help identify HFpEF phenotypes to guide appropriate phenotype-specific therapy.[50]

Two different receptor subtypes of TNF, TNFR1- and TNFR2-mediated signaling pathways have opposing effects on the heart.[18, 51] TNFR1-mediated signaling appears to be the primary cause of deleterious effects of TNF in the heart, including increased oxidative stress and cardiomyocyte apoptosis.[18] In contrast, TNFR2-mediated signaling appears to confer the cardioprotective benefits of TNF.[51] The absence of effect of mutation type or phenotype suggests that TNFR1 and TNFR2 may be sensitive to disease progression of FD independent of genetic makeup. The increase in both TNFR1 and TNFR2 suggest a strong systemic inflammatory component of FD. Novel chronic heart failure therapies targeting these receptors are currently being investigated and accordingly may eventually play a role in the management of FD patients with HFpEF.[52, 53] Importantly, TNFR1 and TNFR2 were associated with late gadolinium enhancement, which represents a prehypertrophic phenotype in FD.[54] These biomarkers may also identify prehypertrophic stages of myocardial involvement in FD patients, triggering further investigations such as cardiac MRI.[55]

Fabry disease patients with LGE and/or diastolic dysfunction had significantly higher levels of BNP and MR-proANP, suggesting the presence of significant long-term pathological

cardiac remodelling and possible eventual progression to heart failure.[56] Elevated plasma troponin I and T provide further evidence for a cardiac-specific involvement in FD[57, 58] and, coupled with assessment of plasma natriuretic peptides, represent important diagnostic and prognostic tools in the evaluation of the cardiomyopathy associated with FD. Matrix metalloprotease (MMP)-2, and MMP-9 are implicated in remodelling of the extracellular matrix (ECM) with increased MMP-2 levels associated with the presence of HFpEF, while increased MMP-9 levels predict LVH and adverse ECM modeling.[59] Increased plasma levels of the gelatinases, MMP-2 and MMP-9, in FD patients suggests that inflammation and extracellular matrix remodelling is a significant component of heart disease in FD. This finding is further supported by the correlation of MMP-2 with MSSI, LVH, LGE, and diastolic dysfunction. The elevation of MMP-9 is consistent with previous work[60] and, together with the elevated galectin-1 levels, confirm a critical role of extracellular matrix remodeling in FD. Galectin-3 correlated strongly positively with MSSI and left-atrial maximum size index, which suggests that galectin-3 may be a marker of advanced disease and the development of heart failure in patients with FD.[61]

Enzyme-replacement therapy was associated with increased levels of TNF, TNFR1, TNFR2, MMP-2, and Lyso-Gb_3. These findings highlight the fact that patients who qualify for and receive ERT typically have more severe disease manifestations. Early detection of FD is especially critical as progression of the disease can be slowed by ERT. Importantly, TNFR1 and TNFR2 were associated with late gadolinium enhancement, which represents a prehypertrophic phenotype in female patients with FD.[62] These biomarkers may also identify prehypertrophic stages of myocardial involvement in FD patients, triggering further investigations such as cardiac MRI.[54] In contrast, patients with advanced FD continue to develop major adverse clinical events despite ERT[63], further underlying the need for early detection and appropriate intervention.

Given that dysregulated inflammation persists in some patients despite ERT, monitoring these patients after initiation of ERT using inflammatory biomarkers may provide valuable information about disease control and long-term prognosis, particularly if used in conjunction with other previously identified biomarkers.[15]

Lyso-Gb_3 is a glycosphingolipid that accumulates in FD and serves as a disease-specific biomarker to identify clinically relevant mutations.[2] Levels of plasma Lyso-Gb_3 and utility in the diagnosis of FD were similar to those reported in prior studies.[2, 64, 65] Plasma Lyso-Gb_3 proved to be a flawless biomarker for the diagnosis of classic FD with both sensitivity and specificity of 100%. The use of a total plasma Lyso-Gb_3 level incorporating the levels of six analogues is novel but did not confer increased accuracy for diagnosis of FD in our cohort. Interestingly, the levels of plasma Lyso-Gb_3 for the three patients with unclassified mutations (G261V, intron2:c.369+5G>T, and V254Gfs) were not statistically different from those of classic mutations, suggesting that these three mutations may be classified as classic rather than cardiac/renal variants. In addition to its utility in diagnosis, Lyso-Gb_3 correlated strongly with LVMI and suggests that it may be a marker of sphingolipid accumulation in the myocardium. This finding is consistent with prior work involving FD patients with cardiac-specific variants.[66] Tumour necrosis factor, TNFR1, TNFR2, MMP-2, and Lyso-Gb_3 were elevated in the plasma of male patients with FD compared to females, which is expected given that the gene for α-galactosidase A follows an X-linked inheritance pattern and, accordingly, hemizygotes typically suffer from a more severe form of the disease.[55, 62] Older patients with FD had elevated plasma biomarkers of remodelling, including BNP, MR-proANP, and galectin-3, without higher levels of inflammatory biomarkers. This finding could have therapeutic implications as ERT may

provide limited benefit in older patients. Indeed, in many patients with advanced FD, ERT does not prevent organ failure and death.[63]

Monitoring markers of cardiac remodelling and systemic markers of inflammation may confer increased sensitivity for early subclinical manifestations for disease, which may indicate the need for aggressive treatment including ERT to prevent progression to ERT-refractory FD and its major associated complications.[63] The presence of systemic inflammation in pediatric FD patients is of special interest since these patients may not display major cardiac or renal manifestations until significant irreversible progression of the disease has occurred. In this case, systemic inflammation may contribute to long-term morbidity and mortality before these patients are symptomatic enough to qualify for ERT. Plasma biomarkers may also be valuable in patients with Fabry polymorphisms or mild mutations, particularly in female patients, where the diagnosis and therapy of choice may be less clear.[67]

3.4.1 Study Limitations

A limitation of this study is the sample size, primarily due to the rarity of diagnosis of FD. Although comparable to other studies involving plasma analysis in FD, the sample size may limit the generalizability of the results, particularly with respect to phenotype (classic versus cardiac/renal variant). In addition, the small sample size limits the quality of the statistical tests and may have resulted in underpowered tests. A large sample size would enable further exploration of the relationship between the clinical and imaging variables and biomarker levels. Another limitation is the multiple comparisons, but we attempted to account for these issues by only testing biologically plausible associations. However, many of the significances are strong enough that even the most stringent corrections would still result in rejection of the null hypothesis. Another, broader limitation of the study is the lack of clinical outcomes. Given the

rarity of FD, multinational registries linked to phenotypic data would be valuable. Future studies linking these biomarkers to clinical outcomes are planned, which will help further define their role in prognostication.

3.5 Conclusions

Plasma levels of inflammatory biomarkers, cardiac remodelling biomarkers, and Lyso-Gb3 are elevated in patients with FD. Patients with more severe disease, assessed via MSSI and its cardiac subset, have higher levels of inflammatory and remodelling biomarker levels. Several inflammatory and cardiac remodelling biomarkers, as well as Lyso-Gb3, were elevated in patients with LVH, while cardiac remodelling biomarkers were elevated in patients with diastolic dysfunction. Markers of cardiac remodelling, ECM turnover and inflammatory biomarkers are significantly elevated in patients with renal dysfunction, suggesting that multisystem disease sequelae of FD are associated with greater states of inflammation. These features are consistent with a phenotype dominated by heart disease with preserved ejection fraction and renal disease and suggest a key pathogenic role of systemic inflammation. Exciting new advances in phenotypic-specific and targeted anti-inflammatory therapy has the potential to revolutionize the management of FD.

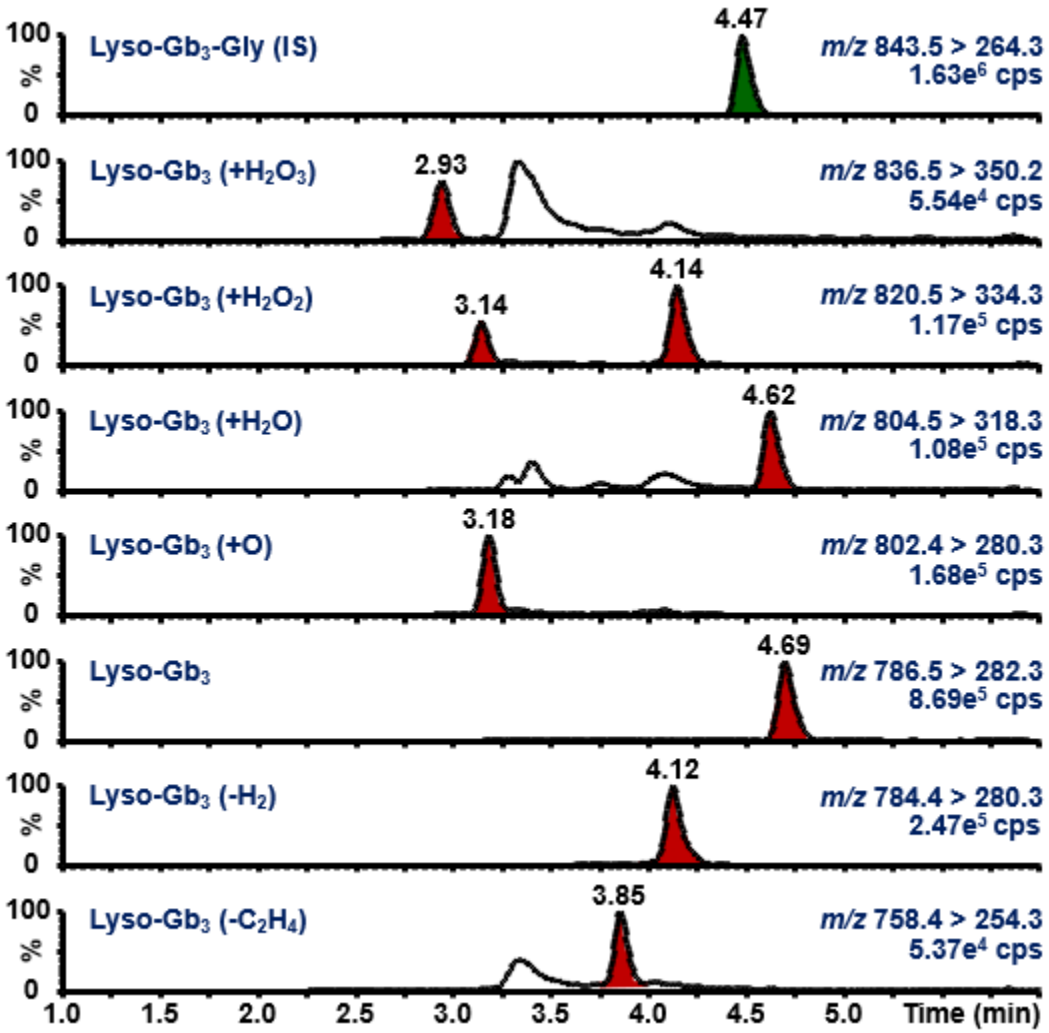


Figure 3.1. Examples of ion chromatograms for Lyso-Gb_3, its 6 analogues and Lyso-Gb_3-Gly (used as the internal standard) detected in plasma from a Fabry patient. The (+H_2O_2) analogue has two structural isomers with retention times of 3.29 and 4.57 min. The areas of these peaks were added together for computation results. Cps = count per second.

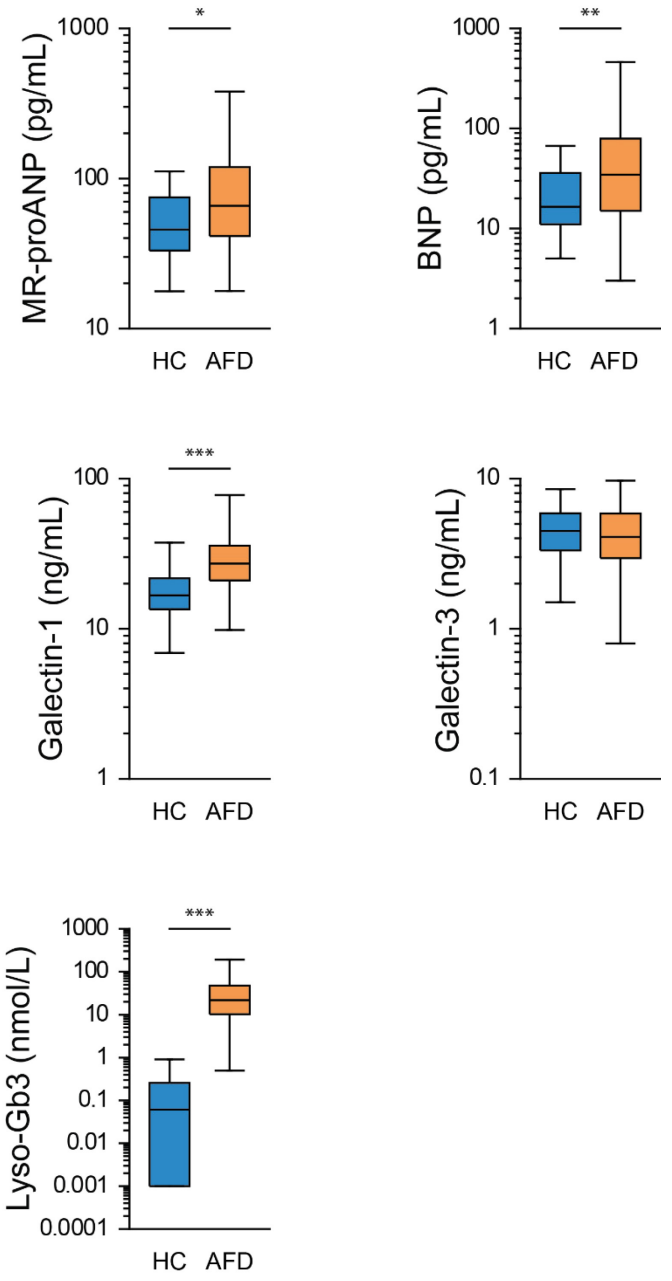
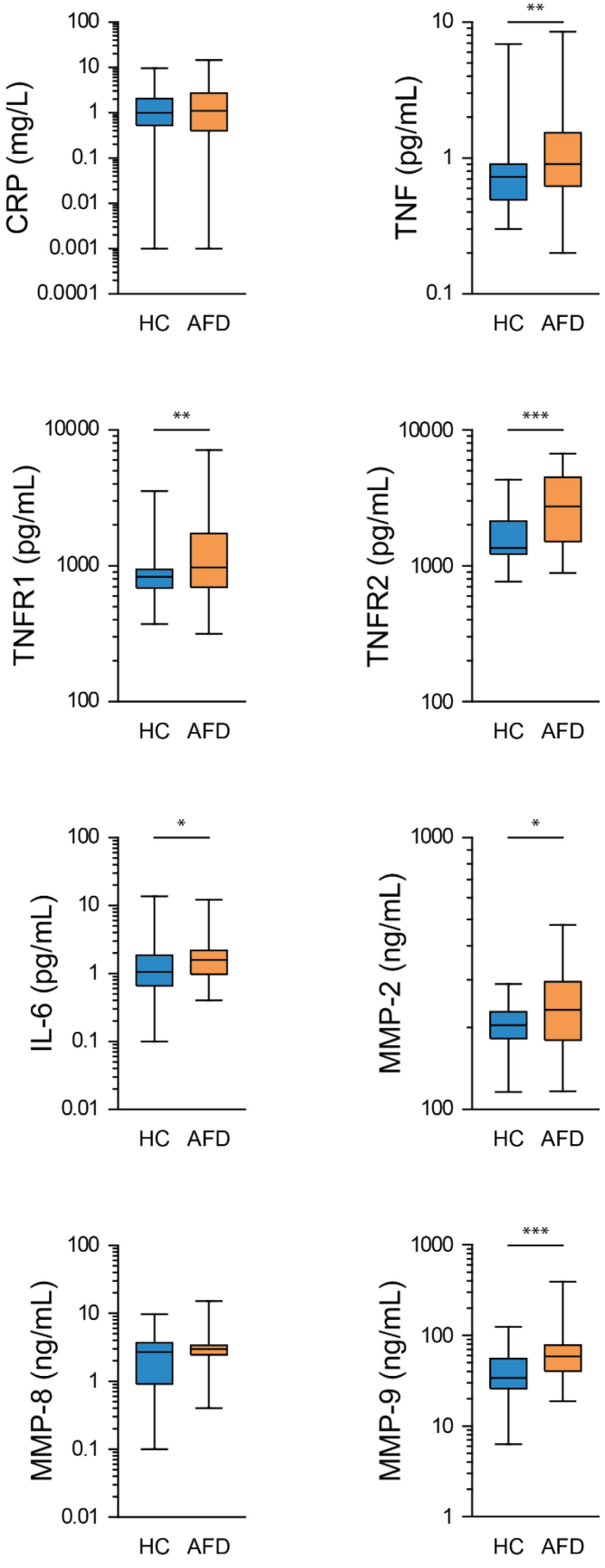


Figure 3.2. Plasma levels of cardiac remodelling biomarkers and Lyso-Gb_3 in cohorts of FD (n=68) and healthy controls (n=40). Biomarkers BNP, MR-proANP, galectin-1, galectin-3, and Lyso-Gb_3 are significantly elevated in the FD cohort relative to healthy controls. FD, Fabry disease; HC, healthy controls. *P<0.05; **P<0.01; ***P<0.001.


CRP (mg/L)
100
10
1
0.1
0.01
0.001
0.0001
HC
AFD
TNF (pg/mL)
**
10
1
0.1
HC
AFD
TNFR1 (pg/mL)
**
10000
1000
100
HC
AFD
TNFR2 (pg/mL)
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1000
100
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1
HC
AFD

Figure 3.3. Plasma levels of inflammatory biomarkers and selected matrix-metalloproteases in cohorts of FD (n=68) and healthy controls (n=40). TNF, IL-6, TNFR1, and TNFR2 are significantly elevated in the FD cohort relative to healthy controls, without significant elevation in CRP. In addition, MMP-2 and MMP-9 are also significantly elevated in the FD cohort relative to healthy controls, while no difference was observed for MMP-8. FD, Fabry disease; HC, healthy controls. *P<0.05; **P<0.01; ***P<0.001.

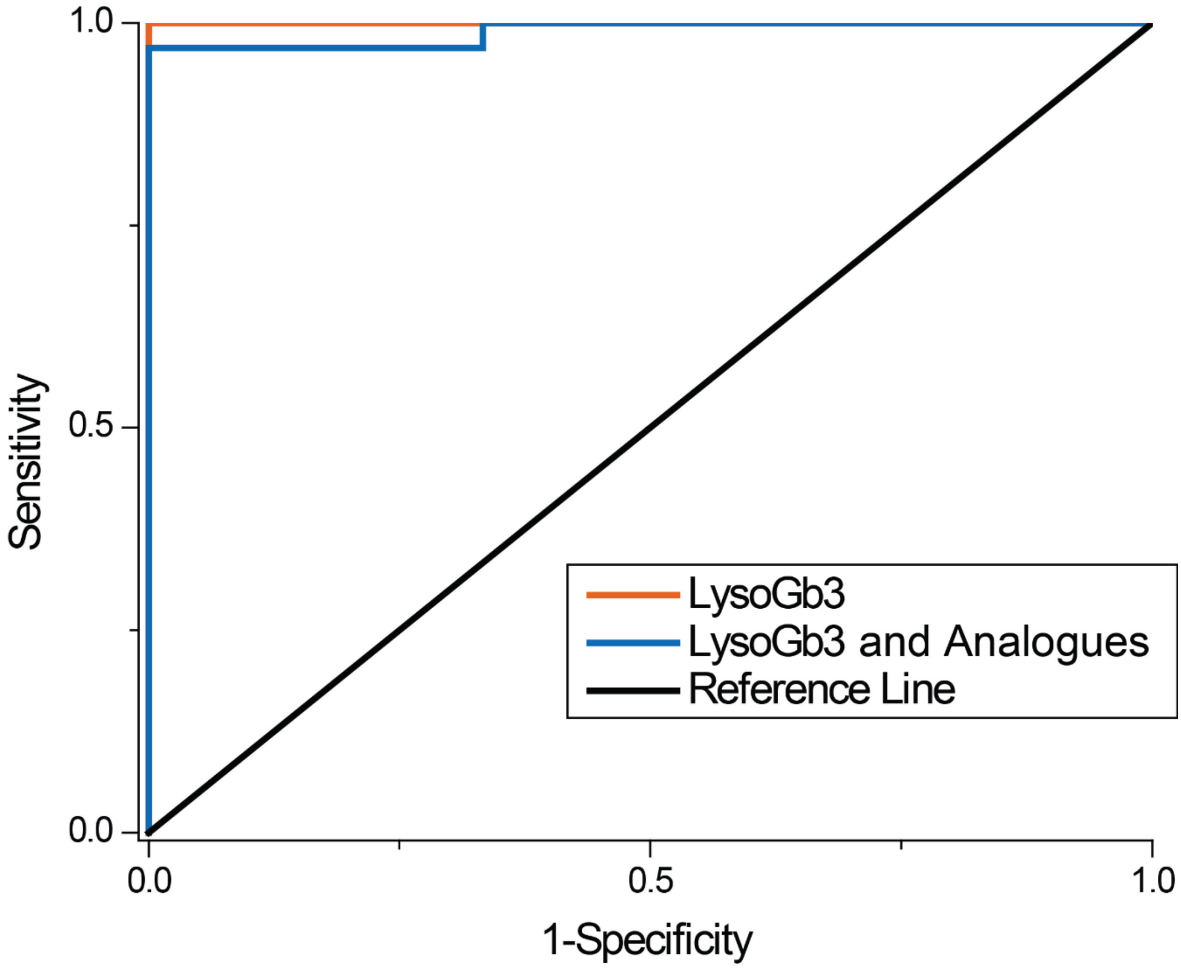

Figure 3.4. Receiver operating characteristic (ROC) curve demonstrating the performance of Lyso-Gb_3 and Lyso-Gb_3 with analogues for the prediction of FD (n=40 healthy controls; n=68 FD patients). Lyso-Gb_3, the gold standard, had excellent performance (AUC=0.998) while Lyso-Gb_3 with analogues had an AUC=0.994.

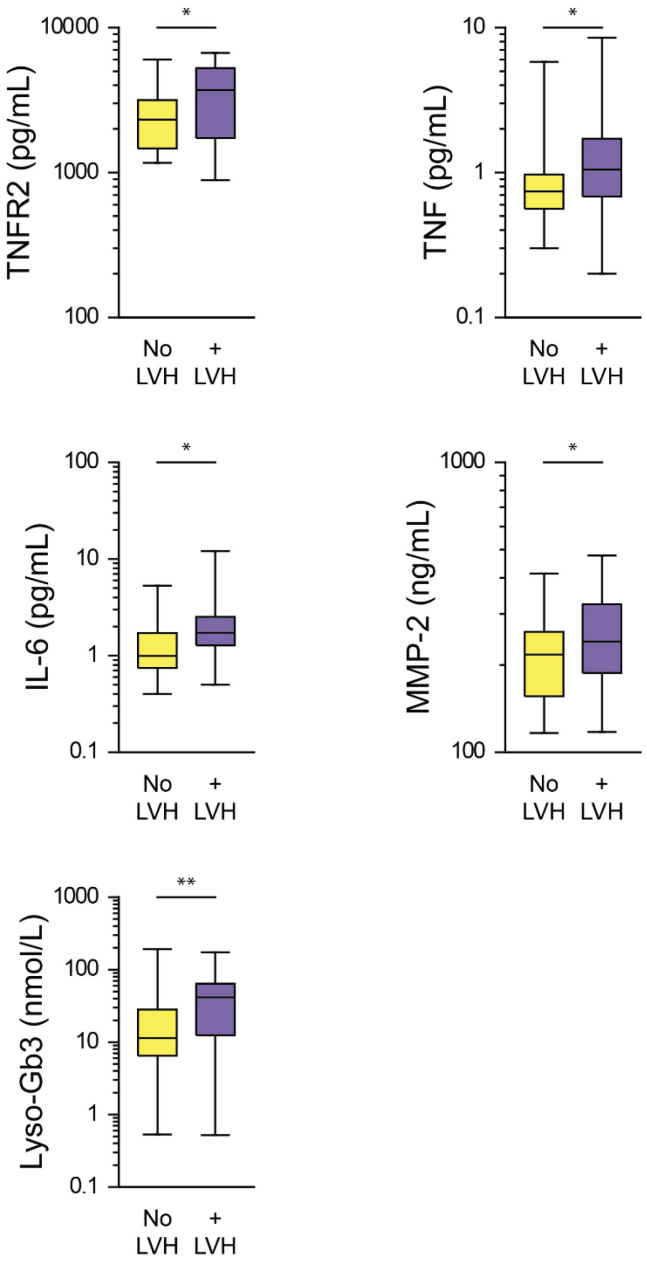

Figure 3.5. Plasma levels of biomarkers in FD patients (n=68) with (n=41) and without (n=27) LVH via imaging criteria. TNFR2, TNF, IL-6, MMP-2, and Lyso-Gb_3 are significantly elevated in FD patients with LVH than those without LVH. FD, Fabry disease; LVH, left ventricular hypertrophy (LVMI ≥85 g/m^2 in males and ≥81 g/m^2 in females). *P<0.05; **P<0.01; ***P<0.001.

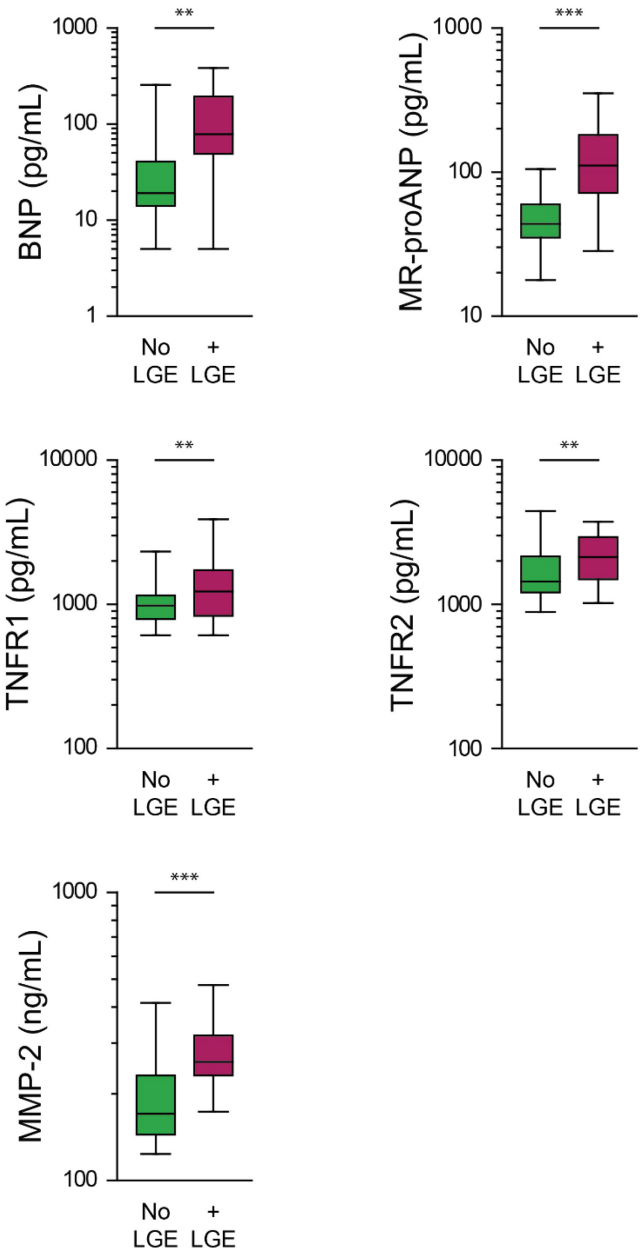

Figure 3.6. Plasma levels of biomarkers in FD patients (n=65) with (n=30) and without (n=35) late gadolinium enhancement (LGE) on cardiac MRI. BNP, MR-proANP, TNFR1, TNFR2, and MMP-2 are elevated in FD patients with LGE. FD, Fabry disease; LGE, late gadolinium enhancement. *P<0.05; **P<0.01; ***P<0.001.

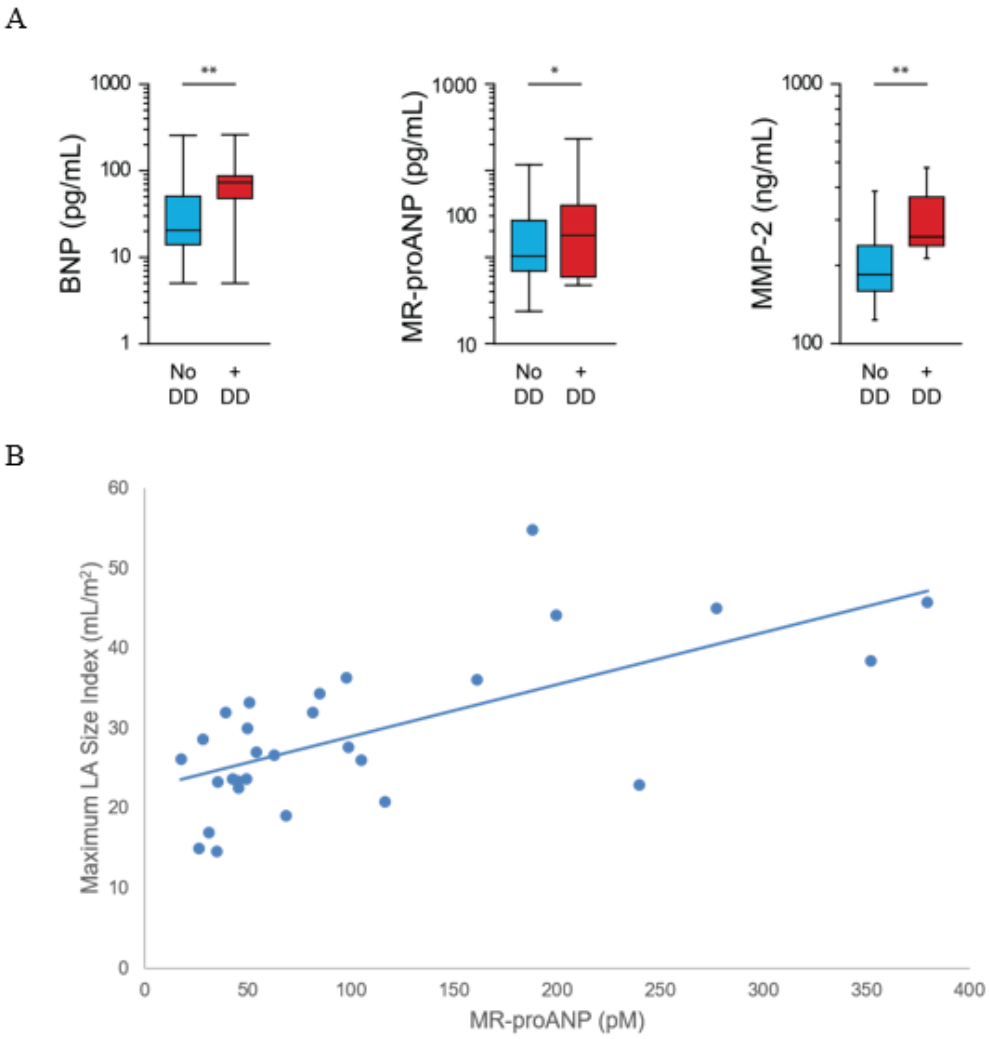

Figure 3.7. Plasma levels of biomarkers in FD patients (n=43) with (n=17) and without diastolic dysfunction (n=26) per echocardiography. BNP, MR-proANP, and MMP-2 are significantly elevated in FD patients with diastolic dysfunction than those without diastolic dysfunction. Diastolic dysfunction could not be assessed in some patients due to arrhythmia (*A*). A correlation plot of MR-proANP versus maximum LA size index (R^2=0.44, p<0.001). MR-proANP and maximum LA size index are positively correlated, as increasing LA size is known to cause atrial cardiomyocytes to release ANP (*B*). DD, diastolic dysfunction; FD, Fabry disease; LA, left atrial. *P<0.05; **P<0.01; ***P<0.001

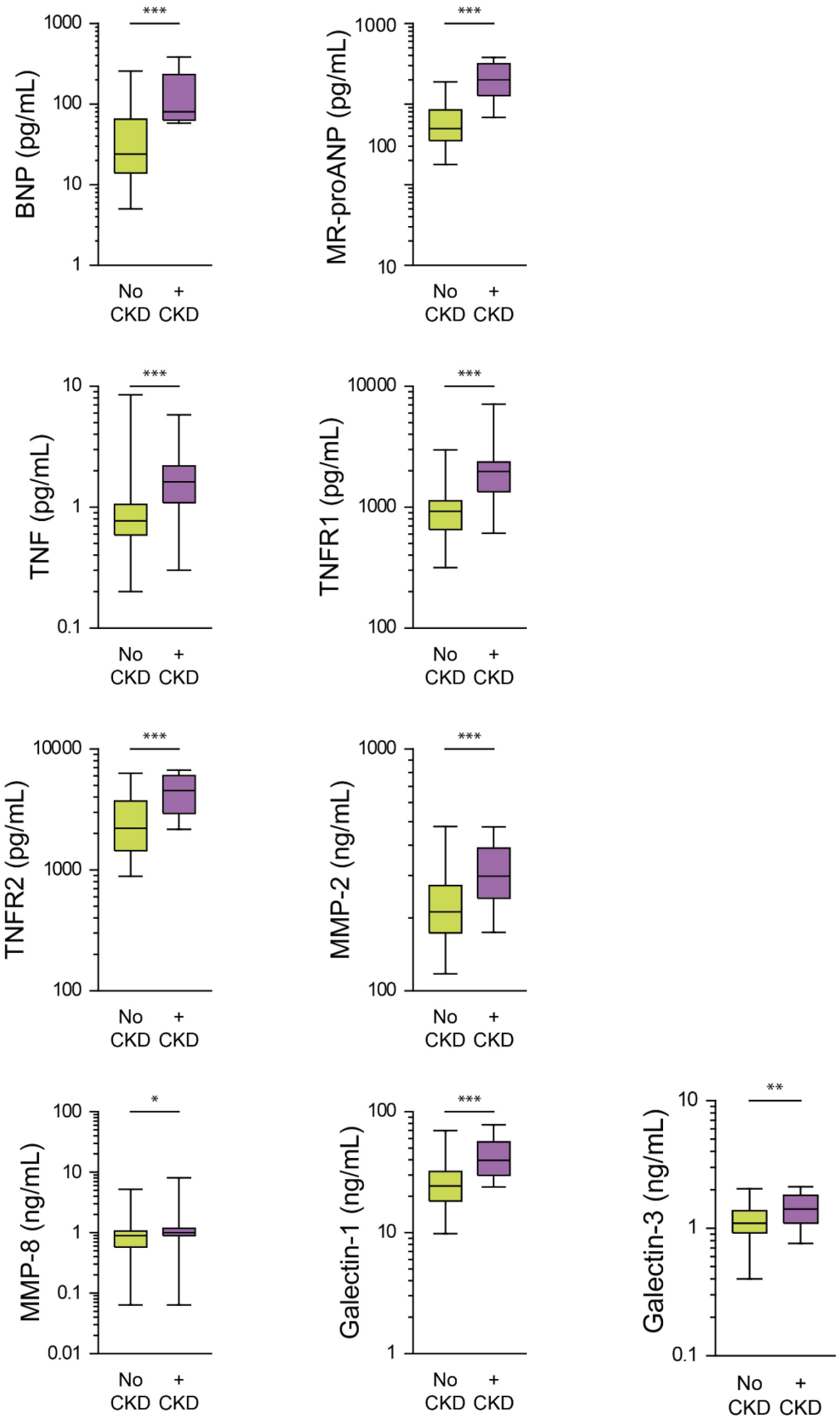
BNP (pg/mL)
1000
100
10
1
***
No CKD
+ CKD
MR-proANP (pg/mL)
1000
100
10
***
No CKD
+ CKD
TNF (pg/mL)
10
1
0.1
***
No CKD
+ CKD
TNFR1 (pg/mL)
10000
1000
100
***
No CKD
+ CKD
TNFR2 (pg/mL)
10000
1000
100
***
No CKD
+ CKD
MMP-2 (ng/mL)
1000
100
***
No CKD
+ CKD
MMP-8 (ng/mL)
100
10
1
0.1
0.01
*
No CKD
+ CKD
Galectin-1 (ng/mL)
100
10
1
***
No CKD
+ CKD
Galectin-3 (ng/mL)
10
1
0.1
**
No CKD
+ CKD

Figure 3.8. Plasma levels of biomarkers in FD patients (n=68) with (n=18) and without significant chronic kidney disease (n=50). BNP, MR-proANP, TNF, TNFR1, TNFR2, MMP-2, MMP-8, galectin-1 and galectin-3 are significantly elevated in FD patients with CKD. FD, Fabry disease; CKD, chronic kidney disease (eGFR<60 mL/min/1.73m^2). *P<0.05; **P<0.01; ***P<0.001.

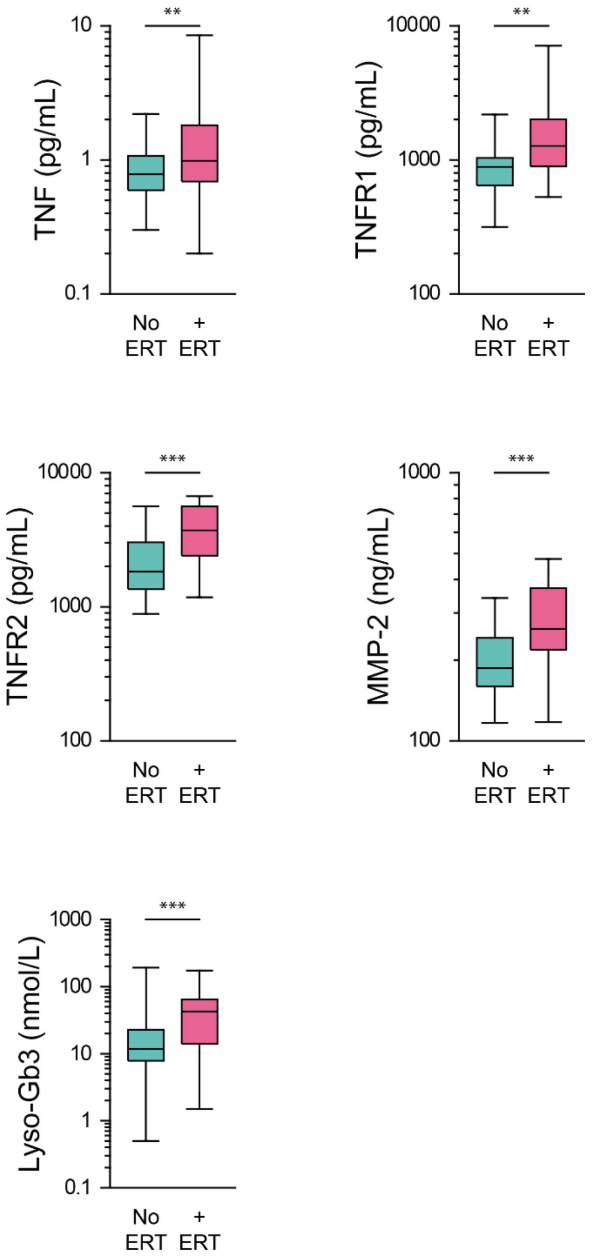

Figure 3.9. Plasma levels of biomarkers in FD patients (n=68), in cohorts of those not receiving ERT (n=31) and those who qualify for and are receiving ERT (n=37). TNF, TNFR1, TNFR2, MMP-2, and Lyso-Gb3 are elevated in FD patients undergoing ERT relative to those not receiving ERT. FD, Fabry disease; ERT, enzyme replacement therapy. *P<0.05; **P<0.01; ***P<0.001.

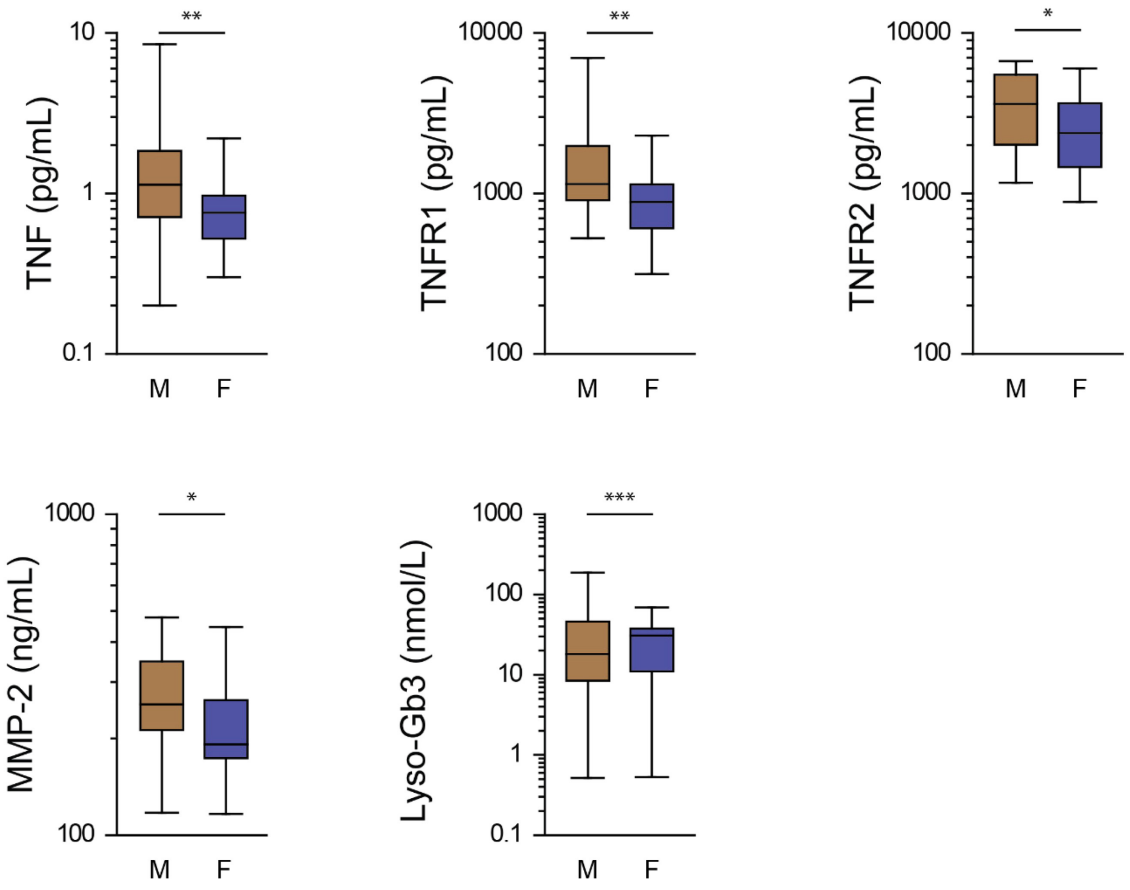

Figure 3.10. Plasma levels of biomarkers in FD patients (n=68) of male (n=34) and female (n=34) sex. TNF, TNFR1, TNFR2, MMP-2, and Lyso-Gb3 are elevated in male FD patients relative to females. F, female; FD, Fabry disease; M, male. *P<0.05; **P<0.01; ***P<0.001.

Table 3.1. Demographic data and cardiac imaging parameters for the Fabry disease cohort.

	Healthy Controls (n=40)	Fabry Disease (n=68)	Fabry Male (n=34)	Fabry Female (n=34)
Demographic & Clinical Information				
Age (years)	46 ±12	42 ±13	40 ±11	44 ±13
Sex (% female)	50	50	0	100
BMI (kg/m^2)	25.0 ±2.7	24.3 ±4.3	24.3 ±3.9	24.4 ±4.8
eGFR ($mL/min/1.73m^2$)	-	85.9 ±32.9	77.8 ±36.7	94.1 ±26.6
Echocardiography Parameters				
LVEF (%)	-	62.6 ±8.4	59.9 ±9.9	66.4 ±3.3
End-diastolic thickness (mm)	-	8.1 ±1.9	8.7 ±2.1	7.6 ±1.3
End-systolic thickness (mm)	-	11.9 ±2.5	12.7 ±2.9	11.1 ±1.6
E-wave velocity (m/s)	-	90.4 ±21.5	94.4 ±23.8	83.8 ±16.2
A-wave velocity (m/s)	-	71.3 ±22.4	71.9 ±23.0	70.4±22.8
E/A ratio	-	1.34 ±0.34	1.36 ±0.23	1.31 ±0.47
e' velocity (m/s)	-	0.089 ±0.026	0.093 ±0.030	0.084 ±0.022
E/e' ratio	-	11.1 ±4.7	11.8 ±5.8	10.2 ±2.7
LA volume index (mL/m^2)	-	29.3 ±9.7	30.9 ±10.4	27.0 ±8.4
Diastolic Dysfunction				
None (%)	-	60	56	69
Grade I (%)	-	12	11	13
Grade II (%)	-	28	33	19
Grade III (%)	-	0	0	0
Cardiac MRI Parameters				
$LVEDV_i$ (mL/m^2)	-	81.6 ±16.4	92.1 ±16.0	71.6 ±9.1
$LVESV_i$ (mL/m^2)	-	30.3 ±8.9	33.8 ±10.1	27.1 ±6.3
LVEF (%)	-	63.6 ±6.7	64.0 ±8.5	63.2 ±4.8
LVMI (g/m^2)	-	78.5 ±21.6	91.4 ±8.5	66.4 ±12.7
T_1 baseline, myo (ms; n=36)	-	1068±39	1041±36	1085±45
T_1 post-contrast, myo (ms; n=34)	-	536±32	551±36	519±41
ECV (%; n=34)	-	22.1±3.0	23.2±3.9	21.4±3.5

Demographic and imaging parameters are expressed as mean±standard deviation. BMI, body-mass index; eGFR, estimated glomerular filtration rate (using the Modification of Diet in Renal Disease equation); LVEF, left-ventricular ejection fraction; E, peak mitral inflow during passive filling in early diastole; A, peak mitral inflow during active filling in atrial systole; e', mitral annular velocity during early diastole; LA, left-atrial; MRI, magnetic resonance imaging; LVEDV, left-ventricular end-diastolic volume; LVESV, left-ventricular end-systolic volume; LVMI; left-ventricular mass-index; myo, myocardium; ECV, extracellular volume.

Table 3.2. List of identified mutations with corresponding phenotype and selected demographic and clinical information in FD cohort (n=68).

Mutation	Phenotype	Age (Sex)	ACE-i or ARB	Statin	ASA	ERT
A143P (n=30) S345P (n=9) Y134S (n=4) E338K (n=3) N215S (n=3) R112H (n=3) R227Q (n=3) Other (n=13)	Classic 87% (n=59) Cardiac variant 4.4% (n=3) Renal variant 4.4% (n=3) Unclassified 4.4% (n=3)	42.0 ± 12.6 years (mean ± standard deviation) Female 50% (n=34)	74% (n=50)	57% (n=39)	68% (n=46)	54% (n=37)
A143P	Classic[69]	15 (M)	Yes	No	No	No
A143P	Classic	19 (F)	No	No	No	No
A143P	Classic	26 (F)	No	No	No	No
A143P	Classic	28 (M)	No	No	No	No
A143P	Classic	29 (F)	Yes	Yes	Yes	No
A143P	Classic	30 (M)	No	No	Yes	Yes
A143P	Classic	33 (M)	No	No	Yes	Yes
A143P	Classic	34 (F)	No	No	Yes	No
A143P	Classic	34 (M)	Yes	No	Yes	Yes
A143P	Classic	35 (M)	Yes	Yes	Yes	Yes
A143P	Classic	35 (M)	Yes	Yes	Yes	Yes
A143P	Classic	35 (M)	Yes	Yes	No	Yes
A143P	Classic	36 (F)	Yes	Yes	Yes	Yes
A143P	Classic	36 (F)	No	No	No	No
A143P	Classic	39 (F)	No	No	Yes	No
A143P	Classic	40 (M)	Yes	Yes	Yes	Yes
A143P	Classic	45 (F)	No	No	No	No
A143P	Classic	46 (F)	No	No	No	No
A143P	Classic	47 (M)	Yes	No	No	Yes
A143P	Classic	48 (M)	No	Yes	No	Yes
A143P	Classic	48 (M)	Yes	No	Yes	Yes
A143P	Classic	51 (F)	Yes	No	Yes	Yes
A143P	Classic	51 (F)	No	Yes	No	No
A143P	Classic	51 (M)	No	Yes	Yes	Yes
A143P	Classic	52 (M)	Yes	Yes	No	Yes
A143P	Classic	54 (F)	Yes	Yes	Yes	No
A143P	Classic	55 (F)	Yes	Yes	No	Yes
A143P	Classic	62 (M)	Yes	Yes	No	Yes
A143P	Classic	66 (F)	Yes	No	Yes	Yes
A143P	Classic	68 (F)	Yes	Yes	Yes	Yes

E338K	Classic[70]	34 (F)	Yes	No	Yes	No
E338K	Classic	36 (F)	Yes	No	Yes	Yes
E338K	Classic	62 (M)	Yes	Yes	Yes	Yes
G261V	Unclassified	36 (F)	Yes	Yes	Yes	No
G43V	Classic[71]	35 (M)	Yes	Yes	Yes	Yes
intron2: c.369+5G>T	Unclassified	41 (M)	Yes	Yes	Yes	No
N215S	Cardiac variant[72]	26 (F)	No	No	No	No
N215S	Cardiac variant	41 (M)	Yes	Yes	Yes	No
N215S	Cardiac variant	66 (F)	Yes	Yes	Yes	Yes
Q321E	Classic[73]	51 (F)	Yes	Yes	Yes	Yes
Q386X	Classic[74]	35 (M)	Yes	Yes	Yes	Yes
Q386X	Classic	46 (F)	Yes	No	Yes	No
R112C	Classic[73]	51 (M)	No	Yes	Yes	Yes
R112C	Classic	55 (F)	Yes	No	No	Yes
R112H	Renal variant[75]	25 (M)	Yes	No	No	No
R112H	Renal variant	48 (F)	Yes	Yes	Yes	No
R112H	Renal variant	50 (M)	Yes	Yes	Yes	No
R220X	Classic[76]	29 (F)	No	No	No	No
R220X	Classic	55 (F)	Yes	Yes	Yes	Yes
R227Q	Classic[76]	19 (F)	No	No	No	No
R227Q	Classic	40 (M)	Yes	Yes	Yes	Yes
R227Q	Classic	55 (F)	Yes	Yes	Yes	No
R227X	Classic[76]	52 (M)	Yes	Yes	Yes	Yes
S345P	Classic[76]	15 (M)	No	No	No	No
S345P	Classic	28 (M)	Yes	No	Yes	No
S345P	Classic	33 (M)	Yes	Yes	Yes	Yes
S345P	Classic	39 (F)	Yes	Yes	Yes	No
S345P	Classic	45 (F)	Yes	Yes	Yes	No
S345P	Classic	47 (M)	Yes	Yes	Yes	Yes
S345P	Classic	50 (M)	Yes	Yes	Yes	Yes
S345P	Classic	51 (F)	Yes	Yes	Yes	No
S345P	Classic	54 (F)	Yes	Yes	Yes	No
V254Gfs	Unclassified	50 (M)	Yes	Yes	No	Yes
W349X	Classic[77]	30 (M)	Yes	Yes	Yes	Yes
Y134S	Classic[74]	34 (M)	Yes	No	No	Yes
Y134S	Classic	35 (M)	Yes	No	Yes	Yes
Y134S	Classic	39 (F)	Yes	No	Yes	No
Y134S	Classic	68 (F)	Yes	Yes	Yes	Yes

ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; ASA, acetylsalicylic acid; ERT, enzyme-replacement therapy.

Table 3.3. Biomarker data for the Fabry disease cohort.

	Healthy Controls (n=40)	Fabry Disease (n=68)	Fabry Male (n=34)	Fabry Female (n=34)
Biomarkers				
BNP (pg/mL)	16.5 (11, 35.75)	34.5 (15, 79.25)	22 (10, 77.75)	39.5 (20, 85.5)
MR-proANP (pM)	45.6 (33.0, 74.7)	65.6 (41.3, 119)	56.7 (39.5, 191)	77.7 (43.9, 117)
TNFR1 (pg/mL)	829 (686, 939)	971 (696, 1726)	1153 (914, 1992)	890 (610, 1148)
TNFR2 (pg/mL)	1350 (1217, 2124)	2730 (1507, 4471)	3608 (2014, 5492)	2376 (1460, 3635)
TNF (pg/mL)	0.73 (0.49, 0.90)	0.90 (0.62, 1.53)	1.13 (0.71, 1.84)	0.77 (0.55, 0.97)
IL-6 (pg/mL)	1.05 (0.66, 1.85)	1.58 (0.97, 2.19)	1.58 (0.99, 2.18)	1.58 (0.96, 2.21)
MMP-2 (ng/mL)	204 (182, 229)	232 (180, 295)	256 (212, 348)	199 (174, 262)
MMP-8 (ng/mL)	2.70 (0.91, 3.68)	2.97 (2.44, 3.36)	3.03 (2.45, 3.38)	2.94 (2.09, 3.36)
MMP-9 (ng/mL)	34.1 (25.8, 55.3)	58.7 (40.4, 78.0)	64.1 (41.8, 83.6)	55.4 (40.1, 74.1)
Galectin-1 (ng/mL)	16.7 (13.5, 21.7)	27.2 (21.0, 35.8)	25.5 (18.2, 38.8)	28.9 (22.5, 35.0)
Galectin-3 (ng/mL)	4.48 (3.33, 5.88)	4.08 (2.95, 5.85)	3.65 (2.81, 6.61)	4.38 (2.95, 5.85)
Lyso-Gb3 (nmol/L)	0.06 (0, 0.25)	21.8 (10.3, 47.2)	47.1 (31.3, 70.8)	11.2 (8.7, 18.3)

Biomarker data are reported as medians (25^{th} percentile, 75^{th} percentile). BNP, B-type natriuretic peptide; MR-proANP, mid-regional pro-atrial natriuretic peptide; TNFR, tumour necrosis factor receptor; TNF, tumour necrosis factor; IL, interleukin; MMP, matrix metalloprotease; Gb3, globotriaosylceramide.

Table 3.4. Plasma concentration of Lyso-Gb_3 and its six related analogues with modified sphingosine moieties (-H_2O_2; -H_2; +O; +H_2O; +H_2O_2, +H_2O_3) by phenotype, classic versus variant.

Biomarker	Classic (n=59) nmol/L	Variant (n=6) nmol/L	Significance
Total Lyso-Gb_3	59 ±64	4.6 ±3.7	P<0.00001
Lyso-Gb_3 (m/z 786)	40 ±40	3.7 ±3.0	P<0.00001
Lyso-Gb_3 analogue (*m/z* 758)	0.71 ±1.6	nd	P=0.11
Lyso-Gb_3 analogue (*m/z* 784)	7.8 ±9.3	0.85 ±0.77	P<0.001
Lyso-Gb_3 analogue (*m/z* 802)	3.2 ±5.7	0.023 ±0.056	P=0.03
Lyso-Gb_3 analogue (*m/z* 804)	4.0 ±4.4	nd	P<0.0001
Lyso-Gb_3 analogue (*m/z* 820)	2.5 ±6.0	nd	P=0.04
Lyso-Gb_3 analogue (*m/z* 836)	0.44 ±1.4	nd	P=0.60

Unclassified mutations are excluded. Values are expressed as mean±standard deviation.
m/z, mass-to-charge ratio; nd, not detected.

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CHAPTER 4

VALVULAR HEART DISEASE IN FABRY PATIENTS

4. Valvular Heart Disease in Fabry Patients

4.1 Introduction

Fabry disease (FD) is an X-linked lysosomal storage disorder in which glycosphingolipids accumulate in various tissues, causing cardiovascular, renal, and neurological impairment. The cardiovascular manifestations include cardiomyocyte hypertrophy, endothelial dysfunction, arrhythmias, and valve disease.[1] FD is classically associated with mild tricuspid, aortic, and mitral valve disease. [2-4] The advent of enzyme replacement therapy, substrate reduction therapy, and aggressive control of cardiovascular comorbidities has resulted in improved outcomes for FD patients and survival.[5] The burden of valve disease in the aging FD population treated with modern medical therapy is unknown. Furthermore, given the accumulation of glycosphingolipids in tissues, a proinflammatory state, and concomitant renal dysfunction often seen in FD, it is possible that valve disease progression will be greater than historical non-FD controls.[6] We describe the burden of valvular disease in a multicenter cohort of Fabry disease with access to contemporary therapy, including enzyme-replacement therapy and aggressive cardiovascular risk reduction therapies.

4.2 Methods

4.2.1 Ethics Statement

Ethics approval was obtained from the Health Research Ethics Boards at the University of Alberta (Edmonton, Alberta, Canada) and the University of Calgary (Calgary, Alberta, Canada). All subjects gave written informed consent.

4.2.2 Patient Population

Fabry disease patients (n=69) were recruited through Canadian metabolic clinics in Edmonton and Calgary between May 2010 and October 2019. Patients received guideline-directed medical therapy and those who qualified under the Canadian Fabry Disease Initiative (CFDI) treatment guidelines were offered enzyme-replacement therapy.[7]

4.2.3 Clinical Data

Demographic information including age, sex, height, and weight were collected. Clinical data including genetic mutation analysis, medical therapy, serum creatinine, and outcome data were also recorded for FD patients.[8] Estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease equation.[9]

4.2.4 Transthoracic Echocardiography

Transthoracic echocardiography was performed for the Fabry disease cohort as described previously.[10, 11] Briefly, echocardiography was performed on commercial ultrasound equipment (M3 S Probe, Vivid 7; GE Vingmed Ultrasound AS, Horten, Norway) using standard acquisition techniques.[12] Echocardiograms were interpreted by experienced cardiologists at the University of Alberta Hospital and Foothills Medical Centre. Chamber quantification and ejection fraction was assessed using guideline-recommended methods.[13] The presence of diastolic dysfunction was graded according to current guidelines.[14] Valvular disease was classified as mild, moderate, or severe using established guidelines.[15] [16] Significant valvular heart disease was defined as moderate or greater regurgitation or stenotic lesions. For the estimation of prevalence, in the case of repeat echocardiograms with discrepant results, the most recent echocardiogram interpretation was used.

4.2.5 Biomarkers

Biomarkers of disease activity, inflammation, and cardiac remodeling were analyzed as previously described for 36 patients.[6] Enzyme-linked immunosorbent assays were used to determine plasma levels of TNF, TNFR1, TNFR2, IL-6, MMP-2, MMP-8, MMP-9, galectin-1, and galectin-3, while BNP and MR-proANP levels were assessed using reagent analysis at provincial health laboratories. Plasma Lyso-Gb_3 was quantified as previously described using ultra-performance liquid chromatography to separate samples and tandem mass spectrometer for analysis.[17]

4.2.6 Statistical Analysis

Statistical analyses were carried out using IBM SPSS Statistics version 20 for Windows (SPSS Inc, Chicago, IL, USA). Discrete variables are presented as count and/or percent and continuous variables with normal distributions are presented as mean ±standard deviation, unless otherwise indicated. Statistical analyses were performed as previously described.[6]

4.3 Results

4.3.1. Demographic Information

Demographic and clinical information, including renal function, New York Heart Association (NYHA) dyspnea scale, and mutation data, for the 68 patients with Fabry disease is listed in Table 4.1. Approximately half the patients were females and most patients had mutations leading to a classic phenotype. Hypertension and dyslipidemia were the most common comorbidities. About a quarter of patients were symptomatic with heart failure (NYHA II or greater). Three patients had reduced ejection fraction. About half of patients were treated with enzyme replacement therapy.

4.3.1. Prevalence of Valvular Disease

Valvular heart disease, especially regurgitant lesions, was present in this population (Table 4.2, Figure 1A). Mitral regurgitation was the most common valve pathology, followed by tricuspid regurgitation. Only two patients had significant (i.e. moderate or greater) valvular stenosis. All reported valve disease was due to primary valvular pathology; two patients in the cohort had heart failure with reduced ejection fraction but did not have any significant valvular disease. One patient had a bicuspid aortic valve. Approximately 38% of patients had at least mild valvular disease, while 10% had moderate or severe disease (Figure 1B).

4.3.1. Characterization of Patients with Valvular Disease

Patients with valvular heart disease were older (p=0.006) but did not differ in BMI (p=0.370) or renal function (p=0.083). Patients with classic phenotypes had worse valvular disease (p=0.036). For the subset of patients with biomarker data, there was no relationship between worsening valvular heart disease and BNP, MR-proANP, TNF, TNFR1, TNFR2, IL-6, MMP-2, MMP-8, MMP-9, galectin-1, galectin-3, or lyso-Gb3. During the enrollment period, approximately one-fifth of patients were hospitalized for heart failure, one patient died, and three separate patients required valvular intervention (Table 4.3). Two aortic valve replacements and one mitral valve repair were performed.

4.4 Discussion

The prevalence of significant valvular heart disease in our Fabry disease cohort was high. The increasing prevalence with advanced age is consistent with prior literature, as is the lack of association with renal function.[4, 18] The prevalence of valvular involvement in Fabry patients

stratified by age was compared to the general adult population (Table 4.4).[18] The overall prevalence of valvular heart disease in the general population was estimated at 2.5% (2.2-2.7%) across all age groups, compared to 10% for the Fabry cohort. In every age group, the proportion of patients with significant valvular heart disease exceeded that of the general population.

The etiology of the increased prevalence of significant valve disease in Fabry patients is likely multifactorial. Direct glycosphingolipid deposition on valve leaflets and apparatuses has been proposed as a potential mechanism.[4] Altered chamber geometry with atrial dilatation, annular dilatation, and/or aortic root dilatation may also contribute to valvular regurgitation.[19, 20] Impaired microvascular function in FD patients may contribute to papillary muscle dysfunction and mitral regurgitation.[21] The higher prevalence of left-sided valvular lesions suggests a pressure-mediated mechanism; however this may be related to clinician inattention to right-sided valve disease.[22]

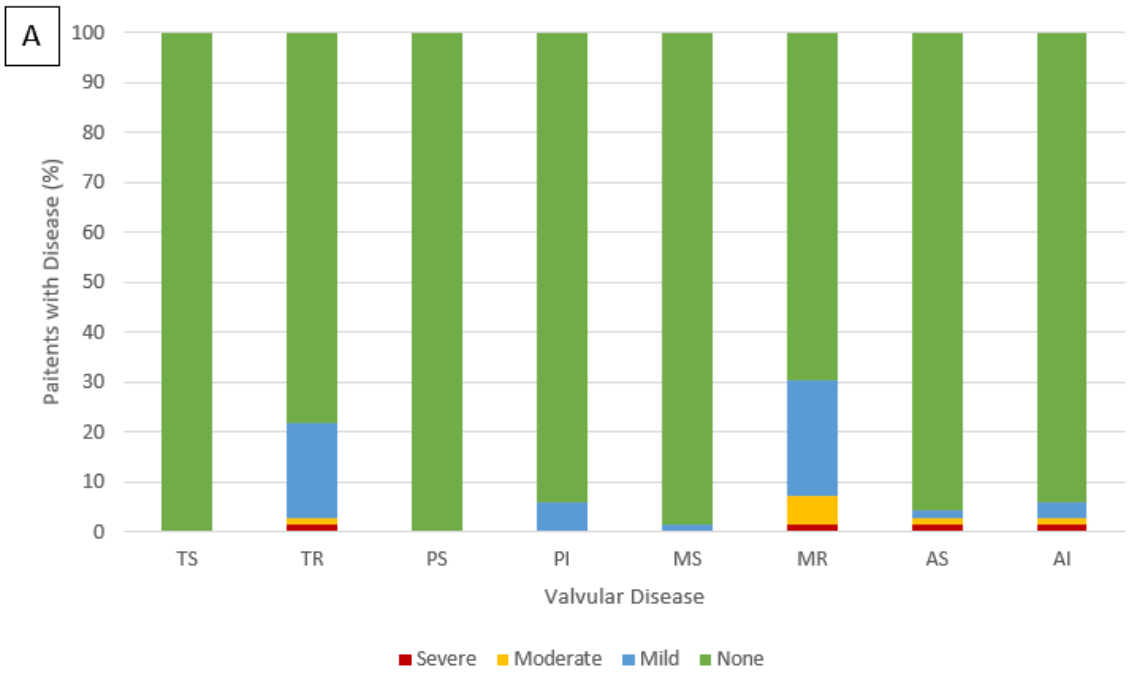
Previous studies of Fabry patients reported a lower prevalence of significant valvular disease (Table 4.5). A prior study of Fabry disease noted thickening of the aortic valve and mitral valve in about a quarter of patients each, but there were no patients with moderate or greater valvular disease.[2] In a more recent study, some moderate disease was noted, but no severe disease was present.[4] This apparent discrepancy can potentially be explained. Firstly, the Kampmann et al. study only enrolled women compared to 48% women in the present study; women are less likely to suffer from the classic version of Fabry disease.[1] Secondly, both prior studies had, on average, younger patients than the present study.

4.4.1 Study Limitations

There are limitations to our study that must be addressed. Patients were recruited through metabolic clinics and cascade screening, which may not capture the entire FD population. Although echocardiography interpretation was performed by Level 3 echocardiographers at an academic institution, they were not interpreted at a core lab and, therefore, the possibility of bias exists. There were instances of valvular disease coded as mild-to-moderate or moderate-to-severe disease. In all these cases, subsequent echocardiography reclassified these lesions. However, clinical information or other cardiac tests could have influenced the interpretation of the severity of these lesions.

4.5 Conclusions

Fabry disease patients have a higher prevalence of valvular disease than the general population, which is dominated by valvular insufficiency. The prevalence of valvular disease in the present study was also greater than that in prior Fabry studies, which may be explained by demographic differences. Older patients and those with classic phenotypes were more likely to have worse valvular disease. Disease requiring valve intervention is not common, but its prevalence may increase over time as FD patients live longer with earlier recognition and improved access to FD therapies such ERT, chaperone therapy, and aggressive cardiovascular risk factor reduction.



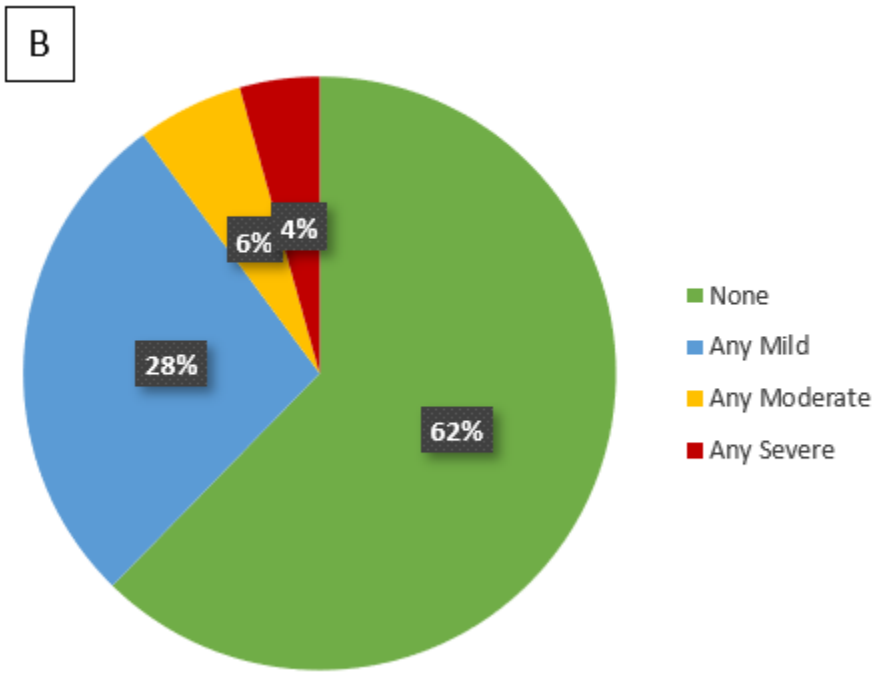


Figure 4.1. (A) Prevalence of valvular heart disease in patients with Fabry disease. A, aortic; M, mitral; P, pulmonic; R, regurgitation; S, stenosis; T, tricuspid. (B) Proportion of patients with at least one valve with indicated severity of disease. 10% of patients had at least moderate valvular disease.

Table 4.1.

Demographics	
Age, years	45 ± 17
Female sex, n (%)	33 (48%)
BMI, kg/m^2	25.6 ± 6.2
Mutation	
Classic	58 (84%)
Variant	11 (16%)
Comorbidities, n (%)	
Hypertension	25 (36%)
Dyslipidemia	20 (29%)
Type 2 diabetes mellitus	1 (1.4%)
Atrial fibrillation	10 (14%)
Renal Disease	
eGFR, mL/min/1.73m^2	93 ± 37
eGFR <60 mL/min/1.73m^2	13 (19%)
Renal replacement therapy	7 (10%)
Cardiac Disease	
NYHA Class, n (%)	
Class I	51 (74%)
Class II	14 (20%)
Class III	4 (5.8%)
Class IV	0 (0%)
LVEF >50%	66 (96%)
LVEF 35-50%	1 (1.4%)
LVEF <35%	2 (2.9%)
Diastolic dysfunction (n=64)	18 (28%)
Medical Therapy, n (%)	
Acetylsalicylic acid	35 (51%)
ACE-i/ARB	37 (54%)
Statin	23 (33%)
ERT	33 (48%)

Demographic and clinical characteristics for the Fabry disease cohort. Estimated GFR data preceding dialysis or renal transplantation were used for these patients. In 5 patients, diastolic dysfunction unable to be assessed due to arrhythmia or was indeterminate. ACE-i, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; BMI, body-mass index; ERT, enzyme-replacement therapy; eGFR, estimated glomerular filtration rate; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association.

Table 4.2.

	Mild	Moderate	Severe
Tricuspid stenosis	0 (0%)	0 (0%)	0 (0%)
Tricuspid regurgitation	13 (19%)	1 (1.4%)	1 (1.4%)
Pulmonic stenosis	0 (0%)	0 (0%)	0 (0%)
Pulmonic insufficiency	4 (5.8%)	0 (0%)	0 (0%)
Mitral stenosis	1 (1.4%)	0 (0%)	0 (0%)
Mitral regurgitation	16 (23%)	4 (5.8%)	1 (1.4%)
Aortic stenosis	1 (1.4%)	1 (1.4%)	1 (1.4%)
Aortic insufficiency	2 (2.9%)	1 (1.4%)	1 (1.4%)

Valvular heart disease in patients with Fabry disease as assessed by echocardiography (n=68).

Table 4.3.

Hospitalization for heart failure	13 (19%)
Mortality (over study period)	1 (1.4%)
Tricuspid valve intervention	0 (0%)
Pulmonic valve intervention	0 (0%)
Mitral valve intervention	1 (1.4%)
Aortic valve intervention	2 (2.9%)

Outcomes for the Fabry cohort (n=68). There were two aortic valve replacements and one mitral valve repair.

Table 4.4.

Age	Adult Population (n=11,911)	Fabry Disease (n=68)
<45	0.7% (31/4351)	2.9% (1/35)
45-54	0.4% (3/696)	19% (3/16)
55-64	1.9% (23/1240)	10% (1/10)
65-74	8.5% (328/3879)	17% (1/6)
≥75	13.2% (230/1745)	100% (1/1)

Percentage of patients with moderate or greater valvular heart disease stratified by age. General population data taken from Nkomo et al.[18]

Table 4.5.

	Kampmann et al.[2]	Weidemann et al.[4]	Present Study
Sample size	n=55	n=111	n=68
Age	40 ±17	39 ±14	44 ±17
Female, %	55 (100%)	60 (54%)	33 (48%)
Valve Disease			
Mild at most	55 (100%)	108 (97%)	63 (90%)
Moderate	0 (0%)	3 (2.7%)*	4 (5.8%)
Severe	0 (0%)	0 (0%)	3 (4.3%)

Comparison of valvular heart disease studies in Fabry disease. *Not specified if valvular lesions occurred concurrently in same patient.

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CHAPTER 5

DISCUSSION

5. Discussion

Fabry disease is an X-linked recessive multisystem disorder and an underrecognized secondary cause of hypertrophic cardiomyopathy. Cardiovascular manifestations of FD include left-ventricular hypertrophy, conduction disease, HFpEF, and valvular heart disease. In this thesis, we explored the contribution of systemic inflammation to heart disease and the prevalence of valvular heart disease in patients with Fabry disease. Chapters 3 and 4 contain detailed discussions, limitations, future directions, and conclusions. This chapter will present an overarching summary of discussion, proposed future directions, and a general conclusion.

5.1 Summary of Results and Conclusions: Chapter 3

Our comparison of patients with FD and healthy controls demonstrated that FD patients have higher plasma levels of inflammatory biomarkers, cardiac remodelling biomarkers, and lyso-Gb3. Patients with more severe clinical disease and renal disease had greater markers of inflammatory and remodelling biomarkers. Patients with LVH had greater levels of inflammatory biomarkers, remodelling biomarkers, and lyso-Gb3. Our data demonstrates that systemic inflammation is implicated in FD and is associated with end-organ dysfunction. These features describe a severe phenotype dominated by HFpEF and renal disease and suggest a key pathogenic role of systemic inflammation.

5.2 Summary of Results and Conclusions: Chapter 4

Our analysis of valvular heart disease in a FD cohort demonstrated that FD patients have a higher prevalence of valvular disease relative to the general population. Specifically, mitral and tricuspid regurgitation were common. Our observed prevalence of significant valve disease was

greater than prior studies but may be explained by demographic differences. Although the overall requirement for valve intervention was low, increasing survival with modern medical management of FD may result in more patients progressing to severe valve disease.

5.3 Limitations

Specific study limitations for Chapters 3 and 4 are discussed therein. Generally, FD is a relatively rare disease and the sample size of our cohort limits detailed analysis. However, our sample sizes are comparable to other previously published literature in FD. Although our analyses were predetermined, the likelihood of type I error increases with number of analyses performed.

5.4 Summary and Future Directions

Cardiovascular disease is a major cause of increased morbidity and mortality in patients with FD. Our work highlights the role of systemic inflammation in FD patients and its association with cardiac structural changes, as well as the high prevalence of valvular disease. The downstream aim of this research is to conceptualize targeted therapies to reduce the burden of cardiovascular disease in FD. As such, there are several future directions that should be explored to translate these findings into clinically significant changes to the management of patients with FD.

Early diagnosis of FD is paramount to prevent disease progression. However, FD is often initially misdiagnosed, leading to a mean duration from onset of symptoms to diagnosis of FD of 16.3 and 13.7 years in females and males, respectively.[1] Our biomarker work, in conjunction

with prior work, may allow for early identification and treatment of FD, especially in females or males with variant phenotypes who may not be diagnosed with α-Gal A activity assays alone.[2-4] Biomarkers may be a useful screening test for further genetic testing, especially in patients with unexplained cardiac hypertrophy.[3, 5]

A major clinical dilemma in FD patients is early identification of patients who may benefit from ERT. Current guidelines for qualification for ERT emphasize the requirement of structural abnormalities.[6] The progression of disease, including the development of LVH, is slowed by ERT.[7, 8] However, in many patients with advanced FD, ERT does not prevent organ failure and death.[9] Our biomarker research may help stratify patients who are predisposed to cardiac structural abnormalities prior to the development of symptoms or abnormalities detected on cardiac imaging. Accordingly, future research elucidating the temporal relationship between elevation of inflammatory, cardiac remodeling, and disease activity biomarkers and clinical outcomes would be valuable. Although excluded by current guidelines, patients without structural abnormalities while being at high-risk for the development of structural abnormalities may derive the greatest benefit from ERT.

Imaging modalities may help refine cardiac risk stratification. Cardiac magnetic resonance imaging (CMR) can provide superior spatial resolution to other cardiac imaging modality.[10-12] In addition, it can allow for the evaluation of myocardial fibrosis. In FD, CMR provides for differentiation from other etiologies of a hypertrophic phenotype.[13] Fibrosis in female FD patients may precede structural abnormalities.[4] However, the presence or absence of fibrosis has not been formally evaluated as a prognostic marker in a large cohort in FD.

Based on our research, valve disease is likely to become a more prominent feature as FD patients are living longer in the modern era of FD management with ERT, SRT, and aggressive

risk factor reduction. As valve outcomes are rare and FD cohorts are small, collaboration with other centers to expand our dataset will better delineate the risk of clinically-significant valve disease in patients with FD. We specifically plan to involve colleagues from the United States to expand our echocardiography cohort. An improved understanding of Fabry patients at risk for valvular disease can allow for earlier qualification of ERT in these patients.

5.5 Conclusions

The research included in this thesis builds upon a body of work describing cardiovascular manifestations in patients with Fabry disease. We demonstrated the presence of systemic inflammation in FD patients and association with structural cardiac disease. We also demonstrated the high prevalence of valve disease in patients with Fabry disease. These findings may lay the groundwork for further targeted therapies to reduce the burden of cardiovascular disease in patients with Fabry disease.

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