Sex differences in autophagy in cardiometabolic syndrome induced heart failure

By

Charlotte Kowall

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Department of Physiology and Pathophysiology
University of Manitoba
Winnipeg

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Abstract

Introduction: Heart failure with preserved ejection fraction (HFpEF) is an extremely prevalent cardiovascular disease driven by a systemic inflammation pathway from co-morbidities such as cardiometabolic syndrome (CMS). CMS includes metabolic disorders such as abdominal obesity, hypertension, and dyslipidemia. Notable features of CMS-induced HFpEF include diastolic dysfunction, hypertrophy, and concentric left ventricular remodeling. Additionally, HFpEF is noted to be sex specific in prevalence and prognosis. Autophagy is a cellular quality control process that involves the recycling of damaged organelles and proteins. When reactive oxygen species, a harmful byproduct of cell functioning, build up, antioxidants and mitochondrial autophagy (mitophagy) must act to clear them. Previous research has connected autophagy to HFpEF; however, information is limited and even less is currently known about cellular mechanisms underlying sex differences in HFpEF. Herein, we sought to investigate sex specific differences in autophagy and oxidative stress mechanisms in the pathogenesis of CMS-induced HFpEF.

Methods: C57BL/6J male and female mice were fed a normal diet, high fat diet (HFD), or HFD + L-NAME for 5 or 8 weeks. Animals were assessed for cardiac function using non-invasive echocardiography prior to sacrifice. Hearts were processed for Western blot analysis and assessed for autophagy markers and antioxidant manganese superoxide dismutase (SOD2), mRNA was used to measure transcription of the mitophagy marker BNIP3 by quantitative real-time PCR (qPCR).

Results and Conclusions: Our findings show by echocardiography that HFpEF, but not heart failure with reduced ejection fraction (HFrEF) was induced in female and male mice fed HFD +

L-NAME and that male mice have worse cardiac parameters than corresponding female mice. In tandem with this finding, female mice fed HFD + L-NAME showed increased autophagy compared to males and lacked the reduction in SOD2 observed in males. Our findings reveal a novel connection between male and female mice in autophagy regulation and cardiac dysfunction in cardiometabolic syndrome induced HFpEF.

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List of Abbreviations

AKT	Protein kinase B
AMPK	Adenosine monophosphate-activated protein kinase
ATG	Autophagy related gene
ATP	Adenosine Triphosphate
BAG3	Bcl-2-associated athanogene 3
BNIP3	BH3-only domain protein
BRD4	Bromodomain containing 4
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
CAD	Coronary artery disease
CMS	Cardiometabolic Syndrome
EF	Ejection fraction
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ETC	Electron transport chain
FS	Fractional shortening

GFF-15	Growth differentiation factor-15
GSHPx	Glutathione peroxidase
HF	Heart Failure
HFD	High Fat Diet
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reduced ejection fraction
HRP	Anti-horseradish peroxidase
iNOS	Inducible nitric oxide synthase
IL-6	Interleukin 6
I/R	Ischemia/reperfusion
LC3	Microtubule-associated protein 1 light chain 3
L-NAME	N [W] - nitro-l-arginine methyl ester
LVIDd	Left ventricular internal dimension in end-diastole
LVIDs	Left ventricular internal dimension in systole
MAPK	Mitogen activated protein kinase
MDVs	Mitochondria-derived vesicles
mRNA	Messenger RNA
mTORC1	Target of rapamycin complex 1

NADH	Nicotinamide adenine dinucleotide
NO	Nitric oxide
NOS	Nitric oxide synthase
iNOS	Inducible nitric oxide synthase
PINK1	PTEN-induced putative kinase 1
PKA	Protein kinase A
PKG	Protein kinase G
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SASP	Senescence associated secreted factors
SAM	Senescence-accelerated mouse model
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
SOD2	Manganese superoxide dismutase
SQSTM1	Sequestosome-1
ST2	Suppression of tumorigenicity 2
TAC	Transverse aortic constriction
TFEB	Transcription factor EB

TNFα Tumor necrosis factor-alpha

TNFR1 Tumor necrosis factor receptor 1

UPR Unfolded Protein Response

ZKSCAN3 Zinc-finger protein with KRAB and SCAN domains 3

Introduction

Heart failure in its most basic sense is the inability of the heart to pump commensurate with the body's metabolic needs. Heart failure presents with symptoms stemming from anomalies in cardiac structure and function and often is associated with systemic or pulmonary congestion with elevation of natriuretic peptide levels (1). Approximately half of patients experiencing heart failure have a form of cardiac dysfunction known as heart failure with preserved ejection fraction (HFpEF). In HFpEF, over 50% of blood entering the heart is ejected (2). Conditions such as cardiometabolic syndrome (CMS), aging, and incidence of myocardial infarction can precede HFpEF (3) with these co-morbidities resulting in systemic inflammation followed by myocardial remodeling and disfunction (4). Due to multiple pathophysiological mechanisms leading to development of HFpEF, it is noted to be a clinical syndrome rather than a discrete disease (5).

Cardiometabolic syndrome (CMS) is characterized by a group of metabolic disorders that are also cardiovascular disease risk factors (6). Etiologies of CMS include abdominal obesity where there are large waist circumferences and/or body mass index, insulin-resistant glucose metabolism, hypertension, and dyslipidemia (low high-density lipoprotein cholesterol concentrations or high serum triglyceride) (7). Individuals with CMS have twice the risk of dying from coronary artery disease and three times more likely to experience a major cardiovascular event such as a heart attack (6). Additionally, CMS is linked to structural and functional alterations in the heart which further increases the likelihood of developing heart failure through CMS-induced low-grade systemic inflammation (8). Previous studies have shown that cardiometabolic syndrome has a stronger association with risk of HFpEF development compared to heart failure with reduced ejection fraction (HFrEF) (8).

The mechanism in which HFpEF results from inflammation from co-morbidities begins with coronary microvascular endothelial inflammation induced by systemic inflammation (4). Systemic inflammation is seen with increased interleukin 6 (IL-6) circulation and tumor necrosis factor alpha (4). Coronary microvascular endothelial inflammation decreases cyclic guanosine monophosphate (cGMP) content, nitric oxide (NO) bioavailability, and activity of protein kinase G (PKG) in neighboring cardiomyocytes (4). Decreased PKG activity leads to heart hypertrophy, causing concentric left ventricular remodeling and cardiomyocyte stiffening (4). Interstitial fibrosis and stiff cardiac myocytes lead to the progression of HFpEF though diastolic left ventricular stiffness (4).

In addition to inflammation, another important player in heart failure, as well as normal heart functioning, is autophagy. Autophagy is a highly regulated cellular quality control process where organelles and proteins are degraded by a lysosomal regulated process (9). A defining feature of autophagy is the development of double-membraned vacuoles called autophagosomes which arise from a phagophore derived by golgi and endoplasmic reticulum membranes that engulf cytoplasmic cargo (10). Once engulfment occurs, autophagosomes undergo docking and fusion with lysosomes (10). This results in the formation of autolysosomes to degrade the contents initially engulfed (10). Autophagy is enacted continuously at low levels in the heart to maintain proper cell functioning; however, in the presence of cardiovascular stressors autophagy can be upregulated as a survival mechanism where cardiomyocytes try to remove damaged organelles and protein aggregates (9,11). Reactive oxygen species (ROS) produced by damaged mitochondria and AMP-activated protein kinase (AMPK) can contribute to autophagy in heart

failure, as well as the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) and mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK1/2) signaling pathways. In these pathways, maladaptive remodeling, fibrosis, and cardiac myocyte death is brought on by chronic high levels of cAMP and MAPK signaling can promote hypertrophy and maladaptive remodeling (12,13). Antioxidants are essential for ROS clearance, with an imbalance leading to excess ROS and detrimental impacts (14). The decrease in number of cardiomyocytes noted in heart failure patients can be connected to autophagy, with excess or insufficient autophagy playing a role in the pathology of heart failure (12). CMS comorbidities, including hypertension, obesity, and diabetes, can lead to autophagy impairment through chronic inflammation, contributing to HFpEF development (15). In addition, excessive autophagy can lead to protein breakdown and atrophy in the context of HFpEF (15), demonstrating a link between CMS, HFpEF, and autophagy.

Notably, cardiometabolic heart failure appears to be sex-specific with a higher prevalence of CMS induced HFpEF in women (8). Additionally, many studies point to increased mortality and worsened prognosis in men (8). Despite these findings, further investigation is needed into the cellular mechanisms underlying sex differences in CMS induced HFpEF development and severity. Studies have recognized autophagy as a response mechanism and key player in heart failure (8), leading us to explore if sex differences in autophagy or antioxidant levels underly sex differences observed in the development and severity of HFpEF.

Literature Review

1. HFpEF classification

Heart failure (HF) can be classified using left ventricular ejection fraction (LVEF), with an LVEF of ≤ 40% being described as HF with reduced ejection fraction (HFrEF), and an LVEF of ≥ 50% being described as HF with preserved ejection fraction (16). HFpEF occurs when ventricular filling, or diastole, is impaired (16). Features of HFpEF include ventricular remodeling, diastolic disfunction, changes in ventricular filling and relaxation, and increased stiffness, which together lead to pressure overload (1). This differs from features of HFrEF which include myocardial remodeling and ventricular dilation, impaired ventricular contraction, and systolic disfunction, which contribute to volume overload in the left ventricle (1). Previous research has suggested that there are differences in the pathophysiology of HFpEF and HFrEF, with neurohormonal activation playing a major role in HFrEF, while microvascular dysfunction is a driving force for HFpEF (17). Additionally, remodeling of the left ventricle differs between the two forms of heart failure (18). Recent studies suggest that systemic inflammation is also a major aspect of HFpEF, with lack of proper myocardial functioning and myocardial remodeling underlying its effect (4). Due to HFpEF etiologies being heterogeneous in therapeutic response, pathophysiologic pathways, and clinical presentation, there is no universal definition of distinct HFpEF phenotypes. Despite this, there are comorbidities that are frequently present in concordance with HFpEF including cardiometabolic syndrome, atrial fibrillation, arterial hypertension, ischemic heart disease, chronotropic incompetence, and right heart dysfunction (19). Previous literature has characterized cardiometabolic HFpEF as having a higher symptomatic burden and lower quality of life compared to other subtypes (20,21).

2. The Pathophysiology of Cardiometabolic Syndrome

The intricate link between cardiometabolic syndrome and HFpEF is defined by the changes that CMS creates in the body which subsequently lead to negative cardiac outcomes. Literature has identified insulin resistance, inflammation, and metabolic disturbance as being major factors that underly the formation of cardiovascular disease.

2.1 Insulin Resistance in CMS

Multi-organ insulin resistance is a characterizing feature of CMS and is thought to be brought on by changes in fatty acid metabolism such as increased fatty acid release into plasma (7). Additional features of insulin resistance include increased circulating catecholamines, growth hormones, and inflammatory cytokines (20). In skeletal muscle and liver, excess fatty acids can interfere with insulin activity, preventing glucose uptake and increasing hepatic glucose production as well as very low-density lipoprotein production (7,22,23). Additionally, fatty acids can lead to increased blood pressure through increased sodium reabsorption and induction of vasoconstriction (7,24).

Both systemic and cardiovascular insulin resistance contribute negatively to heart failure progression (25). Worsening cardiac parameters and cardiac insulin resistance can both be induced by glucotoxicity, lipotoxicity, oxidative stress, and cytokine imbalance (25). In mouse

models of CMS-induced HFpEF, cardiomyocyte lipid accumulation is noted, likely due to insulin resistance (20).

2.2 Inflammation in CMS

Chronic low-grade inflammation is a notable feature of cardiometabolic syndrome, with previous studies indicating that inflammation is the link between HFpEF characteristics and CMS comorbidities (20,26). Inflammatory biomarkers associated with HFpEF incidence and severity include tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), high sensitivity C-reactive protein (hs-CRP), growth differentiation factor-15 (GDF-15), suppression of tumorigenicity 2 (ST2), C-C motif chemokine 20, and TNF-receptor 1 (TNFR1) (26). This inflammation can lead to increased cardiomyocyte hypertrophy and stiffness, oxidative stress, metabolic and mitochondrial dysfunction, and remodeling in the extracellular matrix (27). Inflammation in adipose tissue during obesity can contribute to increased circulating cytokines and myocardial remodeling (27).

2.3 Metabolic Disturbances in CMS

An excess of circulating free fatty acids leads to an overabundance of fuel for cardiomyocytes, prompting change of myocardial energetics and later myocardial dysfunction (28). In CMS-induced HFpEF, inefficient adenosine triphosphate (ATP) production results from a poor ability to adapt substrate use when nutritional status and availability of energy substrates changes (28,29). Contractile reserve and cardiac energetics are impaired by metabolic disturbances, with diastolic dysfunction and ventricular stiffening resulting (28). Oxidative stress and impaired

mitochondrial turnover further activate inflammatory pathways as well as alter immune cell dynamics (28).

3. Sex Differences in Cardiometabolic HFpEF

Multiple studies have found a predominance of HFpEF in women compared to men (30). Currently, there is no consensus to explain the increased prevalence of CMS-induced HFpEF in women; however, multiple hypotheses have been proposed including differences in risk factors and pathophysiological consequences (30).

3.1 Sex Differences in Traditional HFpEF Risks

Notable differences arise when comparing baseline risk factor prevalence in men and women (30). In reports from HFpEF trials, women were found to have a higher prevalence of obesity and history of renal impairment and hypertension when compared to men (31).Visceral adipose tissue, which has been associated with concentric remodeling in the left ventricle, is found to be lower in women compared to men in pre-menopause, but higher during the peri-menopausal transition (32,33). Diabetes mellitus has been found to have an increased risk for heart failure in women compared to men (30,34). Hypertension presents differently in each sex, as in those experiencing HFpEF, hypertension is more prevalent in women compared to men and leads to a greater risk of heart failure in women (30,35). Additionally, in women, hypertension more often leads to concentric hypertrophy, a feature of HFpEF, while in men hypertension more often leads to eccentric remodeling (30,36). Atrial fibrillation shows a higher prevalence in men compared to women with HFpEF; however, women experience worse outcomes with atrial fibrillation and

HFpEF (30). Anatomy also differs between the sexes with females having smaller heart sizes and blood vessels, including a smaller left ventricle (37). This leads to a decreased stroke volume as well as a decreased end-diastolic and end-systolic volume (37).

3.2 Female-specific risk factors

It is hypothesized that HFpEF pathogenesis may involve decreasing estradiol during menopause, as estradiol can regulate nitric oxide signaling, endothelial function, and inflammation (38). During menopause, the risk of renal dysfunction, obesity, hypertension, and diabetes increases due to estrogen decline (38). Hypertensive disorders of pregnancy, such as preeclampsia, as well as pre-term birth and intrauterine growth restriction increase risk of cardiovascular disease, which may include HFpEF (30,39). Studies have shown that both women with infertility and women with more than four births are at higher risk for diastolic dysfunction and incident HFpEF (30,40,41).

3.3 Sex Differences in Mortality

Despite hospital admissions being similar, several studies and meta-analyses report worse survival in men with HFpEF compared to women (30,42,43). Deng *et al.* (2024) report 14 studies with 41,508 HFpEF patients that collectively demonstrate a significantly higher risk of death of mortality for men compared to women. In contrast, some studies report similar mortality between sexes (44) or worse outcomes in females, (45) demonstrating that sex differences in prognosis and mortality are controversial and require further investigation.

4. Experimental Models of HFpEF

Models representing different phenotypic versions of HFpEF allow for the investigation of the pathophysiological processes and potential therapeutic avenues directed towards HFpEF subgroups (46). This section explores common models for HFpEF.

4.1 Obesity and HFpEF

Multiple studies have identified high body-mass index (BMI) as a risk factor for heart failure, with obesity being an independent risk factor for HFpEF (47,48). Previous studies suggest that high BMI has a greater correlation with incidence of HFpEF compared to HFrEF, with obese Class I participants having a 56% higher risk of HFpEF independent of other risk factors compared to those with normal BMI (49,50). Obesity can increase several pathological mechanisms leading to heart disease including oxidative stress, autophagy disruption, lipotoxicity, apoptosis, and inflammation (51). High fat diets often lead to low grade inflammation, with elevated inflammatory cytokines due to adipocyte hypertrophy (52,53). Increased macrophage and dendritic cell infiltration in the liver and adipose tissue occur in both diet-induced obesity in rodents and human obesity and is thought to play a major role in obesity-induced inflammation (52,53).

Diet-induced obesity models in rodents act to mimic obesity in humans, with high-fat diets being the primary method (47,54). This model acts to replicate the increasing levels of obesity in

today's society due to increased availability and consumption of high fat foods (55). These models typically involve 45-60% of caloric consumption being lipid based, with most studies using C57BL/6J mice, which are highly susceptible to the accumulation of fat (53). Markers often used to indicate the presence of an obese state include body weight and fat gain (53). Usually, 4 weeks of a high-fat diet is sufficient to see changes in body weight and glucose levels (55).

4.2 Aging and HFpEF

HFpEF is a major health concern in elderly patients as it is a principal cause of mortality and hospitalization in subjects over 65 years of age (56). Additionally, up to 59% of all heart failure diagnoses occur in patients over 85 years old (57). Aging hearts are more vulnerable to HFpEF with age being an independent risk factor for HFpEF and having a stronger effect than in HFrEF (58). Cell senescence is age-induced cell-cycle arrest brought on by inflammation, DNA damage, and mitogenic signals (59). The accumulation of senescent cells is indicative of aging as cells are damaged faster than the aged body can repair them (60,61). Systemic and cardiac modifications contributing to HFpEF development include inflammation, stiffening, fibrosis, and altered diastolic function and involve molecular mechanisms including mitochondrial disfunction, telomere shortening, and senescence associated secreted factors (SASP) (57). SASP occurs when senescent cells emit pro-inflammatory molecules that induce inflammation and cause tissue remodeling (62). Inflammatory markers seen in HFpEF such as TNF-α and IL-6 are significantly increased in older individuals independently of different cardiovascular risk factors (63).

A murine model often used to examine aging and senescence is the senescence-accelerated mouse model (SAM) (59). This model is made up of senescence-prone (SAMP) and control senescence-resistant (SAMR) strains, where SAMP mice undergo rapidly progressing senescence and experience a 40% shorter lifespan (64). The model was created by selectively inbreeding AKR/J mice and displays many common geriatric disorders (59,64). When exploring cardiovascular disease and oxidative stress, SAMR1 and SAMP8 strains are best-studied and, therefore, usually the strains of choice (59). One study noted, while using SAMR1 and SAMP8 mice, that diastolic dysfunction such as that in HFpEF developed in only SAMP8 mice at 3-6 months of age (59).

4.3 Pressure Overload and HFpEF

Pressure overload can occur in either the left or right ventricle with aortic coarctation, hypertension, or aortic stenosis driving overload in the left ventricle and hypertension or pulmonary stenosis causing pressure overload in the right ventricle (65). In an initial attempt to preserve cardiac output, cardiac hypertrophy occurs; however, this does not hold up against sustained overload of pressure (65). Decomposition involves diastolic disfunction, ventricular stiffening, deposition of fibrotic proteins, and intrusion of inflammatory cells, all of which contribute to poor hemodynamics and reduced myocardial compliance (65,66). The overall result of sustained pressure overload is the development of HFpEF (65).

A model frequently used for examining hypertrophy and heart failure induced by pressure overload is the transverse aortic constriction (TAC) model (67). TAC involves blocking proper

flow out of the left ventricle by constricting the transverse aorta. This results in left ventricular overload which contributes to cardiac remodeling and eventual heart failure. Research suggests that TAC driven remodeling primarily occurs through diastolic dysfunction and interstitial fibrosis, demonstrating TAC induced HF as more like HFpEF than HFrEF (68). A blunt needle is used to guide the suture, which then provides permanent constriction (67). A recent study shows that different levels of constriction tightness, prompted by different needle gauges, may lead to differing cardiac responses; however, further research is needed to confirm this phenomenon (67). L-NAME can be used in tandem with a metabolic stressor to produce HFpEF as it acts to inhibit the vasodilator nitric oxide synthase; however, L-NAME on its own lacks the metabolic, inflammatory, and full systemic embodiment seen in clinical HFpEF, leading it to be an incomplete model (69).

4.4 Myocardial Infarctions/Ischemia Reperfusion and HFpEF

Myocardial infarction (MI) results from partial or complete blockage of blood flow to a portion of the myocardium, also known as myocardial ischemia (70,71). Necrosis is a key form of cell death involved in the identification and diagnosis of MI, as necrosis in the myocardium is typically a defining feature of MI (72). Underlying an MI event is coronary artery disease (CAD) (73). Heart failure occurs following MI primarily due to adverse ventricular remodeling (74). Inflammation is known to manifest following an MI not only from the event itself, but also during reperfusion (73). Injury resulting from reperfusion is important to consider in the context of lasting effects from an acute MI, as it often results in the death of cardiomyocytes, contributing to heart failure progression (75).

Although acute MI is typically viewed through a HFrEF lens, CAD and history of MI are both established risk factors for HFpEF (73). In particular, silent MI, where an MI event is initially undetected, is thought to be important when considering ischemic risk factors for HFpEF (73). The profound inflammatory response produced by MI is especially of note when exploring models of HFpEF (4).

Since ischemia/reperfusion (I/R) is often the root of pathology following acute MI, the most common model for MI stimulates I/R in mice (75). This model involves ligation of the left anterior descending artery for a set period to simulate an ischemic event. The removal of the ligature then permits reperfusion (75). It is essential to ensure that the position of the ligature is consistent across an experiment as changes in ligature positioning can result in differences in the size of the infarct induced (75). Despite mice being often used in experimental settings, this model can also be used in dogs, pigs, and rats (76).

4.5 The "two hit" (HFD + L-NAME) HFpEF Model

Models with a "single-hit", such as those mentioned above in which one stressor is used to emulate HFpEF, are commonly criticized as being unsuccessful in embodying the complex pathophysiology of HFpEF, a multifactorial disease (77). A novel "two-hit" model, developed by Schiattarella et *al.*, combines a high fat diet (HFD) and inhibition of nitric oxide synthase (NOS) through $N^{[W]}$- nitro-l-arginine methyl ester (L-NAME) to enact both metabolic and mechanical stress, recapitulating a main mechanism of HFpEF pathophysiology (78). In this model,

C57BL/6N mice were given a HFD consisting of 60% of calories from lard and drinking water with 0.5 grams L-NAME per liter for 15 weeks (78). L-NAME works by inhibiting nitric oxide synthase, an important component of the body's vasodilation mechanism, leading to systemic vasoconstriction that results in chronic hypertension (78). Features of L-NAME and HFD-treated mice include cardiac hypertrophy and fibrosis, impaired diastolic function, and pulmonary congestion, demonstrating the efficacy of this two-hit model (78). Although HFD alone is recognized as a single-hit HFpEF model, L-NAME is not commonly used as its own model as it does not sufficiently perpetuate major HFpEF characteristics (78).

One feature of HFpEF in humans is the accumulation of misfolded proteins, which is then followed by an unfolded protein response (UPR) that, like autophagy and the ubiquitin-proteasome system, help to alleviate stress on the myocardium (78,79). A regulator of cardiomyocyte stress responses is the IRE1α-Xbp1 signaling pathway, which has been shown to be inactive in both human HFpEF and in experimental HFpEF using the two-hit model (78,80). This pathway acts to sense the accumulation of proteins that are incorrectly folded in the endoplasmic reticulum (ER) and upregulate protein folding genes, ER-associated degradation, and lipid/secretory capacity (81). Previous studies demonstrated systemic inflammation in HFD + L-NAME mice as well as increased inducible NOS (iNOS), a feature of human HFpEF hearts (78). Since iNOS can induce S-nitrosylation of cysteine residues, harming protein function, further investigation connecting iNOS and the IRE1α-Xbp1s axis revealed that Xbp1s generation is hindered through an iNOS-mediated increase in S-nitrosylation of IRE1α (78,82). Through experiments comparing HFD or L-NAME only to HFD + L-NAME, it was found that the HFD + L-NAME group best recapitulated key features of clinical HFpEF, including lung congestion,

exercise intolerance, and increased natriuretic peptides, by causing NO dysregulation and impacting the IRE1α-Xbp1 pathway (78).

Previous studies using the two-hit HFD + L-NAME model demonstrate that male mice exhibit worsened clinical outcomes compared to females (83). Through experimentation, it was concluded that sex hormones did not play a role in these differences, prompting a further investigation into sex differences at a tissue level (83). Akin to other cardiovascular conditions, it is suggested that in using this model, females exhibit a protective effect, leading to an attenuated cardiac phenotype (83).

4.6 Other multi-factorial models

As pre-clinical research regarding HFpEF progresses, new multi-factorial models including two-hit and three-hit models emerge.

Most two-hit models, like the HFD + L-NAME model, combine metabolic and mechanical stress (69). Two examples are ZSF1 obese rats and DSS obese rats (69). Breeding female Zucker diabetic fatty rats with spontaneous hypertensive heart failure male rats develops ZSF1 obese rats (84). These rats have a leptin receptor with two mutations and emulate arterial remodeling, exercise intolerance, and other features of HFpEF (69,84). DSS obese rats result from breeding DSS and Zucker rats and initially emulate structural changes seen in clinical HFpEF however, these rats are protected against obesity-induced vascular dysfunction, prompting criticism that

this model may not sufficiently emulate characteristics in human HFpEF (69,85,86). An additional two-hit model uses obese ZSF-1 rats and administers SU5416, a vascular endothelial growth factor receptor blocker, to enhance pulmonary hypertension; however, this model is only shown in male rats (69,87).

Recently, a three-hit model has been developed that adds cardiac myocyte-specific calcium stress to the metabolic and mechanical stressors seen in two-hit models (88). This model uses HFD + L-NAME as well as an increased influx of calcium via cardiomyocyte-specific expression of the β2a-subunit in the L-type calcium channel (88). The three-hit model has been reported to have a more profound HFpEF phenotype when compared to the two-hit HFD + LNAME model due to the additional stressor (88). Major mechanisms of note in this model are necrosis, myocardial fibrosis, fibroblast activation, cardiomyocyte hypertrophy, fibroblast activation, and increased profibrotic M_2-macrophage populations (88). Current limitations of this model include a lack of information on sex-specific effects and a lack of testing to determine occurrence of exercise intolerance, a hallmark feature of HFpEF (88).

5. Autophagy in Cardiac Physiology and Disease

5.1 Autophagy as a Cellular Process

Autophagy is an essential mechanism for the degradation and recycling of cellular waste products and damaged organelles. It is noted to be involved in many bodily processes including differentiation, development, innate and adaptive immunity, cell death, and aging (89). The process of autophagy begins with stimuli that activate phosphatidylinositide-3-kinase (PI3K),

which forms a complex with autophagy-related gene (ATG)14L, as well as Beclin 1 (90). ATG14L is an essential component of this interaction as it bridges together this complex and allows for functioning autophagy by directing the complex to the preautophagosomal structure (PAS). In both mammals and yeast, ATG14L has been found to enhance autophagic activity when overexpressed, demonstrating its role in autophagy regulation (91). Additonally, ATG14L has been identified as a limiting factor in the process of autophagy (91). After complex formation and direction, a phagophore is formed that, with the help of other ATG products, engulfs cytoplasmic cargo with sequestosome-1 (SQSTM1/p62) and fuses at the ends to create an autophagosome (90,92). Phagophore synthesis occurs at locations within mammalian cells called omegasomes, which are known to be near the cell's endoplasmic reticulum (93). An autolysosome is formed when an autophagosome then fuses with a lysosome, which then digests cargo into elemental building blocks through resident hydrolases (90). These building blocks are then used for energy or new macromolecule biosynthesis (93).

ATGs underly the main molecular mechanisms of autophagy (94). ATG protein is essential for autophagy regulation, vesicle formation, and lysosomal transmission (94). ATG1 complexes aide in autophagy initiation along with the inhibition of mammalian target of rapamycin complex 1 (mTORC1) activation, which reduces ATG13 to form an ATG-14-ATG1-ATG17 complex to begin autophagy (94). The PI3K-ATG6 (Beclin-1) complex acts to gather the ATG-12-ATG 5 complex and ATG16 polymer, along with LC3 molecules which induce phagocyte expansion (94). Additionally, the ATG12-ATG5-ATG16 ubiquinated connective system leads autophagy during the process of autophagosomal membrane expansion (94). ATG7 and ATG3 help turn microtubule-associated protein 1 light chain 3 (LC3)-I into its active form, LC3-II, where it can

bind to the double membrane of autophagosome vesicles (94). When cells and tissues are deficient in ATG7, they are noted to be “autophagy deficient” due to the requirement of ATG7 for ATG12-ATG5-ATG16L1 complex formation and LC3 lipidation, both of which allow for phagophore expansion and cargo engulfment (95). Even other ATG proteins being overexpressed cannot compensate for a loss of ATG7 (95). Over expression of ATG7 is noted to increase basal autophagy in cardiomyocytes which acts to attenuate misfolded proteins that have accumulated, demonstrating its importance in the context of cardiovascular disease (96).

The main types of autophagy consist of macroautophagy, microautophagy, and chaperone-mediated autophagy. Despite differences, all forms of autophagy possess a common goal of delivering cargo to a lysosome for degradation and recycling (97). Chaperone-mediated autophagy is a solely selective type of autophagy and consists of chaperones first recognizing a specific amino acid sequence on protein substrates and then initiating unfolding and lysosomal translocation (92). Both a cytosolic cargo-recognition complex and a lysosomal cargo-translation complex are necessary for chaperone-mediated autophagy in mammalian cells (98). Microautophagy consists of substrate sequestration through the lysosome membrane, followed by substrate uptake (92). Protrusions from the membrane are used to take in these substrates (97). Macroautophagy is the most well studied autophagy process and the type that is referred to when the term “autophagy” is used (92). Contrary to other types of autophagy, macroautophagy consists of substrate sequestration away from the lysosome and involves *de novo* synthesis of autophagosomes (97,99). Research has shown that macroautophagy can be highly selective, including being organelle-selective and targeting lipid droplets (lipophagy), endoplasmic reticulum (ERphagy or reticulophagy), peroxisomes (pexophagy), or mitochondria (mitophagy)

(98,100). This is proposed to be done through LC3-II at the phagophore acting as a receptor and interacting with the target's adaptor molecules, such as p62, to encourage substrate uptake and degradation (101). P62 is essential to the process of autophagy as it not only acts as mechanism for recruiting ubiquinated cargo, but it also acts as a signaling molecule that participates in many signaling pathways such as the Keap1–Nrf2 pathway (102). Manipulation of the cell's p62 level is noted to change ubiquitination patterns and impact cell survival (103). Autophagy activation correlates with decreased p62 levels due to p62 clearance occurring along with the cargo it shuttles to the proteasome (103). Overexpressed p62 levels can impede autophagy from being successful due to delays in cargo delivery (103).

5.2 Autophagy in Normal Cardiac Physiology

Since the heart has a very limited regenerative ability, autophagy can be an essential tool for myocyte function and survival (90). Autophagy allows for substrate recycling, energy replenishing, and maintained viability through degrading and recycling dysfunctional, damaged, or aged organelles and proteins (90). A study exploring the results of gene deletion of autophagy-related gene 5 (Atg5) in mice demonstrated that removing this gene resulted in disrupted cardiac functioning, including contractile dysfunction, cardiac hypertrophy, and left ventricular dilatation (104). Additionally, results showed disorganized sarcomere structure and increased levels of ubiquination, demonstrating the importance of functional autophagy in cardiac homeostasis (104).

Currently, there are three recognized master transcriptional regulators of autophagy in the heart including transcription factor EB (TFEB), zinc-finger protein with KRAB and SCAN domains 3 (ZKSCAN3), and bromodomain containing 4 (BRD4) (98). These regulators act on cardiomyocyte autophagy, and their actions depend on the stressor at hand to induce or inhibit activation of autophagy genes (98).

5.2 Autophagy in Cardiovascular Disease

Changes in autophagic activity has been noted in cardiomyocytes under stressors such as pressure overload, starvation, chronic ischemia, and ischemia/reperfusion injury (90). Whether these changes are beneficial or detrimental is heavily debated; however, exploring components of the autophagy process such as the level of autophagic activity, the substrates being degraded, and the events triggering autophagy can help provide insight into whether autophagy is aiding or hindering cell survival (90). One way that autophagy can promote survival is through the clearance of damaged proteins and organelles to replenish the cell's nutrient supply and lower cellular stress (105).

In atherosclerosis, moderate autophagy can prevent cell death and stabilize an inflammatory reaction; however, abnormal autophagy can promote inflammation and lead to further disease development (94,106). In ischemia reperfusion, autophagy can be protective but can also be overly activated, leading to myocardial cell death (94,107). After myocardial infarction, cardiac dysfunction can be lessened through activation of transcription factor EB (TFEB), a transcription

factor that helps to coordinate autophagy, lysosome biogenesis, and cell stress adaptation responses (108).

Stress and nutrient-sensing pathways play a major role in autophagy in cardiovascular disease and most notable of these pathways is the PI3K/protein kinase B (AKT)/mammalian target of rapacycin (mTOR) pathway (94). mTOR is a key inhibitory signal for autophagy that phosphorylates ATG13, preventing autophagosome binding (109). mTOR is regulated by the PI3K/AKT cell survival signaling pathway (109). Literature has demonstrated that this pathway is essential in oxidation, apoptosis, proliferation, and cell growth and it has been shown to be highly activated in cardiovascular diseases (94). Additionally, multiple studies have connected the PI3K/ATK/mTOR signaling cascade to cardiac hypertrophy (110).

5.3 Autophagy in Heart Failure

Autophagy is a major physiological process in heart failure and is interwoven with cellular death mechanisms such as apoptosis and necrosis (111). Like many conditions, autophagy's role in heart failure and whether it helps or hinders progression depends on cellular context and the stage of the disease (14). A notable feature of early heart failure is cardiomyocyte hypertrophy, where damaged organelles and misfolded proteins accumulate and then are cleared through autophagy as a protective mechanism (111). Autophagy can also clear inflammatory factors and ROS, to help maintain heart function, demonstrating that insufficient autophagy can lead to waste build up and worsening heart function (111). The mechanism by which autophagy removes ROS is through degradation of oxidatively damaged macromolecules, such as proteins,

and ROS-producing organelles (14). Additionally, previous experimentation suggests that extracellular matrix buildup can be mitigated by autophagy (14).

Impairment of mitophagy works in tandem with autophagy downregulation to progress pressure overload induced heart failure (108). Overactivation of autophagy can also be harmful in heart failure as non-waste proteins and organelles can be cleared in addition to waste, leading to impaired cell functioning and cardiomyocyte cell death (111). In the acute decompensated phase of heart failure, normal cell contents can be damaged by high levels of autophagy (14).

Literature on autophagy in HFpEF is both limited and conflicting with some studies discussing excess autophagy as a harmful aspect of heart failure, while other studies show that insufficient autophagy is at play. Despite this, most literature indicates insufficient autophagy as a likely attributing factor to HFpEF. One study demonstrated that male HFD + L-NAME mice have lower autophagy levels compared to control mice (112) and a recent review paper attributed HFpEF progression to impaired autophagy (15). A contrasting study using male mice with HFpEF induced by D-aldosterone infusion found that autophagy is enhanced in HFpEF (113). Regardless of whether autophagy is thought to be detrimentally augmented or impaired, sources agree that autophagy plays a significant role in heart failure.

Currently, exact mechanisms of autophagy regulation in each cell type in a failing heart are not well understood, prompting the need for further research of cell-specific autophagic activity (108). Autophagic flux is difficult to reliably measure in live animals and humans; however,

measurement through electron microscopy or biochemical methods examining autophagosomes can help provide insight (108).

5.4 Sex Differences in Autophagy in Cardiovascular Disease

Previous literature has demonstrated that sex differences in autophagy exits; however, cell type, organism type, or tissue type can determine what changes occur (114). Some circumstances can lead to a more robust autophagic response in females, while others may lead to a greater response in males (114). A previous study showed that in mouse hearts, basal expression of ATG5 is higher in males than females (114). In the context of cardiovascular disease, autophagy can be modulated by androgens during myocardial infarction, leading to sex differences in the incidence of ischemic heart disease (115). Some research has been done to explore sex differences in autophagy in HFrEF, with Wood et al. (2024) showing increased skeletal muscle autophagy gene expression in men compared to women, potentially explaining greater muscle wasting in men. Research on sex differences in autophagy in HFpEF is currently extremely limited, demonstrating a knowledge gap that requires further investigation.

6. Autophagy, Mitochondria, and Oxidative Stress in Heart Failure

Oxidative stress links autophagy, mitochondria, and heart failure. Dysfunctional mitochondria notably contribute to heart failure development though their association with oxidative stress with build up from insufficient autophagy further worsening the heart's condition.

6.1 Oxidative Stress and Reactive Oxygen Species

Oxidative stress occurs when more reactive oxygen species (ROS) are produced than can be cleared by antioxidants (14). ROS are highly reactive oxygen-based chemical species such as hydrogen peroxide, hydroxyl radical, and superoxide (116). ROS are generated during normal physiological processes; however, pathophysiological processes such as damage to mitochondrial electron transport chain (ETC) complexes, may lead to overproduction (14). Neutrophils, cardiac myocytes, and endothelial cells all produce ROS in the heart, with mitochondria, NAD(P)H oxidase, uncoupled NOS, and xanthine oxidase generating ROS within cardiac myocytes (116). In heart failure, ROS production in mitochondria has been found to increase, demonstrating a link between oxidative stress and failing mitochondria (116). Antioxidants are instrumental in maintaining cell functioning when ROS accumulate (117). Antioxidants act by neutralizing unstable free radicals, such as ROS, to decrease oxidative cell and tissue damage (117). Examples of notable antioxidants include glutathione peroxidase (GSHPx), catalase, and superoxide dismutase (SOD); however, many more antioxidants exist (116). Manganese superoxide dismutase (SOD2) is present in the mitochondria to turn free radicals into hydrogen peroxide and then water, working in tandem with catalase, glutathione peroxidases, or peroxiredoxins (118,119). The importance of SOD2 in the context of cardiovascular functioning can be seen in a study by Sharma *et al.* where they found that a strain of SOD2 deficient mice died at approximately 4 months old due to dilated cardiomyopathy followed by heart failure (118). Consequences of oxidative stress include DNA damage, lipid and protein peroxidation, and cellular dysfunction, all of which can cause cells damage and death (116). In the heart, myocardial remodeling and endothelial dysfunction are notable effects of oxidative stress (116).

6.2 Mitophagy in the Heart

Due to the high energy demands of the heart, cardiac cells have many mitochondria to generate large amounts of ATP (120). Mitophagy is a specialized type of autophagy that recycles dysfunctional components of mitochondria into amino acids, fatty acids, and nucleotides (120). Low levels of mitophagy can be beneficial in mitigating the effects of heart failure; however, excessive mitophagy can be harmful and lead to further heart failure progression (120). Mitophagy adaptors, such as mitochondrial proapoptotic BH3-only domain protein (BNIP3) help connect mitochondria and autophagosomes in the mitophagy process (120,121). BNIP3 helps promote autophagy, with increased expression showing increased mitophagy flux (120,122).

6.3 Mitochondria and Heart Failure

In physiological conditions, mitochondria allow for normal cardiomyocyte functioning by maintaining calcium homeostasis and redox balance and using oxidative phosphorylation to make ATP (123). More energy is needed to support a failing heart, leading to increased mitochondrial ETC activity and therefore more ROS leakage (14). Heart failure can be further progressed by impaired oxidative metabolism, induction of cell death signaling, and mitochondrial DNA damage (123). It is thought that exercise intolerance, a key feature of HFpEF, may be due to impaired oxygen utilization stemming from mitochondrial abnormalities (124). Impaired mitochondrial functioning is also thought to be a crucial aspect of decreased cardiac output seen in heart failure (120).

6.4 Links Between Autophagy and Mitochondria

Autophagy and mitophagy can help rescue a cell with damaged mitochondria through the PTEN-induced putative kinase 1 (PINK1)/Parkin pathway, where autophagy receptors degrade and recognize dysfunctional mitochondria, or by using mitochondria-derived vesicles (MDVs) to shuttle mitochondrial components to lysosomes (123,125,126). An important player in heart failure is Rab7, a lysosome associated protein involved in regulatory function. Rab7 is involved in Bcl-2-associated athanogene 3 (BAG3) mediated chaperone-assisted selective autophagy (CASA), which helps remove dysfunctional mitochondria (123,127). Cardiomyocyte death, along with defects in myocardial energy metabolism, are tied to mutated BAG3 and subsequent changes in CASA regulation (123,127). Myocardial remodeling can be shaped through lysosomal activity and its impact on mitochondrial homeostasis as lysosomes are essential for the autophagic removal of damaged mitochondria (123,128).

Rationale and Hypothesis

Rationale:

Although there are several available treatments to treat HFrEF, there are currently no available treatments for HFpEF due to lingering knowledge gaps regarding the heterogeneous array of pathophysiological mechanisms that can lead to HFpEF (129). Even less is known about cellular mechanisms underlying sex differences in HFpEF prevalence, clinical presentation, and mortality. With autophagy and its interplay with mitochondrial dynamics being recently identified as an important physiological aspect of HFpEF development, we sought to investigate the possibility that sex differences in autophagy and mitochondrial dynamics underly sex differences in HFpEF progression and severity.

Hypothesis:

We hypothesize that mice with CMS induced HFpEF will exhibit sex specific differences in autophagy regulation and oxidative stress responses.

Materials and Methods

1. Animals

Male and female C57BL/6J mice were fed *ad libitum* a normal chow diet (ND; Prolab RMH 3000) or HFD (60% of calories from fat; Research Diets D12492) in the absence and presence of L-NAME (0.5 g/L, Sigma Aldrich) in drinking water for 5 weeks (ND and HFD + L-NAME groups) to induce CMS-induced HFpEF (78) or 8 weeks (HFD only groups). Animals were housed at an animal facility with standard laboratory conditions, 12-12 hr light-dark cycle. Animal experiments for this study were approved by the University of Manitoba's animal care committee and follow guidelines for the protection of animals used for scientific purposes by Canadian Council for Animal Care, directive 2010/63/EU.

2. Echocardiography

Animals were assessed after 5-8 weeks of treatment or 5 weeks of a normal diet under light anesthesia (1.5% isoflurane) with a GE HealthCare Vivid E95 Ultrasound System (GE Medical Systems) and a L8-18i linear-array transducer. Mid-papillary level measurements were taken to determine % fractional shortening (% FS), left ventricular internal dimension in end-diastole (LVIDd) and left ventricular internal dimension in systole (LVIDs) and parasternal long axis measurements were used to determine % ejection fraction (% EF).

3. Western Blot Analysis

Sacrifice was performed through administration of 5% isoflurane and cervical dislocation. Hearts were then lysed in Radioimmunoprecipitation assay (RIPA) buffer (1% triton x [Sigma-Aldrich, X100-1 L]-0.5 mL, 0.1% SDS-250 µL of 20% SDS, 140 mM NaCl-1.4 ml 5 M NaCl, 10 mM Tris pH 8, sodium deoxycholate-0.5 g, pH of RIPA 7.7) with protein inhibitors (10µL of NaF, 10µL of PI, 10 µL of Na3VO4, and 3.4µL of PMSF added per 1mL of RIPA) later added. Protein was extracted from hearts and protein concentrations of each sample were measured by bicinchoninic acid (BCA; Thermo Fisher Scientific, 23225) assay. For western blot analysis, lysate containing 20 µg of protein was denatured at 100ºC for five minutes. Protein was then resolved on denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, followed by transfer onto nitrocellulose membranes (Bio-Rad, 1620094). Membranes were probed at 4ºC overnight with primary antibodies made using a 1:1000 or 1:2000 dilution with 2.5% skim milk in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.3% Tween- 20, pH 7.4). Primary antibodies were targeted against GAPDH, p62/SQSTM1, ATG7, ATG14L, and SOD2. Membranes were then washed using TBST for 10 minutes three times and then incubated with secondary anti-murine or anti-rabbit antibodies conjugated to anti-horseradish peroxidase (HRP) conjugate for one hour at room temperature. Bands were detected using enhanced chemiluminescence (ECL) substrates and visualized using an Amersham Imager 680 (GE Healthcare).

4. Quantitative real-time PCR

After sacrifice, hearts were excised and then rinsed in PBS prior to being stored at -80ºC. To extract RNA, each heart was homogenized in 1 mL of Invitrogen TRIzol Reagent (15596-018). Chloroform (200 µL, Sigma Aldrich, B9673-200 mL) was used. The iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad, 172-5035) was used to synthesize complementary DNA (cDNA), followed by amplification of the quantitative Real-Time PCR reaction via iQ SYBR Green Supermix (Bio-Rad, 14-222-777) with Bnip3 and housekeeping gene L32. Primers included *Bnip3* forward 5'-GGGTAGAACTGCACTTCAGCAA-3' and reverse 5'-CCCTGTTGGTATCTTGTGGTGT-3' and *L32* forward 5'-TAAGCGAAACTGGCGGAAAC-3', and reverse 5'-GCTGCTCTTTCTACGATGGCTT-3' (Sigma, Accession number: XO 6483). The CXF96 qPCR detection system (Bio-Rad) was used, with fold change in gene expression calculated using the ΔΔCT method.

5. Statistical Analysis

Quantitative results are presented as the mean result ± the standard error of the mean (SEM). Comparisons between groups were completed using an unpaired two-tailed student's t-Test or one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Graph Pad Prism 6 and 9 software was used to measure statistical significance. Differences that were statistically significant were indicated by $*p < 0.05$, or $**p < 0.01$. In all studies, data was obtained from at least n = 3 mice for each condition tested.

Results

1. Weight Gain and Echocardiographic Parameters Indicate Cardiometabolic HFpEF:

This study focused on CMS and development of HFpEF, therefore, we examined parameters relating to these syndromes and ensured that HFpEF, not HFrEF is perpetuated in our model. In order to verify there was a metabolic difference in mice fed HFD (or HFD + L-NAME), we completed end-points that include mouse weight recording throughout the study. As shown in Figure 1.1, mice in both the HFD and HFD + L-NAME groups had a higher end weight than mice in the ND groups. Interestingly, males fed HFD had a higher end weight than mice fed HFD + L-NAME.

To confirm that HFpEF resulted from the combined HFD + L-NAME diet, we assessed ejection fraction and left ventricular internal dimension in end-diastole (LVIDd) using M-Mode echocardiography in mice fed normal or HFD with and without L-NAME. Preserved ejection fraction is the most notable feature of HFpEF and our results, shown in Figure 1.2 (A-B), showed that ejection fraction did not significantly change with the HFD + L-NAME model. LVIDd in HFpEF is shown in previous literature to either stay similar to normal conditions or slightly increase (130). This contrasts HFrEF, where a large increase of approximately 4.5-5 millimeters, due to ventricle dilation, is noted (130). As demonstrated in Figure 1.2 (C-D), our results resemble those expected with HFpEF.

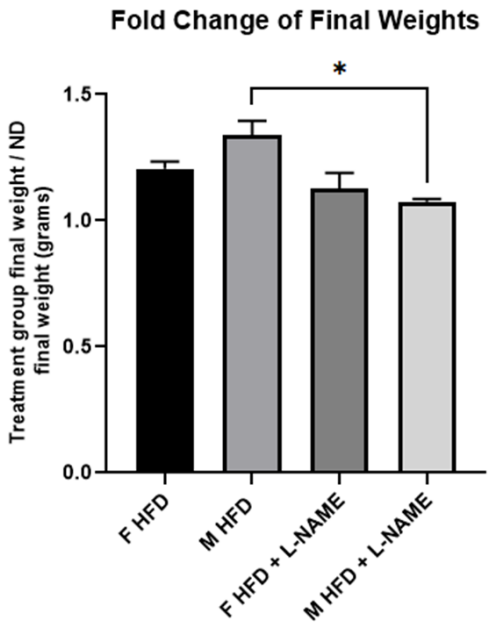


Figure 1.1. HFD and HFD + L-NAME treatments result in weight increase.

Fold change of weight measurements of HFD or HFD + L-NAME groups relative to ND controls. Measurements were taken at week 8 for HFD and week 5 for HFD + L-NAME. Data is expressed as mean ± SEM.

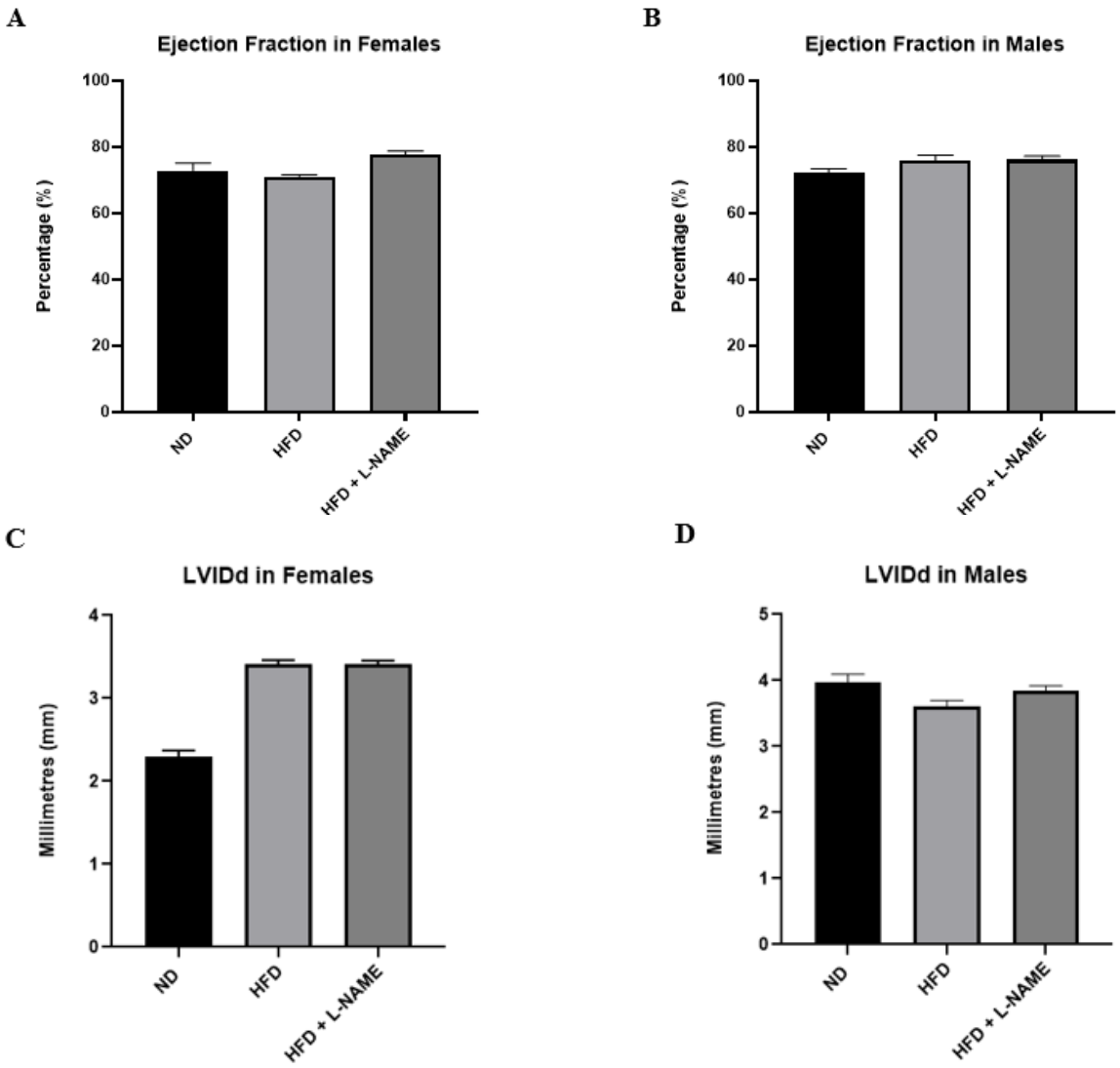


Figure 1.2. Ejection fraction and LVIDd assessment in female and male mice treated with HFD or HFD + L-NAME.

Panels A-B: Ejection fraction in female and male mice fed ND, HFD, and HFD + L-NAME. Ejection fraction calculated from M-Mode echocardiography. Data is expressed as mean ± SEM.

Panels C-D: Left ventricular internal dimension in diastolic end (LVIDd) in female and male mice fed ND, HFD, and HFD + L-NAME. LVIDd calculated from M-Mode echocardiography. Data is expressed as mean ± SEM.

2. **Fractional shortening and left ventricular diameter in systole (LVIDs) change in male HFD + L-NAME mice:**

Fractional shortening (FS) and LVIDs are two cardiac parameters that were examined to understand how overall heart function was impacted by HFD + L-NAME.

FS measures the change between LVIDd and LVIDs and acts as a measure of how well the left ventricle is functioning (131). Decreased FS indicates worse cardiac performance (131). Figure 2.1 demonstrates that fractional shortening remains similar across all groups in females (Figure 2.1 A), but decreases significantly in male HFD + L-NAME mice compared to male ND mice (Figure 2.1 B).

Left ventricular internal diameter in systole (LVIDs) is a measure of the ability for the heart to efficiently contract (132). With worsening HFpEF severity, increased wall thickening can lead to a narrowed left ventricular cavity and decreased LVIDs (132). Our results show a similar LVIDs in female mice across groups (Figure 2.2 A), but a decreased LVIDs in male mice fed HFD + L-NAME compared to ND male mice.

Collectively, these parameters (Figure 2.1 and Figure 2.2) show worsening cardiac function in male HFD + L-NAME mice compared to female HFD + L-NAME mice.

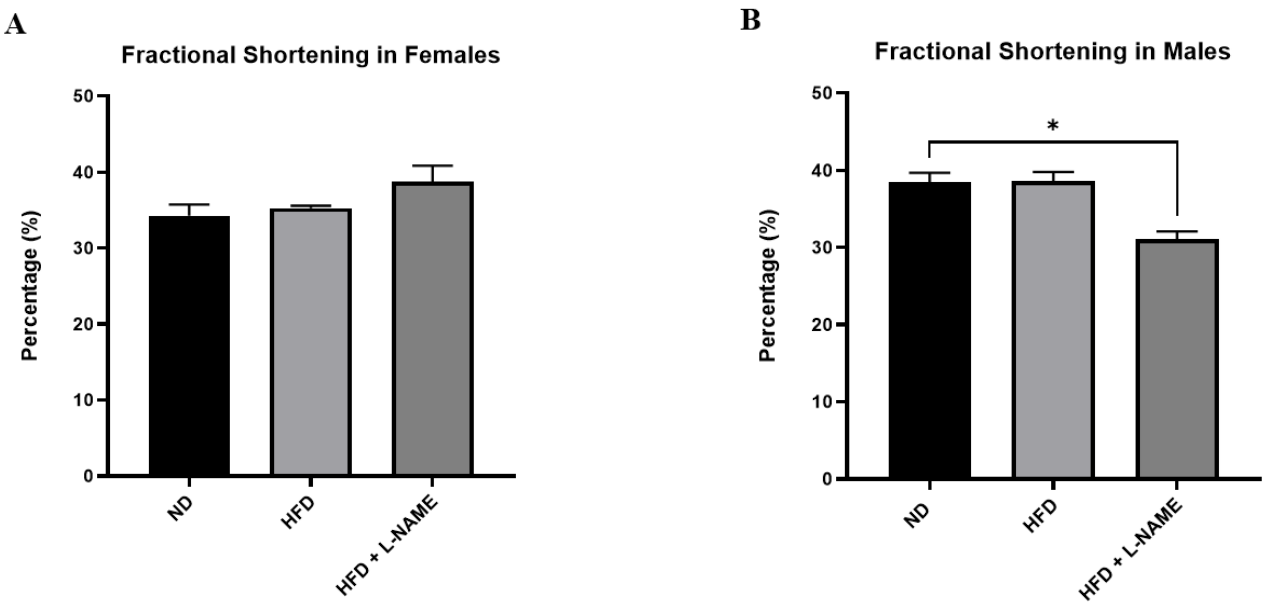


Figure 2.1. Fractional shortening decreases in female and male mice treated with HFD + L-NAME.

Panel A. Fractional shortening in female mice fed ND, HFD, and HFD + L-NAME. Fractional shortening calculated from M-Mode echocardiography. Data is expressed as mean ± SEM.

Panel B. Fractional shortening in male mice fed ND, HFD, and HFD + L-NAME. Fractional shortening calculated from M-Mode echocardiography. Data is expressed as mean ± SEM, *$p < 0.05$.

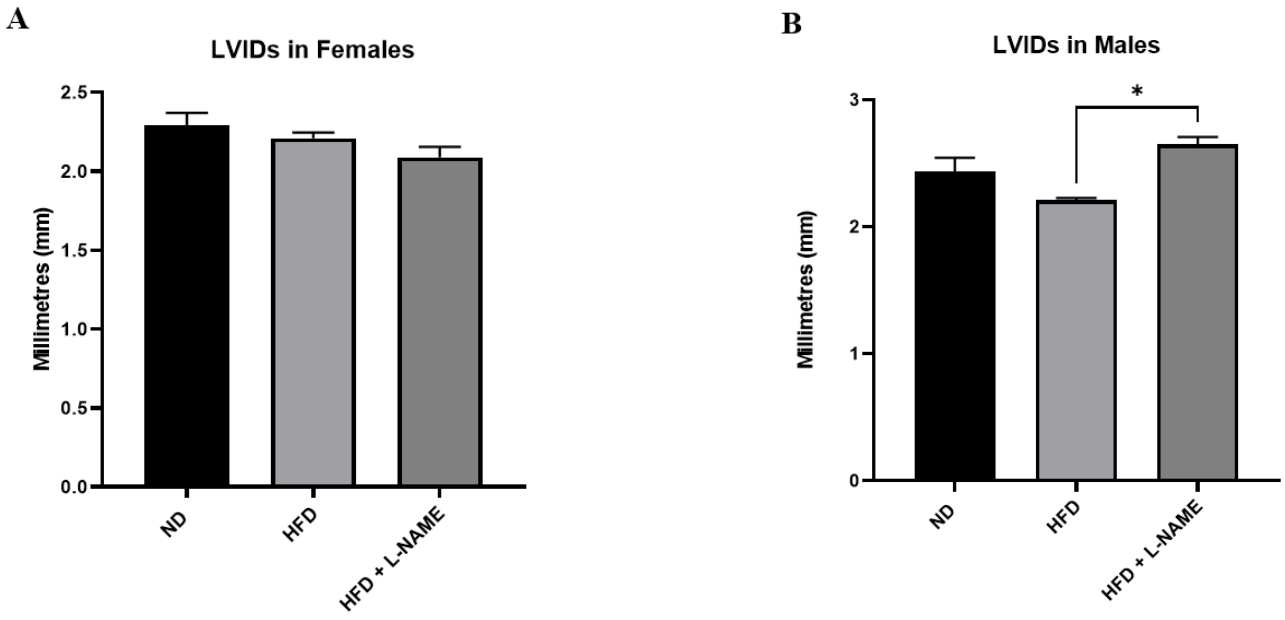


Figure 2.2. LVIDs assessment in female and male mice treated with HFD or HFD + L-NAME.

Panel A. LVIDs in female mice fed ND, HFD, and HFD + L-NAME. LVIDs calculated using M-Mode echocardiography. Data is expressed as mean ± SEM.

Panel B. LVIDs in male mice fed ND, HFD, and HFD + L-NAME. LVIDs calculated using M-Mode echocardiography. Data is expressed as mean ± SEM, *$p < 0.05$.

3. Mitophagy is Increased in HFD + L-NAME Females

We sought to gain more insight on mitochondria-specific autophagy as literature has indicated mitochondrial dynamics as an essential aspect of HFpEF development. Previous literature indicates that mitophagy may increase in HFD + L-NAME as an attempt to mitigate detrimental effects from the accumulation of dysfunctional mitochondria (133).

To measure mitophagy, we assessed the levels of Bnip3 in using qPCR. Analysis results of BNIP3 demonstrated a marked increase in Bnip3 in female mice treated with HFD + L-NAME compared to other groups (Figure 3). This observation may indicate a compensation response to dysfunctional mitochondria in female HFD + L-NAME mice, that is not present in males.

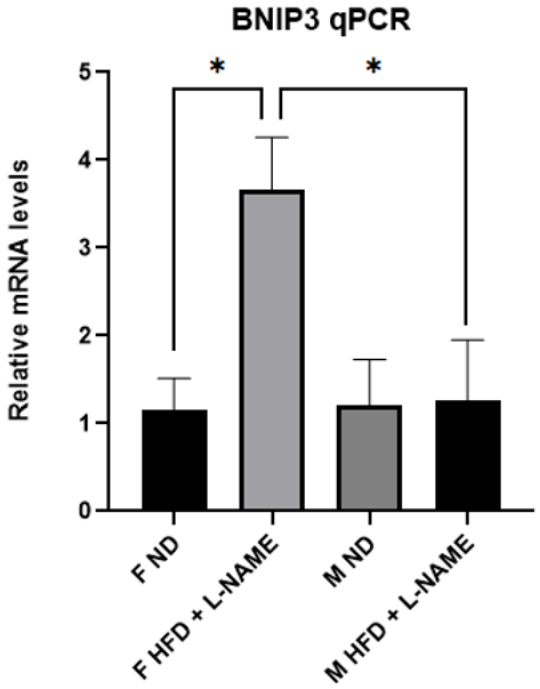


Figure 3. BNIP3 mRNA increases in female HFD + L-NAME mice

Relative mRNA levels of BNIP3 in female and male mice fed ND or HFD + L-NAME measured using qPCR. Data is expressed as mean ± SEM, *$p < 0.05$.

4. Autophagy is Increased in Female Mice Treated with HFD + L-NAME Compared to Male Mice

Autophagy markers p62/SQSTM1, ATG14L, and ATG7 were examined to assess differences in autophagy between ND, HFD, and HFD + L-NAME in male and female mice. For these experiments, p62, ATG14L, and ATG7 were assessed by Western blots analysis.

As shown in Figure 4.1 p62 levels were decreased in female HFD + L-NAME mice compared to female ND and female HFD mice, but male HFD + L-NAME mice had unchanged p62 levels compared to their ND counterpart. As p62 clearance is proportional to autophagic clearance (92), these results demonstrate increased autophagic clearance in female mice under HFpEF conditions compared to males. An increase in p62 in male HFD only mice may indicate impaired autophagy that is not as prominent in female fed HFD.

As shown in Figure 4.2, ATG14L levels were increased in both male and female mice compared to ND to HFD + L-NAME; however, we had only observed an increase in female mice that was statistically significant. Increases were also noted when comparing ND to HFD only mice in both males and females.

Notably, as shown in Figure 4.3,

ATG7 levels were increased in both male and female mice when compared ND to HFD + L-NAME, with the difference in female mice having greater statistical significance. Female mice also demonstrated a difference between ND and HFD groups.

Overall, female mice undergoing HFD + L-NAME treatment demonstrate statistically significant increases in autophagic markers, while male HFD + L-NAME mice show increases with less, or no, statistical significance when compared to ND mice. HFD mice exhibit increased ATG7 and

ATG14L markers, however, they also exhibit increased p62, indicating a lack of completion of the autophagic process.

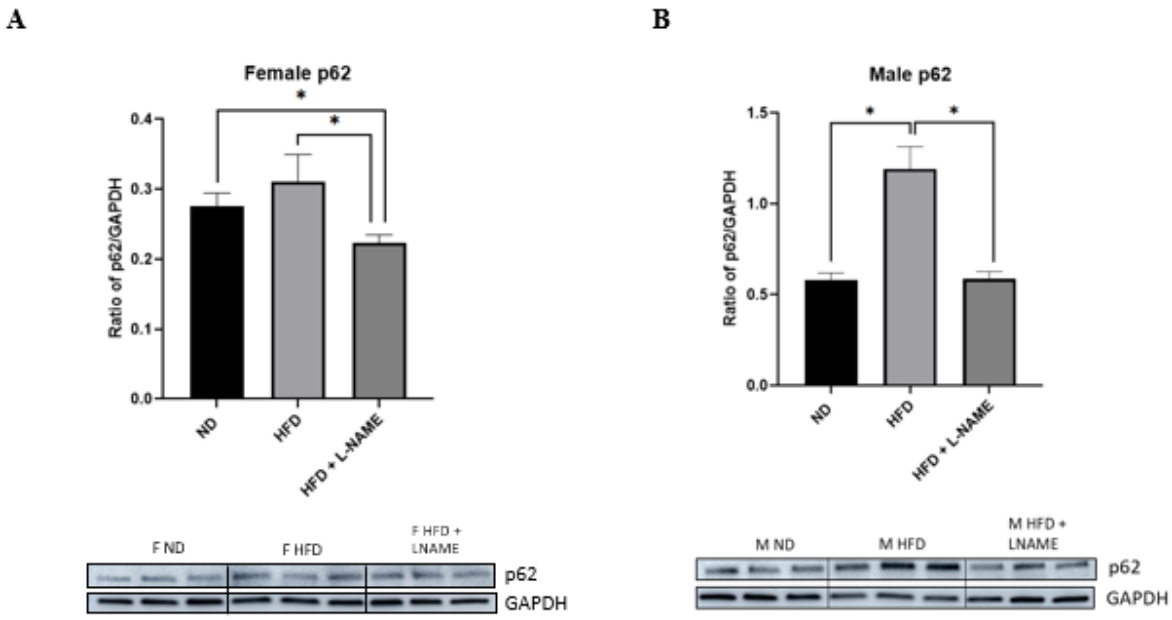


Figure 4.1. p62 levels decrease in male mice treated with HFD + L-NAME

Panel A: Western blot analysis of lysate from female mice fed ND, HFD, or HFD + L-NAME. The western blot was probed with primary antibody directed towards p62, with GAPDH as a loading control. Data is expressed as mean ± SEM, *$p < 0.05$.

Panel B: Western blot analysis of lysate from male mice fed ND, HFD, or HFD + L-NAME. The western blot was probed with primary antibody directed towards p62, with GAPDH as a loading control. Data is expressed as mean ± SEM, *$p < 0.05$.

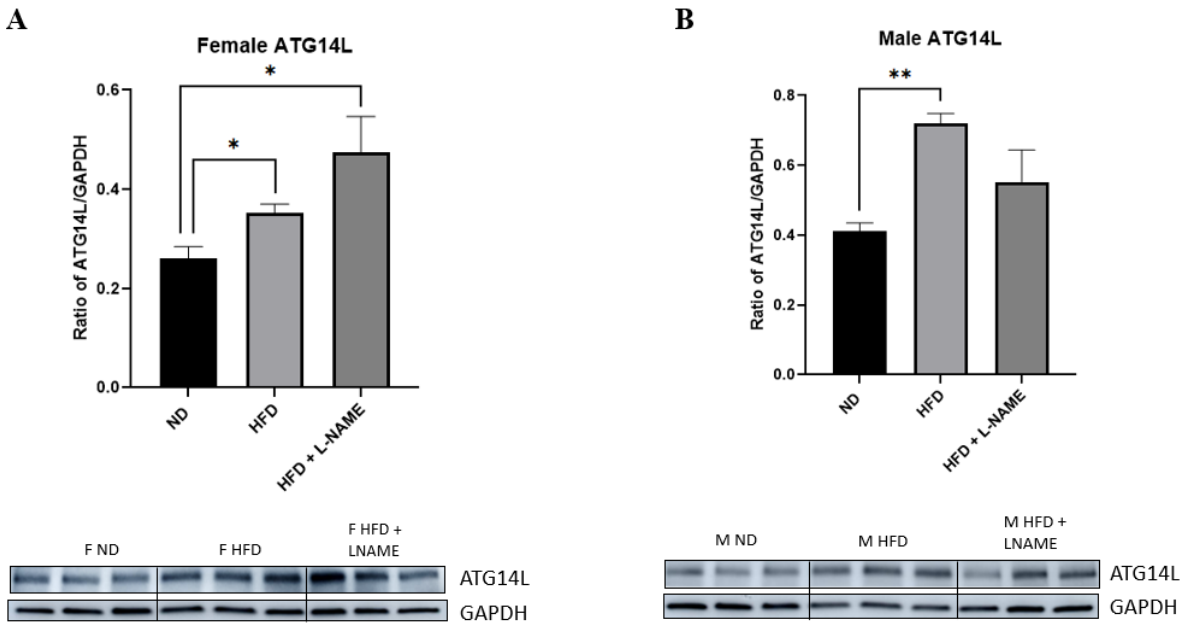


Figure 4.2. ATG14L increases significantly in female HFD + L-NAME mice compared to normal diet

Panel A: Western blot analysis of lysate from female mice fed ND, HFD, or HFD + L-NAME. The western blot was probed with primary antibody directed towards ATG14L, with GAPDH as a loading control. Data is expressed as mean ± SEM, *$p < 0.05$.

Panel B: Western blot analysis of lysate from male mice fed ND, HFD, or HFD + L-NAME. The western blot was probed with primary antibody directed towards ATG14L, with GAPDH as a loading control. Data is expressed as mean ± SEM, **$p < 0.01$

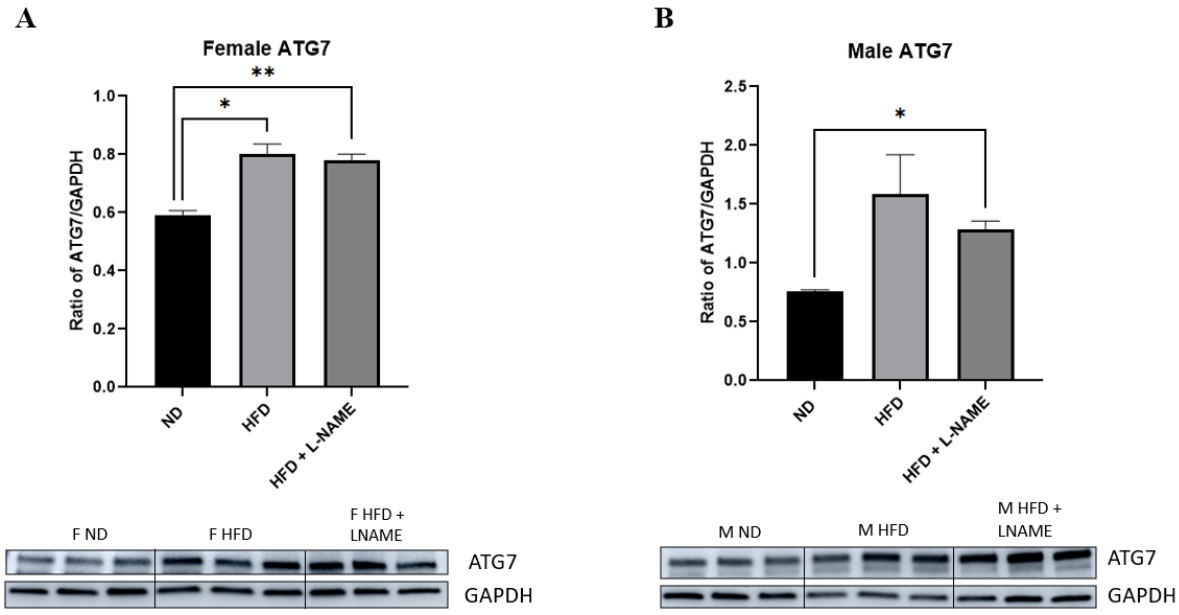


Figure 4.3. ATG7 increases are more statistically significant in female HFD + L-NAME mice compared to ND

Panel A: Western blot analysis of lysate from female mice fed ND, HFD, or HFD + L-NAME. The western blot was probed with primary antibody directed towards ATG7, with GAPDH as a loading control. Data is expressed as mean ± SEM, *$p < 0.05$, **$p < 0.01$

Panel B: Western blot analysis of lysate from male mice fed ND, HFD, or HFD + L-NAME. The western blot was probed with primary antibody directed towards ATG7, with GAPDH as a loading control. Data is expressed as mean ± SEM, *$p < 0.05$.

5. **Antioxidant SOD2 decreases in male mice but not in female mice treated with HFD or HFD + L-NAME.**

To examine changes in anti-oxidant systems and mitochondrial dynamics, we examined SOD2 protein content by Western blots. SOD2, an essential antioxidant in the mitochondria that acts to neutralize superoxide anion radicals, has been noted to decrease with worsening HFpEF conditions (134,135). Decreased SOD2 is also connected to worsening mitochondrial functioning (134), making it a notable marker to examine when considering ROS, oxidative stress, and HFpEF.

As shown by Western Blot analysis in Figure 5, SOD2, was decreased in male mice fed HFD + L-NAME, but not in corresponding female mice (Figure 5). This demonstrates that antioxidant levels are only impaired in male HFD + L-NAME mice, not in females and may point to sex-specific changes in mitochondria producing ROS. Any changes in HFD mice compared to other groups were not statistically significant (Figure 5).

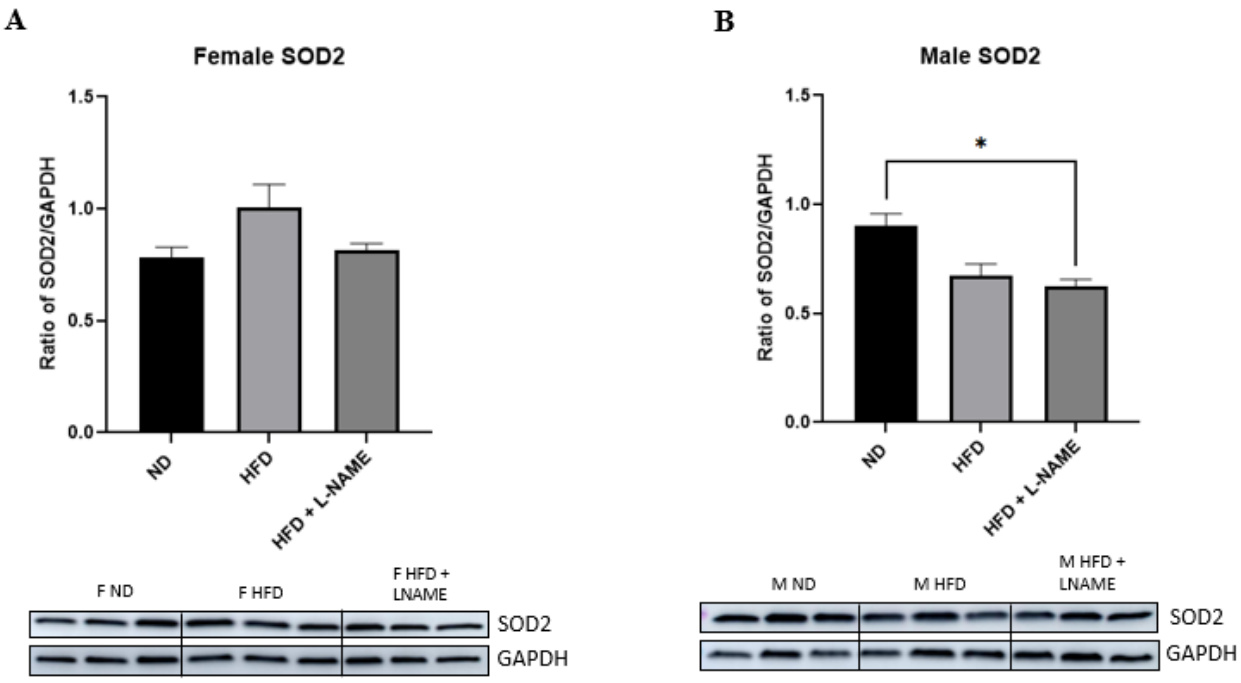


Figure 5. SOD2 decreases in HFD + L-NAME male mice

Panel A: Western blot analysis of lysate from female mice fed ND, HFD, or HFD + L-NAME. The western blot was probed with primary antibody directed towards SOD2, with GAPDH as a loading control. Data is expressed as mean ± SEM.

Panel B: Western blot analysis of lysate from male mice fed ND, HFD, or HFD + L-NAME. The western blot was probed with primary antibody directed towards SOD2, with GAPDH as a loading control. Data is expressed as mean ± SEM, *$p < 0.05$.

Discussion

Heart failure is an ongoing health issue worldwide, and trends have noted that HFpEF prevalence is increasing, especially in women (136). This increase is connected to increased risk factors within populations such as metabolic syndrome, obesity, and chronic kidney disease as well as increased HFpEF awareness and diagnosis (136). Compared to HFrEF, HFpEF is under-researched as in 2019, only 20% of heart failure trials focused on HFpEF despite similar prevalence of HFpEF and HFrEF (137). HFpEF patients have notably shorter life expectancy; however, there is currently no cure for HFpEF other than a heart transplant, leading to a significant strain on impacted individuals, families, and healthcare systems (138). Within the realm of HFpEF research, a vital question that still remains unanswered is: Why is HFpEF more prevalent in women and yet men are shown to have worse survival? Since many cellular mechanisms surrounding HFpEF pathology are still not well understood, further exploration is needed to explore these cellular mechanisms in a sex-specific context.

HFpEF is known to be a multisystemic disease as its etiologies and later impacts are found to impact not only the heart but also the body systemically (139). Low grade inflammation along with metabolic dysregulation drive HFpEF, with obesity, hypertension, diabetes, and other co-morbidities leading to proinflammatory cytokine secretion and profibrotic mediators (139,140). From these events arises increased myocardial stiffness, diastolic impairment, structural cardiac remodeling, elevated left ventricular filling pressures and exercise intolerance (139). In addition, the bioavailability of NO for cardiomyocytes is reduced by an inflammation-induced hindering of endothelial NOS activity (78). Multiple studies have confirmed autophagy as a key player in

HFpEF development, but whether overactivation or under activation is the driving force towards disease progression remains a heavily debated topic.

In this study we use the novel two hit model to induce HFpEF in mice by combining HFD and L-NAME treatments to provide both metabolic and mechanical stress associated with HFpEF in humans (78). This model has been shown to closely reproduce clinical features of HFpEF, both in the heart and systemically (78). Our findings demonstrate weight gain, preserved ejection fraction, and no changes in LVIDd following HFD + L-NAME treatment for 5 weeks. Interestingly, weight gain was higher in HFD groups than HFD + L-NAME groups, with this difference being statistically significant in male mice. Literature has shown that in rodents, L-NAME can impede appetite and food intake due to NO acting as an appetite stimulant (141,142), providing a potential explanation for differences we have seen. L-NAME administration was shown to centrally impact appetite regulation pathways in the brain, leading to decreased food intake (142).

A common parameter for measuring left ventricular functioning is echocardiography derived fractional shortening (FS) (143). Although FS is a measure of systolic function, many studies have demonstrated abnormalities in systolic functioning in HFpEF patients despite ejection fraction being preserved (130). Literature reveals strong associations between left ventricular systolic abnormalities and adverse outcomes in HFpEF patients as well as connections between worsened systolic function and impaired diastolic reserve in HFpEF (130,144). An increase in LVIDs can be indicative of decreased overall heart function and increased cardiac mortality

(145). Previous research regarding sex-differences in heart failure progression indicate impaired contractile reserve in men compared to women, and although HFpEF mechanisms remain elusive, researchers believe sex differences are present in these mechanisms as well (146,147). In our study, we show worsening FS and increased LVIDs in male mice fed HFD + L-NAME, but not female mice, demonstrating worsening cardiac functioning in males. This aligns with many clinical HFpEF observations of worsening outcomes in men (43). Interestingly, L-NAME on its own has been shown in literature to increase fractional shortening (148), indicating that the decrease seen in males is likely due to overall worse cardiac functioning, not just the addition of L-NAME. Additionally, L-NAME itself is not known to cause compensatory hypertrophy or increased LVIDs due to compensatory mechanisms (149). This allows us to conclude that an increase in LVIDs in males given HFD + L-NAME is not solely due to the effects of L-NAME, but more likely due to overall impaired heart function in the HFpEF model.

Mitophagy is an essential mechanism for cell maintenance and overall mitochondrial functioning. An increased reliance on fatty acid oxidation during HFD conditions promotes oxidative stress that leads to mitochondrial stress, requiring mitophagy to help mitigate further damage (150). qPCR analysis demonstrates increased BNIP3 mRNA levels, a marker of mitophagy initiation, in female HFD + L-NAME mice compared to other groups, indicating that damaged mitochondria may be better cleared in females with HFpEF than males. In order to examine BNIP3 at a protein level, further studies should include western blots exploring ND, HFD, and HFD + L-NAME groups for males and females.

Autophagy marker ATG7 provides insight on autophagy initiation in the cell as it processes LC3-I to LC3-II and conjugates ATG12 to ATG5, allowing for elongation of the phagophore (151,152). Literature has shown that increased ATG7 levels increase autophagic flux and functioning, but do not increase cytotoxicity in the cell (151). Although studies have yet to show if autophagy upregulation via ATG7 is beneficial in the context of HFpEF, previous studies have examined Atg7 upregulation in instances of doxorubicin-induced cardiomyopathy, cardiomyocytes with autophagy deficiency, and cardiac proteinopathy (129–131). Another marker of note, ATG14L, plays a major role in regulating autophagosome and lysosome fusion through regulation of the class III phosphatidylinositol 3-kinase complex (153,154). Contrary to ATG7, ATG14L acts later in the process of autophagy (154) and has yet to be tested as a therapeutic target for heart disease.

Sequestosome 1 (SQSTM1/p62) is an essential protein in autophagy that bridges damaged and ubiquinated proteins to the autophagosome and is then engulfed with cargo to be degraded (155,156). This protein is an avid marker for successful autophagy as defective autophagy leads to p62 accumulation (157). Recent literature on autophagy in HFD mice has noted hindered autophagosome degradation, through increased p62 levels, leading to autophagosome accumulation (158). Although HFD + L-NAME conditions can potentially also lead to hindered degradation due to cellular impairment it is noted that initial autophagic responses during early disease often lead to successful autophagy as an attempted rescue response (159).

A particularly notable part of our findings is that sex differences in autophagy markers are present when analyzing protein content of male and female mice fed ND, HFD, and HFD + L-NAME. Despite both males and females having increased autophagy with HFD + L-NAME conditions, the differentiating factor is increased autophagic clearance in females, and a higher statistical significance in female measurement, indicating a lower likelihood that results are due to chance. Our results show increased p62 in female and male HFD mice, with male mice showing an especially marked increase. This may indicate increased impairment of degradation in male mice under metabolic conditions. When examining HFD + L-NAME groups we found that only female mice had a decreased in p62 levels compared to ND mice, indicating, along with other autophagy markers, increased successful autophagy. When examining markers ATG14L and ATG7, we show a more statistically significant increase in autophagy in female HFD + L-NAME mice compared to males, with male HFD mice interestingly showing increased levels compared to HFD + L-NAME. Although ATG7 increases in both males and females, this marker is early in the autophagic pathway, indicating that autophagy may be initiated but is not as successful in males compared to females. Previous literature indicates that differences between HFD and HFD + L-NAME in mice may be due to L-NAME blunting successful autophagy (133); however, we do not see the same effect in females, raising a question of if mechanical stress is more detrimental in males than females. In tandem, our novel findings pave the way for future studies exploring further if sex differences in autophagy underly sex differences in cardiometabolic HFpEF.

Another interesting finding from our study is that the antioxidant SOD2 is found to be decreased significantly in male mice undergoing HFD + L-NAME but not female mice. In male mice, we

see a non-significant decrease in the HFD group compared to ND mice, demonstrating that obesity may be an important factor in the decrease seen in HFD + L-NAME mice. Studies have shown that decreased extracellular SOD is correlated with increased oxidative stress in chronic heart failure, leading to ventricular hypertrophy, ventricular remodeling, and systolic dysfunction (160,161). The cardiac dysfunction we have reported in male HFD + L-NAME mice may therefore be connected to increased oxidative stress, which decreased successful autophagy may further exacerbate.

Previous research has shown that mortality is increased and reported quality of life is decreased in men with HFpEF compared to women (36). A study by Tong et *al.* exploring sex differences in HFpEF experimentally also noted an attenuated cardiac phenotype in female mice compared to males and concluded that sex hormones did not bring about this difference (83). Current research on the reason for this difference or further sex-difference related studies is sparse; however, our current study can add to existing knowledge. Our findings show decreased cardiac functioning in males undergoing HFD + L-NAME treatment compared to females and show that successful autophagy may be increased in females. Additionally, antioxidant levels are only maintained in females, not males. This suggests that successful autophagy may be cardioprotective in females, leading to decreased oxidative stress and improved cardiac parameters. A previous study, conducted by Quarles et *al.* demonstrated that increased autophagy through administration of rapamycin, an inhibitor of the autophagy inhibitor mTOR, can greatly improve diastolic function and myocardial stiffness in mice (162). Independently, our current findings indicate increases in autophagy alongside improved cardiac function in females, adding to emerging evidence that links autophagy to cardiac performance under conditions of

diastolic dysfunction. Collectively, our research demonstrates a potential explanation for sex differences seen in cardiometabolic-induced HFpEF prognosis, quality of life, and mortality.

Conclusion and Future Directions

In conclusion, our study provides a starting point for exploring sex-differences in autophagy and mitochondrial mechanics as a potential explanation for sex differences seen in cardiometabolic syndrome induced HFpEF. We show that males fed HFD + L-NAME show worse FS and LVIDs compared to a normal diet whereas females do not and demonstrate that successful autophagy may be increased overall in females relative to males. We also demonstrate that SOD2 is decreased in males given HFD or HFD + L-NAME but not in females. Together, our results connect autophagy and cardio protection in females with HFpEF. The implications of these findings lie in the potential for increased knowledge of the currently elusive pathway contributing to sex differences in HFpEF and may contribute to future therapies that are effective for both men and women.

Moving forward, it is essential to explore these changes in autophagy and oxidative stress further. Our treatment plan consisted of 5 weeks of HFD + L-NAME; however, new HFpEF models exploring more stressors may lead to increased differences between groups in western blotting experiments and uncover other sex differences within the autophagic pathway. Additionally, further studies could explore more antioxidant markers to determine if SOD2 is just one of many antioxidants with sex differences. Despite this, our novel approach demonstrates the first step towards understanding these sex differences and highlights the important role of autophagy in cardiometabolic HFpEF progression. These findings can contribute to the development of HFpEF therapies that target autophagic pathways or oxidative stress regulation in order to improve HFpEF prognosis, mortality, and quality of life.

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