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Role of Ataxia Telangiectasia Mutated Kinase in Western-type Diet-induced Cardiac Outcomes under Basal and Ischemic Conditions

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ABSTRACT

Role of Ataxia Telangiectasia Mutated Kinase in Western-type Diet-induced Cardiac Outcomes under Basal and Ischemic Conditions

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Ataxia-telangiectasia mutated kinase (ATM), a serine/threonine kinase, plays a role in DNA damage repair, redox sensing, and metabolism. In the heart, ATM contributes significantly in the myocardial infarction (MI)-induced cardiac remodeling with effects on fibrosis, hypertrophy, apoptosis and inflammation. This study investigates the role of ATM deficiency in 14 weeks Western-type diet (WD)-induced cardiac outcomes prior to and 1-day post-MI in a sex-specific manner using wild-type (WT) and ATM heterozygous knockout (hKO) mice. In male mice, ATM deficiency induced rapid body weight gain and preload-associated dysfunction, while WT mice displayed afterload-associated dysfunction 14 weeks post-WD. Myocyte apoptosis and hypertrophy were higher in hKO-WD versus WT-WD. WD increased fibrosis, and expression of collagen-1α1, MMP-2 and MMP-9 only in WT-WD. AMPK activation was higher, while activation of mTOR and NF-kB was lower in hKO-WD versus hKO-NC. Serum levels of IL-12(p70), eotaxin, IFN-γ, MIP-1α, and MIP-1β were higher in hKO-WD versus WT-WD. Conversely, female hKO-WD mice exhibited an attenuation of weight gain and maintenance of heart function. Cholesterol, triglyceride, and glucose levels were higher in female hKO-WD. WD-induced apoptosis and Bax expression were lower in hKO-WD vs WT-WD. Collagen-1α1 expression was higher in hKO-WD vs WT-WD. MMP-2 and MMP-9 expression increased only in WT-WD. MI decreased cardiac function in both male and female mice versus their WD counterparts. The cardioprotective effects of ATM deficiency in terms of heart function were abolished in female mice 1 day post-MI. MI led to a similar infarct size and increase in apoptosis in the two WD-MI groups of both sexes. These data suggest that – 1) ATM deficiency associates with systolic and preload associated diastolic dysfunction, and exacerbates apoptosis, hypertrophy, and fibrosis in male mice in response to WD; 2) In female mice, ATM

deficiency plays a cardioprotective role with preserved systolic function and decreased apoptosis in response to WD; 3) the sex-specific cardioprotective effects of ATM deficiency in females were abolished 1day post-MI. Thus, ATM deficiency affects cardiac structure and function in a sex-specific manner in response to WD and early post-MI.

DEDICATION

To my son Charlie: The world is a big, beautiful place. I promise we will have adventures and see the world together. You are my sunshine. Reach for the stars little man. Mommy loves you.

ACKNOWLEDGMENTS

To my advisor and mentor Dr. Singh: I am very grateful that you allowed me to join the lab. Thank you for being a wonderful example of how women in science can successfully balance work and family. I am very grateful for all your support and guidance.

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CHAPTER 1. INTRODUCTION

Western Type-Diet, Obesity, and the Heart

Approximately two-thirds of the American population consumes a diet high in fat, sugar and carbohydrates called the western-type diet (WD)[50,81]. The majority (~60%) of American adults are overweight or obese[23,79]. Obesity or lifelong consumption of a WD can lead to chronic inflammation, increased oxidative stress, and metabolic disorders[60,69,70]. Consequently, continuous consumption of WD paired with sedentary lifestyle can induce cardiac structural and functional abnormalities[15,60]. Interestingly, diet-induced adiposity has an independent association with the development of heart failure[4]. The duration and severity of obesity, especially with concomitant comorbidities such as hypertension, insulin resistance and diabetes mellitus (DM), contribute to the myocardial disorder called obesity cardiomyopathy[1,4,91,105]. The hemodynamic changes associated with adiposity directly contribute to myocardial morphological and mechanical dysfunction observed in obesity cardiomyopathy and can include left ventricular (LV) dilation, right ventricular dysfunction, eccentric/concentric hypertrophy, cardiac output, fibrosis, atherosclerosis, and systolic dysfunction[1,4,17]. Further, prolonged increases in blood volume and pressure can cause preload and afterload dysfunction[4,6,91,105]. A variety of diet-induced cardiovascular abnormalities similar to humans have been noted in animal models (rats, mice, rabbits and dogs) which include ventricular stiffness, apoptosis, hypertrophy, fibrosis, atherosclerosis, and an increase of arrhythmic and ischemic events[1,15,21,45].

Western-Type Diet, Obesity, and Cardiac Remodeling

Cardiac remodeling results from a variety pathophysiological stimuli and is defined as a series of cellular and molecular changes that alter the size, mass, geometry and function of the heart [11,72]. Many distinct and complex molecular processes such as fibrosis, hypertrophy and

apoptosis are hallmarks of cardiac remodeling[1,93,113]. While early cardiac remodeling serves as a protective compensatory mechanism from the pressure and volume overload caused by adiposity, the alterations in myocardial structure can lead to cardiac dysfunction and heart failure[4,93]. Adiposity or obesity is shown to alter cardiac performance through changes in cardiometabolic demands, circulating blood volume, pressure overload, and wall tension[5,17]. Therefore, excessive adipose tissue is associated with hemodynamic-induced changes in cardiac function and morphology[4,99].

Western-Type Diet, Obesity, and Cardiac Fibrosis

Following cardiac injury, the heart as a limited regenerative capacity. While fibrosis most commonly occurs as a protective mechanism after myocardial infarction, other stimuli can also promote the development of cardiac fibrosis including oxidative stress, inflammation, apoptosis, hypertension, diabetic cardiomyopathy, and dilated cardiomyopathy[29,53,66]. Increased inflammation, oxidative stress and metabolic abnormalities observed with obesity and WD cause deleterious effects of the myocardium[60,69,70]. Therefore, cardiac fibroblast can become activated as a result of the cardiac damage caused by WD and obesity[44,107]. To preserve myocardial structure, the fibroblasts transdifferentiate into myofibroblasts, which secrete elevated levels of extracellular matrix (ECM) proteins which serve as a protective scaffold[39,103]. However, with obesity and WD, continual and excessive ECM remodeling can occur, causing an accumulation of collagen with associated increase in tensile scar strength[1,44,72]. Unfortunately, increase in cardiac fibrosis has also been linked to decreased electrical conductance, mechanical function, and is a primary cause of heart failure[53].

Western-Type Diet, Obesity, and Cardiac Hypertrophy

Visceral adiposity and WD induce multiple pathological alterations including cardiac hypertrophy[45,84]. An increase in circulating blood volume, stroke volume and cardiac output can occur with excessive adipose tissue, leading to left ventricle enlargement, hypertension, and increased wall stress[4]. As LV wall stress increases, compensatory LV hypertrophy may occur to temporarily preserve systolic function[4,45]. LV hypertrophy seems to occur after cardiac fibrosis development, and also corresponds to the severity of obesity[4]. Right ventricular hypertrophy as well as eccentric or concentric LV hypertrophy have been reported with obesity cardiomyopathy[4,5]. Cardiac hypertrophy has been noted after only six weeks of WD in mice[45]. Though hypertrophy is initially protective, prolonged increase in blood volume may cause LV dilation and lead to decreased systolic and diastolic function[4,6]. Studies have also associated the metabolic and neurohormonal alterations such as increased sympathetic nervous system tone and insulin resistance observed in WD and/or obesity with the development of LV hypertrophy[1,3,62].

Western-Type Diet, Obesity, and Cardiac Apoptosis

Myocyte apoptosis is linked to decreased cardiac function and the development of heart failure[14]. WD and/or obesity-induced hemodynamic alterations, interruption/activation of molecular pathways, decreased metabolic flexibility, lipotoxicity, increase in oxidative stress and inflammation promote cardiac apoptosis in animal models and human subjects[3,13,22,45,62,111,117]. Interestingly, high fat diets have also been shown to directly cause cardiac injury through the induction of apoptosis, dysregulation of autophagy, and endoplasmic reticulum stress[52]. The lack of metabolic flexibility observed with WD and obesity also promotes cardiac cell apoptosis through the exacerbation of toxic byproducts[64,113]. Further, adiposity with concomitant

triglyceride accumulation in animal models associates with cardiac cell lipotoxic apoptosis[111]. Lastly, WD and obesity associate with increased oxidative stress and inflammation. The increased oxidative stress with constant low-grade inflammation promote and exacerbate cardiac cell apoptosis[60,69,70].

Western-Type Diet, Inflammation, and the Heart

Inflammation is a vital defense mechanism that is triggered by a variety of stimuli[20]. The immune system activates the inflammatory response to remove the injurious stimuli and initiate healing[27]. Therefore, acute inflammation serves to minimize injury and restore homeostasis. Intake of excessive fat affects several organs such as the intestines, pancreas, liver, brain and adipose tissue and elicits a low-grade systemic inflammatory response[19,36]. The chronic low-grade inflammation that has been observed with WD and obesity is injurious and linked to the development of diabetes and cardiovascular disease[27,44,70,78,88,110]. Interestingly, adiposity is also linked to increased oxidative stress, endothelial dysfunction, immune dysfunction with associated decrease in white blood cells, altered phagocytosis abilities and increased circulating levels of proinflammatory cytokine [36,62,78]. The inflammatory cascade is initiated by activation of toll-like receptors and the nuclear factor signaling pathways[44,48,65]. Traditionally, neutrophils and macrophages are recruited to the site of cellular damage to remove the damaged cells. After this occurs, the tissue can begin to heal. Unfortunately, a constant presence inflammatory response exacerbates damage to arteries, cardiac tissue, cardiac remodeling, fibrosis, atherosclerosis, and apoptosis[33,97]. Therefore, when an ischemic event occurs, there is an increase in cardiac insult [1,2,33,67,68,97].

Western-Type Diet, Metabolism, and the Heart

Cardiometabolic flexibility is essential for maintaining adequate ATP synthesis[26,57]. The myocardium possesses the ability to utilize multiple substrates for ATP production. The majority of myocardial energy requirements is provided by fatty acid oxidation (50-70%), while glucose provides approximately 10% of cardiac energy production[26,54]. While glucose metabolism only offers 10% of cardiac ATP, insulin signaling plays a critical role in myocardial homeostasis through the phosphoinositide 3-kinase (PI3K)/Akt signaling cascade which provides the majority of glucose uptake through the translocation of GLUT 4[55,61]. WD and obesity can induce metabolic dysfunction can reduce the storage, transport and use of nutritional energy, leading to insulin resistance, hyperglycemia and DM[64,113]. Therefore, hyperglycemia, insulin resistance, and hyperlipidemia can alter the natural balance of glucose or fatty acid utilization, which may lead to poor metabolic adaption[71,82,113]. Key metabolic signaling cascades, such as PI3K/Akt and AMP-activated protein kinase (AMPK), are altered with insulin resistance and DM, further inhibiting metabolic flexibility, decreasing ATP production, accumulating toxic byproducts and increasing oxidative stress[55,61,82,95]. Consequently, myocardial structural and functional alterations, exacerbated inflammation, DNA damage, myocyte death and an acceleration of heart failure have been noted[61,82,113].

Western-Type Diet, Obesity, and Myocardial Infarction

Heart disease is the leading cause of death in the United States with 1 in 4 deaths caused from heart disease[106]. MI is a major contributor of heart failure (HF) and heart disease[106,118]. Several factors increase the risk of MI including obesity, cardiac remodeling, diabetes, increased cholesterol, and triglycerides[118]. WD has been linked to the development of obesity and increased risk of cardiovascular events[96]. In fact, the risk of MI increases by 35% with WD[8].

Adiposity with and without concomitant metabolic syndrome increases the risk of MI[100]. Diabetic patients also have an increased risk of MI and ~40% increased risk of subsequent MI's[30]. In fact, MI is the leading cause of death in non-insulin dependent diabetic patients[30]. MI occurs when the oxygen supply to the myocardium is severely decreased or totally obstructed, causing significant myocardial damage[92,101]. Shortly after the myocardium becomes ischemic (~30 minutes), cardiomyocytes can experience structural and functional alterations. Cardiac ischemia causes deleterious effects on the cardiomyocytes, activation of the inflammatory cascade leading to cell death[18,101]. During the inflammatory phase of MI, damage associated molecular patterns initiate cascades that lead to the recruitment of neutrophils and macrophages to the area of infarction[40]. The dead cardiomyocytes and damaged ECM are then digested and removed from the area of injury[40,85]. During the reparative/proliferative phase, promotion of inflammatory resolution occurs, fibroblasts activate and transdifferentiate to myofibroblasts, and collagen deposition (fibrosis) begins[85,109]. The final phase of cardiac repair post-MI is the maturation phase. During this final phase, the ECM becomes crosslinked, causing the scar to mature, and myofibroblasts deactivate[85,109]. The mature scar can decrease myocardial contraction and promote LV dilation and the development of HF[85,109]. Similar to adiposity, MI can lead to chronic systemic inflammation and increased circulating inflammatory chemokines[1,40]. In animal models, diets high in fat have been seen to increase nitric oxide, oxidative stress and exacerbate infarct size and cardiac damage following MI[36].

Ataxia Telangiectasia Mutated Kinase

Ataxia telangiectasia mutated kinase (ATM) is a high molecular weight (370kDa) serine/threonine kinase which belongs to the PI3K protein family[113]. ATM has a multitude of functions as it is located in the nucleus, cytoplasm, and mitochondria[16,38,101]. Studies have shown

that ATM is involved in regulation of cell cycle checkpoints, DNA damage response, telomere maintenance, and unfolding chromatin in the nucleus[9,47]. The cytoplasmic functions of ATM include regulation of autophagy, angiogenesis, glucose metabolism, and mitochondrial and peroxisome functions during oxidative stress[9,16,28,49,56]. However, the primary function of ATM is to maintain cellular homeostasis[113]. During homeostasis, ATM is an inactive dimer/tetramer[7,47]. However, ATM can become rapidly activated in response to DNA double stranded breaks, increased production of reactive oxygen species, oxidative damage, ionizing radiation and other genotoxic agents[101,113]. When DNA double stranded breaks occur, a histone (H2AX) located within the chromatin becomes phosphorylated and serves as a binding platform for repair[76]. ATM is recruited to the site of double stranded breaks by the MRE11-RAD50-NBS1 (MRN) complex[7]. ATM then binds to the MRN complex, undergoes autophosphorylation of ser1981 to activate the monomer form of ATM, which is important for homologous recombinant DNA repair[7,47]. A series of other accessory proteins (BRCA1, MDC1/NFBD1, 53BP1) colocalize with the complex and are phosphorylated in an ATM-dependent manner[76]. After which, ATM regulates the cell cycle to prevent cell division occurring concomitantly with DNA repair. Studies have shown that ATM coordinates/regulates G1-S checkpoint by phosphorylation of p53 at Ser15, G2/M through the phosphorylation of protein kinase Chk2 at Thr68, and intra-S checkpoint through phosphorylation of SMC1 at Ser957 and Ser966[7,86]. Aside from DNA damage repair, other functions of ATM include oxidative stress signaling, being a redox sensor, regulating metabolic control and mitochondrial function[35].

Ataxia-Telangiectasia

When ATM kinase function is reduced or abolished due to *ATM* gene mutations on chromosome 11q22-23, multisystem dysfunction occurs[38]. A variety of *ATM* gene mutations such as nonsense, missense and splicing site variations have been observed[9,113]. However, nonsense mutations are the primary *ATM* gene mutation, which results in nonfunctional ATM kinase funciton[35,51]. Ataxia-telangiectasia (A-T) is an autosomal recessive disorder, occurs due to a mutation in the *ATM* gene, and is characterized by multiorgan system dysfunction which may include cardiovascular, immune, endocrine, and neurological abnormalities[80,83,90,94]. The severity of the AT phenotype is based on the type and quantity of mutations which occur[9]. For example, some individuals (~2% of the populations) are A-T carriers, with a heterozygous (hKO) mutation in the *ATM* gene[113]. While patients with an heterozygous (hKO) ATM mutation generally display less severe systemic dysfunction, they are still at an exponentially higher risk of the development of cancer, insulin resistance, DM, ischemic heart disease and HF[7,16,49,113]. ATM heterozygous mutations also associate with increased cardiovascular related morbidity and mortality[9].

ATM and the Heart

ATM and Cardiovascular Functional Alterations

It is well documented that AT associates with the development of cardiovascular disease[7,9,47]. Previously, our laboratory investigated the cardiac functional alterations that occur during baseline and ischemic conditions using ATM knock-out (KO) and ATM hKO (heterozygous knockout) mice. In baseline conditions, lack of ATM in mice associates with decreased septal thickness and left ventricular mass, which likely contributed to the growth retardation that occurs with classic AT[43]. KO mice also had a proportional decrease in left

ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), left ventricular end diastolic volume, and left ventricular end systolic volume (LVESV) with no changes in percent ejection fraction (%EF) and fractional shortening (%FS)[43]. It should be noted that the maintained %EF and %FS observed in KO mice occurred concomitantly with increased cardiac hypertrophy. As previously stated, cardiac hypertrophy serves as a compensatory mechanism, and therefore may be contributing to the preserved %EF and %FS[4,6,43]. Additionally, diastolic impairment with increased E/A ratio and decreased deceleration time was observed in KO hearts[43].

MI significantly contributes to the development of HF and heart disease, and ATM heterozygous mutations increase cardiovascular related death[9,106,118]. Our laboratory investigated the functional alterations in ATM deficient (hKO) mice 4 hrs, 1, 3, 7, 14, and 28 days following MI[31,32,42,102]. Echocardiographic measurements 4hrs following MI showed a similar decrease in %EF and %FS between genotypes[102]. While MI significantly decreased LVEDD, LVESD, LVEDV and LVESV in hKO-MI group vs. WT-MI[102]. This suggests that ATM deficiency may slightly attenuate the cardiac dysfunction, with relatively maintained ventricular diameters and volumes 4hrs following MI. Similarly, ATM deficiency associated with cardioprotective effects 1-day following MI, with slightly higher %EF and %FS, and decreased LVESV and LVEDV in hKO-MI group vs WT-MI[31]. LVESV and LVEDV remained significantly lower in hKO-MI group vs WT-MI 3-days post-MI. However, there was no significant difference in the systolic function as %EF and %FS was decreased to similar extent in both genotypes[31]. ATM deficiency continued to attenuate cardiac dysfunction 7-days following MI with increased %EF and %FS, and decreased LVESD and LVEDD when compared to WT-MI groups[42]. Interestingly, there was also an increase in infarct thickness in the hKO-MI group which may have contributed to the

decreased severity of MI-induced LV chamber dilation[42]. Conversely, the protective effects associated with ATM deficiency were abolished (14- and 28-days post-MI) with decreased %EF and %FS, and increased LVESV in hKO-MI vs WT-MI. Mortality was also lower (~14%) in hKO-MI mice vs WT-MI 28 days post-MI[32].

ATM and Cardiac Fibrosis

The heart as a limited regenerative capacity. Deposition of fibrosis is a common occurrence in the heart following myocardial injury[29,53,66]. Previously our lab examined the structural effects of the ATM null and ATM heterozygosity during baseline and ischemic conditions. It was observed that baseline ATM KO mice have exponentially (4-fold) increased cardiac fibrosis compared to WT[43]. Similarly, cardiac fibrosis was significantly increased in hKO[31]. In addition, cardiac fibrosis was significantly increased in hKO-MI versus WT-MI 3 and 7 days post-MI[31,42]. Expression of α-smooth muscle actin (α-SMA), a marker of differentiation of fibroblasts to myofibroblasts, was found to be significantly increased 3, 7, and 28 days following MI[31,32,42]. This increase in α-SMA may explain why there was an increase in fibrosis in the hKO-MI group. Of note the increase in fibrosis was clearly evident in the non-infarct LV region of hKO-MI hearts 28 days post-MI[32].

ATM and Cardiac Hypertrophy

Cardiac hypertrophy occurs as a compensatory mechanism with changes in hemodynamics as well as cardiac structure[4,6]. Our laboratory has shown that baseline ATM KO mice have an increase in cardiac hypertrophy compared to WT mice. This compensatory increase in hypertrophy may help to explain why systolic function in terms of %EF and %FS was

maintained in KO mice[43]. Similarly, an increase in cardiomyocyte cross sectional area (a measurement of cardiac hypertrophy) was observed in the hKO mice and the non-infarcted region of the ATM deficient heart 28-days following MI[32]. While hypertrophy can be protective early following cardiac injury, it can lead to cardiac dysfunction late post-MI[4,6,32]. Therefore, the increase in cardiac hypertrophy in the non-infarcted areas of the 28-day hKO-MI group may be one of the reasons for exacerbation of cardiac function with ATM deficiency late post-MI.

ATM and Cardiac Apoptosis

ATM is well known for its facilitation of double stranded DNA damage repair[113]. Our laboratory investigated if reduction or total abolishment of ATMs kinase function plays a role in myocyte apoptosis during baseline and ischemic conditions. Interestingly, lack of ATM did not associate with increased levels of apoptosis in the heart. Apoptosis was found to be significantly increased in hKO-MI group 1- and 3-days following MI[31]. It was suggested that the increase in apoptosis could have contributed to the increased myofibroblast activation and associated exacerbation of cardiac fibrosis[31]. Conversely, total cardiac cell apoptosis was significantly reduced in the infarct LV region of hKO-MI when compared to WT-MI 7-days following MI. Interestingly, myocyte apoptosis along the border area of the LV infarction in hKO-MI group was significantly greater when compared to WT-MI[42]. Apoptosis was found to be lower in the infarct LV region of the ATM deficient group vs WT 28-days post-MI. Collectively these data suggests that ATM deficiency affects cardiac cell apoptosis during ischemic conditions.

ATM and Inflammation

Acute inflammation serves to mediate the eradication of damaging agents, however, chronic inflammation can be injurious[27,115]. Pathological inflammation is often observed in AT patients and has been linked to adaptive and innate immune defects[63,74,75,80,116]. While the role of inflammation in the pathogenesis of AT is not fully elucidated, the increased inflammation observed in AT patients may directly contribute to the development of cardiovascular disease[73,115]. Our laboratory has provided evidence that ATM deficiency associates with decreased neutrophil and macrophage infiltration in the infarct LV region 1-day post-MI[31]. TGF-β1, an anti-inflammatory signal, was also found to be decreased 3-days post-MI. This suggests that ATM deficiency has the potential to delay the inflammatory response during early MI[31].

Collectively, our laboratory has provided evidence that ATM deficiency is cardioprotective up to 7-days following cardiac ischemic injury with less deleterious effects on cardiac systolic function. It is possible that the increased αSMA expression and concomitant increase in fibrosis served as a compensatory mechanism following cardiac injury and thereby preserving some systolic function. However, ATM deficiency is no longer protective 14-to 28-days following MI.

Specific Aims

WD, adiposity and ATM deficiency independently associate with the development of insulin resistance, cardiometabolic dysfunction, increased oxidative stress, inflammation, increased risk of MI, excessive cardiac remodeling, heart disease and the development of HF[60,69,70,80,83,90,94]. While the majority of AT patients are considered underweight, one study showed that AT patients prefer to consume diets that are high in fat and sugar similar to WD[89].

However, little is known regarding how ATM deficiency affects cardiac outcomes prior to and post-MI in response to WD feeding. The objective of this study was to investigate the role of ATM deficiency in 14 weeks WD-induced cardiac outcomes prior to and 1 day post-MI using male and female WT and ATM hKO mice. The specific aims of the study were to - 1) investigate the role of ATM deficiency in WD-induced cardiac functional and biochemical parameters using male WT and ATM deficient mice; 2) examine the role of ATM deficiency in WD-induced cardiac functional and biochemical parameters using female WT and ATM deficient mice; 3) assess if WD-feeding plays a role in MI-induced ischemic heart disease during ATM deficiency using male and female mice.

CHAPTER 2. HEART FAILURE AND DIABETES: ROLE OF ATM

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Abstract

Heart failure is a leading cause of death in the United States. Diabetes, also known as diabetes mellitus (DM), exponentially increases the risk of heart failure. The increase in oxidative stress and metabolic dysfunction caused by DM can lead to DNA damage and the development of diabetic cardiomyopathy. Ataxia telangiectasia mutated kinase (ATM) is a DNA damage response protein with a primary nuclear function to regulate cell cycle progression in response to double-strand DNA breaks, acts as a redox sensor, and facilitates DNA repair. ATM deficiency associates with the development of insulin resistance and DM. Consequently, patients with Ataxia telangiectasia, a rare autosomal recessive disorder, have an increased risk of developing heart failure. The main objective of this review is to summarize the shared metabolic and cardiac abnormalities associated with DM and ATM deficiency, with a focus on the development of heart failure.

Introduction

Patients with diabetes mellitus (DM) have an increased risk of developing heart failure. An astonishing fact is that 30 million Americans are diabetic and 90 million are prediabetic[1]. As average life expectancy rate increases, the prevalence of patients with DM and heart failure has progressively accelerated in western and developing countries[2]. DM is a metabolic disorder characterized by the inability to properly use, transport and store nutritional energy, resulting in hyperglycemia[3]. There are two main types of DM, insulin-dependent type 1 DM and non-insulin-dependent type 2 DM. This review is focused on type 2 DM which accounts for ~90-95% diabetic diagnosis[1,4]. In addition to hyperglycemia, DM associates with insulin resistance, inflammation, mitochondrial dysfunction, endothelial dysfunction and increased oxidative stress[5,6].

Ataxia telangiectasia mutated kinase (ATM) is a 370kDa serine/threonine kinase that belongs to the PI3-kinase (PI3K) protein family. ATM is a regulator of the G1/S checkpoint, and is present in the nucleus, cytoplasm and mitochondria[7]. Activation of ATM occurs in response to double-strand DNA (dsDNA) breaks, increased reactive oxygen species (ROS) production, oxidative damage, and other genotoxic stressors[8] . In response to dsDNA breaks, ATM and accessory proteins (MDC1, 53PB1, and BRACA1) are recruited to the site of dsDNA breaks where ATM becomes activated via auto-phosphorylation of Ser1981[9,10]. Upon activation, ATM terminates cell cycle progression until DNA repair has been completed[10]. ATM is also a redox sensor and is activated in response to oxidative stress. While dsDNA breaks cause ATM to become activated in monomer form, oxidation of ATM results in a covalently bound dimer form[11]. The *ATM* gene is located on chromosome 11q22-23[11]. While many different types of mutations have been identified on the *ATM* gene, the majority are nonsense mutations which cause premature stop codons resulting in truncation, and dysfunctional ATM protein[12,13]. Mutations in the ATM gene result in a multisystem disorder called Ataxia telangiectasia (AT) which causes cardiac, neurological, immunological, and endocrinological abnormalities[14–17]. Approximately 2% of the population has a heterozygous mutation in the *ATM* gene. Individuals with a heterozygous mutation exhibit a less severe AT phenotype. However, they are at a higher risk of developing DM, heart failure and cancer [7,8,10,18].

DM and ATM deficiency share many risk factors for the development of heart disease such as insulin resistance, increased oxidative stress, endothelial dysfunction and mitochondrial dysfunction[6,11]. DM and ATM deficiency independently associate with the development and acceleration of heart failure[6,7,19,20]. Since ATM contributes to both nuclear and cytoplasmic signaling, disruption in ATM function can directly contribute to metabolic complications such as

DM and the development of heart disease[11]. Not much is known about the role of ATM in the development of diabetic cardiomyopathy (DCM). This review article summarizes the role of ATM in myocardial remodeling using myocardial infarction (MI) as a model, and discusses the shared metabolic abnormalities during DM and ATM deficiency, and how this may exacerbate the progression of heart failure.

DM and Heart Failure

DCM is a complex, multifactorial heart failure syndrome that is characterized by cardiac structural and functional abnormalities independent of hypertension, coronary artery disease and dyslipidemia[3,6]. The risk of developing heart failure is doubled in DM males and four times as likely in DM females as compared to nondiabetic patients[4]. Approximately 20-30% of diabetic patients have heart failure which greatly increases the risk of morbidity and mortality[6,21]. Modifications in cardiac energy metabolism and insulin signaling are the key metabolic abnormalities associated with hyperglycemia and the development and progression of DCM[6]. Hyperglycemia also causes altered calcium homeostasis, increased glycation end products, ROS production, mitochondrial dysfunction and cardiac metabolic dysfunction[4,6], which can contribute to the development of the structural and functional abnormalities that are hallmarks of DCM[6,19]. The early stages of DCM are generally asymptomatic. However, diastolic dysfunction, left ventricular hypertrophy, fibrosis and cardiac remodeling have been noted[3,6]. The later stages of DCM associate with systolic dysfunction, heart failure and increased morbidity and mortality[3,6]. When cardiomyopathy and DM occur concomitantly, the development of heart failure is greatly accelerated[4]. While DCM occurs independent of hypertension, 80% of diabetic patients are hypertensive, which further exacerbates the risk of heart disease[6,22,23].

ATM, DCM and Heart Failure

ROS-mediated DNA oxidation and fragmentation have been observed in the myocardium during DM[24]. Since ATM is activated in response to dsDNA breaks and oxidation, ATM may play a role in the development and progression of DCM. The DNA damage incurred during DM directly contributes to the cardiac structural and functional alterations, which are the hallmarks of DCM[24,25]. Decreased LV compliance, increased atrial filling, lengthened isovolumetric relaxation, variations in left ventricular end diastolic volume (LVEDV), decreased early diastolic filling, cardiomyocyte stiffness, and diastolic dysfunction are often observed in early and advanced stages of DCM[6,26]. During late stages, an increase in left ventricular diameter, decreased percent ejection fraction (%EF), increased filling pressures and advanced systolic dysfunction are observed[6,27]. Complications from advanced systolic dysfunction in DCM often lead to the acceleration of heart failure. Previous work from our laboratory has shown that stimulation of β-adrenergic receptor (β-AR) increases ATM expression in the heart and cardiac myocytes[28]. ATM expression was also found to be higher in the infarct left ventricular region of mice 1 and 3 days post-MI[29]. Similar to DCM, ATM deficiency associates with cardiac structural and functional abnormalities. Mice lacking ATM (KO) exhibited decreased LV mass, LV end diastolic diameter (LVEDD), LV end systolic diameter (LVESD), LVEDV, and LV end systolic volume (LVESV) versus wild-type (WT) mice. In addition, ATM KO mice displayed an increased E/A wave ratio and decreased deceleration time for the E-wave[30]. Using heterozygous KO (ATM deficient) mice, it was demonstrated that ATM plays an important role in β-AR-stimulated myocardial remodeling with respect to heart function, apoptosis and fibrosis[28]. ATM deficiency differentially affected myocardial remodeling in response to early (1 and 7 days post-MI) versus late (14 and 28 days post-MI) phase. During early MI phase, MI-mediated decrease in %EF and

percent fractional shortening (%FS) was significantly lower in ATM heterozygous KO mice versus their wild-type (WT) counterparts. Additionally, LVESD and LVEDD were lower in ATM deficient hearts 4 hrs,1,3 and 7 days post-MI[29,31,32]. In contrast, exacerbated LV dysfunction was observed in ATM deficient mice as evidenced by a greater decrease in %EF and FS, and increase in LVESV 14 and 28 days post-MI. Mortality post-MI was also higher in ATM deficient hearts post-MI[33,34].

Increased cardiac fibrosis is a common feature in the diabetic and ATM deficient heart[6,8]. Fibrosis and cardiac remodeling are initial indicators of DCM and lead to the development of cardiac dysfunction and heart failure[3,35]. Cardiac fibrosis is caused by extracellular matrix (ECM) remodeling in response to cardiac damage. While ECM deposition is protective in early cardiac injury, excessive ECM deposition causes myocardial stiffening and cardiac dysfunction[36]. Consequently, myocardial fibrosis is a major cause of heart failure[8,36]. Cardiac interstitial, perivascular and replacement fibrosis has been observed in diabetic patients independent of hypertension and coronary artery disease[37]. Further, DM associates with left and right ventricle interstitial fibrosis with increased type I and III collagen deposition[37]. Additionally, increased fibrosis in the heart has been observed in DM mice and rat models. Of note, severity of cardiac fibrosis may depend on comorbidities, species, age, and sex [37]. ATM deficiency also associates with augmented cardiac fibrosis[8]. ATM deficient mice exhibit increased cardiac fibrosis at basal levels, and following β-AR stimulation[28]. Similarly, ATM deficient mice display increased cardiac fibrosis 3, 7 and 28 days post-MI compared to WT[29,31,34].

Cardiac hypertrophy is another major contributor to the development of heart failure and occurs during ATM deficiency and DM[7,11,27,30,37]. Myocyte hypertrophy occurs to compensate for the increased hemodynamic load caused by the progressive decrease in cardiomyocytes during cardiac injury[8,38]. Similar to fibrosis, hypertrophy is beneficial during early cardiac injury, but progressively leads to cardiac dysfunction and heart failure[27]. Left ventricular hypertrophy is noted during the early stages of DCM[27]. Interestingly, hypertrophy as indicated by an increase in myocyte cross sectional area and increased expression of atrial natriuretic peptide was observed in the myocardium of ATM KO mice [29,30]. ATM deficient mice also exhibit increased myocyte cross-sectional area prior to and 28 days post-MI[34].

Angiogenesis promotes myocyte survival during ischemia through the mitigation of hypoxia. DM associates with two aberrant angiogenic responses. The kidneys and retina display excessive angiogenesis, while decreased angiogenesis occurs in the heart and peripheral limbs[39]. Impaired angiogenesis is suggested to be a major contributor of DM-induced ischemic heart disease (IHD). Cardiomyocytes may exert anti-angiogenic effects in the heart via the exosomal transfer of miR-320 into endothelial cells[40]. ATM deficiency also associates with decreased cardiac angiogenesis post-MI[33]. Since angiogenesis is an important protective mechanism post-MI, ATM deficiency may negatively affect this protective mechanism during development of DCM.

The impairment of insulin metabolic signaling observed in DM and ATM deficiency is known to accelerate the development and progression of heart failure[8,20,27,41]. Insulin signaling promotes the activation of endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) production, which are critical for optimal cardiac function[27]. Consequently, decreased myocardial eNOS activation and NO production is observed during DM, which can lead to cardiac

macro and microvascular disease. This may further promote cardiac fibrosis through the upregulation of collagen cross linking enzymes[27]. Moreover, insulin resistance and ATM deficiency associate with downregulation of the PI3K/Akt signaling in cardiovascular tissues which may result in decreased NO production, a critical vasodilator[6,11,27,42]. Though future studies are needed, the altered PI3K/Akt pathway, insulin resistance and decreased NO bioavailability may also explain why DM and AT patients exhibit vascular complications such as macro-vascular and microvascular disease[5,9,11]. The role of ATM specifically in DCM using ATM deficient mice remains to be investigated. However, changes in structural and functional parameters of the heart, fibrosis, angiogenesis and hypertrophy in ATM deficient hearts post-MI clearly indicate that ATM has the potential to play a critical role in the development of DCM.

Cardiac Energy Metabolism during DM and ATM Deficiency

Myocardial tissue has the highest metabolic requirement in the body. The primary metabolic pathway utilized for myocardial ATP production is oxidative phosphorylation using long chain fatty acids and glucose[43]. Given that fatty acid oxidation accounts for 50-70% of cardiac energy production[3], cardiomyocytes possess multiple proteins for fatty acid transport such as fatty acid transport protein (FATP), fatty acid binding protein (FABP), and cluster of differentiation 36 (CD36) also known as fatty acid translocase (FAT)[3]. Glucose oxidation provides approximately 10% of myocardial energy production[43]. Cardiac basal glucose uptake is provided by glucose transporter 1 (GLUT1), which is constitutively expressed on the sarcolemma membrane[43], whereas the majority of cardiac glucose uptake is facilitated through insulin-dependent glucose transporter 4 (GLUT4), which translocates to the plasma membrane in response to insulin and contraction[11,43]. The natural competition/balance of glucose or free fatty acid substrate

utilization was first described in 1963 and is known as the Randle cycle, where increased blood glucose inhibits free fatty acid oxidation[44]. Therefore, the utilization of fatty acid or glucose is dependent on the substrate availability[45]. Studies suggest that cardiac metabolic flexibility is essential in maintaining adequate ATP synthesis under normal and hypoxic conditions[43]. Unfortunately, insulin resistance, DM and heart failure significantly affect metabolic adaption during starvation and cardiac injury[46].

Hyperglycemia and hyperlipidemia observed in DM increase the storage and use of free fatty acid substrates in the myocardium, thereby inhibiting glucose utilization and decreasing metabolic flexibility[3,46]. DM further associates with impaired metabolic flexibility through attenuation in signaling that regulates the translocation of GLUT4 to the cardiomyocyte cell membrane, impairing glucose uptake, NO production and calcium homeostasis in the DM myocardium [20,35,45]. Moreover, a downregulation of AMP-activated protein kinase (AMPK), a master regulator of energy homeostasis, is noted in DCM resulting in inhibited glucose uptake and upregulation of FAT and FATP1, shifting metabolism towards fatty acid oxidation[4,6]. However, excessive utilization of free fatty acids can lead to an accumulation of toxic byproducts and cause cardiac lipotoxicity[4,45,47] In addition, this rise in fatty acid uptake and oxidation potentially contributes to the mitochondrial dysfunction, increased ROS production and development of DCM[3,43]. This may lead to decreased ATP production, decreased cardiac contractility, altered calcium handling, inflammation and exacerbation of myocyte death[4,45]. Interestingly, an increase in glucose and free fatty acids in the cytosol of myocytes have been observed during heart failure[46]. However, substrate entry into mitochondria is decreased, resulting in gluco- and lipo-toxicity and an increase in ketone substrate utilization which further decreases metabolic flexibility[46] contributing to worsening of DCM.

Although, the role of ATM in the development of DCM needs further investigation, evidence from non-cardiac cells and tissue suggest that ATM deficiency may share several similar pathways to DM when it comes to shifting cardiac energy metabolism. In L6 muscle cells transfected with ATM, insulin caused a dramatic increase in GLUT4 translocation[18]. Increase in cell surface GLUT4 may help shift the balance toward glucose metabolism[48]. Since DM and ATM deficiency associate with decreased AMPK activity, this suggests that AMPK activation may serve as a potential target to mitigate some of the metabolic changes that occur in the diabetic heart. Interestingly, some anti-diabetic drugs such as metformin are shown to decrease hepatic gluconeogenesis, increase insulin sensitivity and stimulate AMPK activation, which in-turn, decreases lipid and cholesterol synthesis[46]. Although the mechanism isn't well understood, metformin treatment also activates AMPK, which reduces insulin resistance and increases glucose uptake in cardiac tissue, skeletal muscle and insulin resistant cardiomyocytes[27,46]. Interestingly, a genome-wide association study identified an association for a SNP (single-nucleotide polymorphism) at a locus containing ATM gene for the treatment success of metformin[13]. Further, inhibition of ATM using KU-55933 inhibited metformin-induced AMPK activation in hepatic cells, suggesting that ATM plays a role in the glucose lowering effects of metformin upstream of AMPK[49]. Collectively, these studies provide a role for ATM in the regulation of glucose homeostasis and metformin response. Of note, ATM heterozygous knockout mice (hKO) with ApoE null mutation (ATM +/-/ApoE-/-) display abnormal lipid metabolism and hypercholesterolemia as compared to the WT control[50,51]. Increased ROS derived from mitochondrial dysfunction may serve as the driving force behind the development of metabolic abnormalities observed during ATM deficiency. Treatment with mitochondrial-targeted antioxidant MitoQ successfully reversed multiple features of metabolic abnormalities detected in

ATM +/-/ApoE-/- mice[52]. Oxidative activation of ATM causes a glucose metabolic shift from glycolysis to the pentose phosphate pathway, thereby causing anti-oxidative response which increases the survival and proliferation of cardiac progenitor cells in diabetic hearts[11,53]. Together, these studies provide evidence that ATM activation may play a protective role in the development of metabolic syndrome associated with DM. However, future investigations are needed to clarify the role of ATM in the development of DCM.

Insulin Resistance during DM and ATM Deficiency

Insulin signaling plays an important role in cardiac cellular homeostasis[4]. During normal cardiac insulin metabolic signaling, insulin binds to the insulin receptor, activating insulin receptor substrates, PI3K and Akt (Fig 2.2). This signaling cascade stimulates the activation of IRS1/2 transcription, inactivates Akt substrate 160 and translocates GLUT4 to the plasma membrane, stimulating glucose uptake and preventing lipolysis[43,45]. Insulin resistance or insufficient insulin production are two primary causes of diabetic hyperglycemia[54]. In DM, insulin resistance results in overproduction and release of insulin by the pancreatic β-cells, which causes hyperinsulinemia. With time, the β-cells become dysfunctional and cannot produce the quantity of insulin necessary to compensate for the insulin resistance[3,45], further complicating therapy. As a result, a decrease of insulin-mediated glucose uptake and increased free fatty acid oxidation is observed (Fig 2.2) [3,45].

ATM is established as an insulin responsive protein that plays a significant role in the glucose metabolic pathway[9,18]. Metabolic abnormalities such as hyperglycemia, insulin resistance and diminished/delayed insulin secretion have been observed in AT patients and ATM deficient mouse model[9,41,55]. Altered mitochondrial function and associated oxidative stress observed in

cardiomyocytes during ATM deficiency are implicated (but not fully elucidated) in the development of metabolic dysfunction and insulin resistance[7,9,11].

Alteration in PI3K/Akt signaling is a major contributor to the development of insulin resistance and type 2 DM. Akt stimulates the transcription and translocation of GLUT4, which mediates the majority of cardiac glucose transport. Decreased PI3K/Akt signaling and associated decrease in GLUT4 expression has been observed in diabetic hearts and is linked to the acceleration of heart failure[4,6]. ATM KO hearts exhibit lower Akt activation at basal levels and in response to β-AR stimulation. Akt activation was also lower in the myocardium of ATM deficient mice 4hrs post-MI[30,32]. Therefore, the attenuation of PI3K/Akt signaling during ATM deficiency may contribute to the decrease in GLUT4 translocation, glucose uptake and insulin resistance observed in DM[6,18,20]. While ATM plays a large role in glucose metabolism, the effect of ATM deficiency on GLUT1 expression are still being elucidated. In skeletal muscle, ATM deficiency upregulated GLUT1 transcription with resultant increase in basal glucose uptake and insulin resistance[56]. Conversely, inhibition of ATM decreased cell surface GLUT1 with concomitant reduction of glucose transport in L6 myoblasts[57].

Increased JNK activation may also play a role in the development of insulin resistance by directly phosphorylating IRS 1 and 2, and reducing Akt activation and GLUT4 translocation[6,58]. JNK activation induces rat myocyte apoptosis, and is suggested to promote the development of the cardiovascular abnormalities seen in DM and DCM[6,58]. Enhanced JNK activation is also common during ATM deficiency, and is known to cause insulin resistance through the disruption of insulin signaling[11,59–61]. Chloroquine (an ATM activator) reduced metabolic abnormalities,

JNK activation and the development of atherosclerosis while improving glucose tolerance in ATM +/-/ApoE-/- mice[60].

Activation of mitogen activated protein kinases (MAPK) pathway is suggested to contribute to cardiac hypertrophy, fibrosis, functional alterations, cardiomyocyte insulin resistance, acceleration of DCM and heart failure[6,62,63]. Extracellular signal-regulated kinase 1 and 2 (ERK1/2) belong to the MAPK family and play a role in transmitting extracellular signals to the intracellular molecular targets. ERK1/2 signaling contributes to cell survival, growth, differentiation, and proliferation [64,65]. Activation of ERK1/2 has been observed in response to hyperglycemia-induced stress and insulin signaling[64,65]. Interestingly, selective inhibition of ERK pathway is suggested to ameliorate insulin resistance [64] and may be a potential therapeutic target for DM. Lack of ATM associates with decreased ERK1/2 activation in neurosphere cells [66]. Mouse embryonic fibroblasts exhibit decreased ERK1/2 activation and defective differentiation into adipocytes[67]. DNA damage is suggested to activate ERK1/2 in an ATM-dependent manner in fibroblasts[68]. ERK1/2 activation was higher in the myocardium of WT mice 4 hr post-MI. However, ERK1/2 activation remained unchanged in mice with ATM deficiency[32]. Future investigations are warranted to investigate the role of ERK1/2 signaling in the development of insulin resistance and DM during ATM deficiency.

Conclusion

ATM is a versatile kinase involved in regulation of cell cycle progression, coordination of DNA damage response, vesicle/protein transport mechanisms, glucose metabolism, oxidative stress and mitochondrial function[7,8]. Consequently, ATM deficient individuals have an enhanced susceptibility to developing metabolic disorders, IHD and cancer[8]. Additionally, disruption of

cytoplasmic signaling initiated by ATM contributes to metabolic complications, insulin resistance, DM, heart disease and heart failure[8,11]. Cardiovascular alterations observed during ATM deficiency further demonstrate the importance and versatility of ATM. ATM deficiency associates with increased cardiac fibrosis, hypertrophy, and an array of structural and functional abnormalities[8,29,31,34]. Further, ATM deficiency and DM independently associate with insulin resistance, metabolic impairment, hyperglycemia, hyperinsulinemia, dyslipidemia, hypertension, increased atherosclerosis, and cardiac structural and functional abnormalities (Fig 2.1). Additionally, DM and ATM deficiency exhibit alterations in metabolic associated proteins, including decreased AMPK, PI3K/Akt and ERK1/2 activation, and increased oxidative stress and JNK activation (Fig 2.2). Taken together, this information suggests that activation of ATM and ATM-dependent mechanisms may serve as potential therapeutic targets to protect against the metabolically-induced cardiovascular dysfunction observed during DM, and potentially reduce the development of heart failure. This is consistent with the observation of decreased ATM activation in muscle cells during insulin resistance[18]. Since DM and ATM deficiency share many metabolic and cardiac abnormalities (Fig.2.1), it can be speculated that DM may cause a decrease in the expression of ATM, disturbing ATM-dependent metabolic mechanisms and causing cardiac structural and functional abnormalities leading to DCM and heart failure. Therefore, future studies are crucial to the understanding of the role of ATM and other signaling pathways involved in DM-mediated development and progression of DCM, and to the development of therapeutic approaches to decrease the risk of heart failure in diabetic patients.

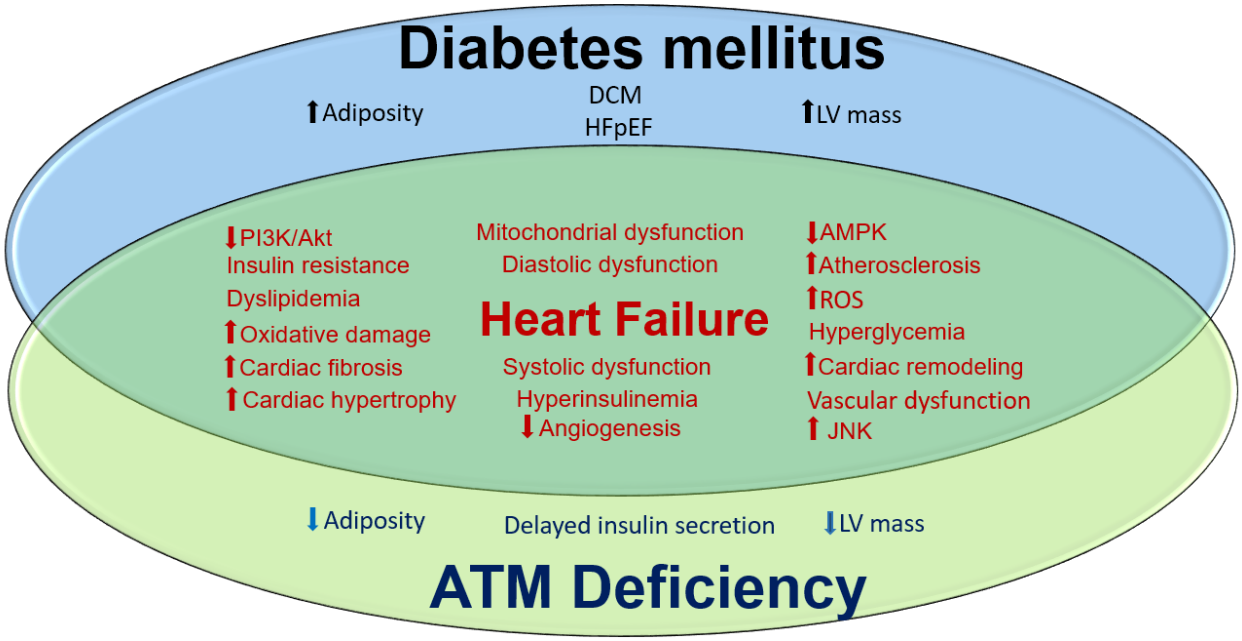

Figure 2.1. Shared metabolic and cardiovascular abnormalities of diabetes mellitus (DM) and Ataxia telangiectasia mutated kinase (ATM) deficiency.

Alterations of AMPK, PI3K/Akt, and JNK independently associate with DM and ATM deficiency, and have been implicated in the inhibited glucose uptake and insulin resistance observed in both conditions. Further, aberrant metabolic conditions such as dyslipidemia, hyperinsulinemia, hyperglycemia and insulin resistance are features of DM and ATM deficiency, and contribute to vascular dysfunction. Mitochondrial dysfunction, increased ROS production and associated oxidative damage are also present in DM and during ATM deficiency. Lastly, DM and ATM deficiency independently associate with cardiac structural and functional abnormalities including increased cardiac fibrosis, hypertrophy, systolic and diastolic dysfunction. Collectively, all of the aforementioned conditions contributes to the development and progression of diabetic cardiomyopathy and heart failure.

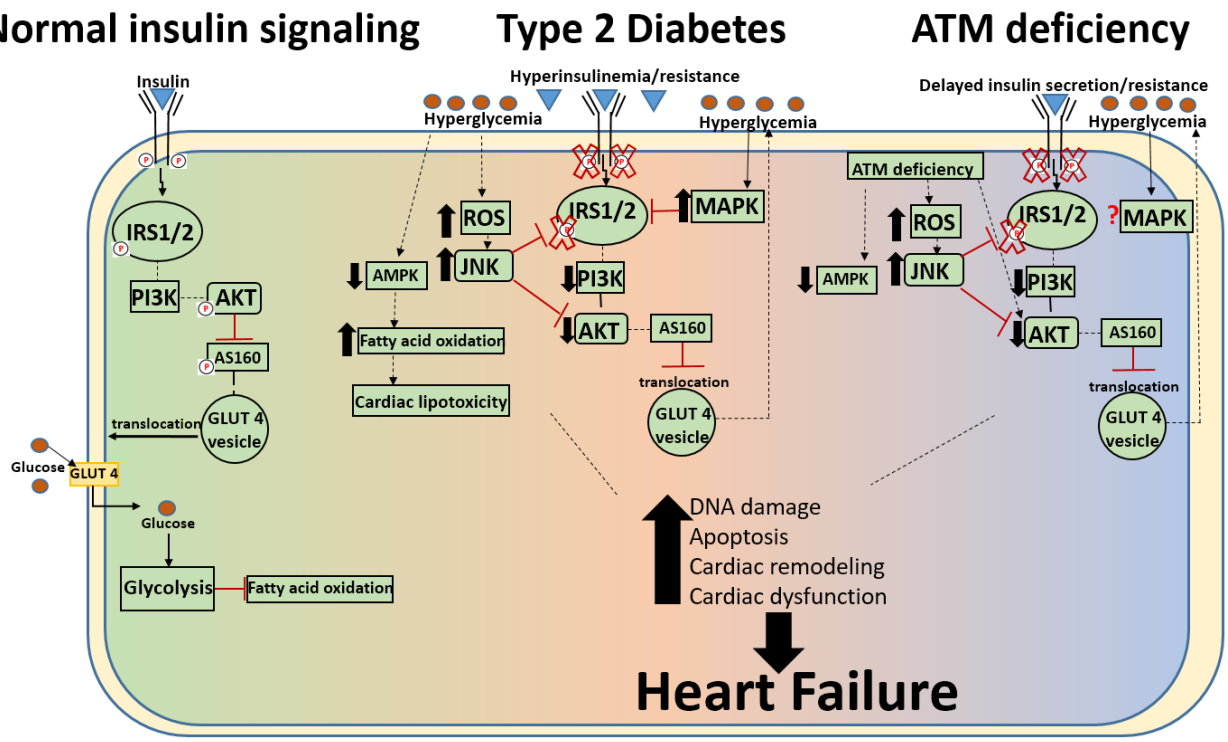

Figure 2.2. Schematic depiction of insulin signaling during Diabetes and ATM deficiency.

Left: Insulin responsive signaling: The intracellular signaling cascade begins with the binding of insulin to the insulin receptor which activates IRS1/2 (insulin receptor substrate proteins 1 and 2) and downstream targets such as Akt. Active Akt may phosphorylate Akt substrate 160 (AS160), and stimulate GLUT4 translocation to the plasma membrane and allowing glucose uptake. *Center:* Type 2 diabetic insulin signaling: Overproduction/release of insulin causes hyperinsulinemia. As insulin resistance increases, decreased insulin-mediated glucose uptake occurs resulting in decreased activation of IRS1/2 and Akt, phosphorylation of AS160, GLUT 4 translocation and glucose uptake. Persistent hyperglycemia then causes an increase in reactive oxygen species (ROS), and may activate JNK and decrease AMPK activation. These changes may shift the metabolism toward fatty acid oxidation and cardiac lipotoxicity. Together, these alterations can cause DNA damage, apoptosis, cardiac remodeling and cardiac dysfunction, leading to heart failure. *Right:* Insulin resistance during ATM deficiency: Delayed insulin secretion and insulin resistance is observed during ATM deficiency with decreased activation of IRS1/2 and Akt. This may decrease AS160 phosphorylation and glucose uptake. ATM deficiency independently associates with mechanisms suggested to cause insulin resistance such as decreased AMPK activation, increased JNK activation and higher oxidative stress. ATM deficiency also associates with increased DNA damage, apoptosis, cardiac remodeling and cardiac dysfunction.

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CHAPTER 3. DEFICIENCY OF ATAXIA-TELANGIECTASIA MUTATED KINASE MODULATES FUNCTIONAL AND BIOCHEMICAL PARAMETERS OF THE HEART IN RESPONSE TO WESTERN-TYPE DIET

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Abstract

Ataxia telangiectasia mutated kinase (ATM) deficiency exacerbates heart dysfunction late after myocardial infarction. Here, we hypothesized that ATM deficiency modulates Western-type diet (WD)-induced cardiac remodeling with an emphasis on functional and biochemical parameters of the heart. Weight gain was assessed in male wild-type (WT) and ATM heterozygous knockout (hKO) mice on weekly basis, while cardiac functional and biochemical parameters were measured 14 weeks post-WD. hKO-WD mice exhibited rapid body weight gain at weeks 5, 6, 7, 8 and 10 vs WT-WD. WD decreased percent fractional shortening and ejection fraction, and increased end systolic volumes and diameters to a similar extent in both genotypes. However, WD decreased stroke volume, cardiac output, peak velocity of early ventricular filling and aortic ejection time, and increased isovolumetric relaxation time (IVRT) and Tei index in vs WT-NC (normal chow). Conversely, IVRT, isovolumetric contraction time and Tei index were lower in hKO-WD vs hKO-NC and WT-WD. Myocyte apoptosis and hypertrophy were higher in hKO-WD vs WT-WD. WD increased fibrosis and expression of Collagen-1α1, MMP-2, and MMP-9 in WT. WD enhanced AMPK activation, while decreasing mTOR activation in Hko. Akt and IKK-α/β activation, and Bax, PARP-1 and Glut-4 expression were higher in WT-WD vs WT-NC, while NFκB activation and Glut-4 expression were lower in hKO-WD vs hKO-NC. Circulating concentrations of IL-12(p70), eotaxin, IFN-γ, MIP-1α and MIP-1β were higher in hKO-WD vs WT-WD. Thus, ATM deficiency accelerates weight gain, induces systolic dysfunction with increased preload, and associates with increased apoptosis, hypertrophy and inflammation in response to WD.

New & Noteworthy

Ataxia telangiectasia mutated kinase (ATM) deficiency in humans associates with enhanced susceptibility to ischemic heart disease. Here, we provide evidence that ATM deficiency accelerates body weight gain, and associates with increased cardiac preload, hypertrophy and apoptosis in mice fed with Western-type diet (WD). Further investigations of the role of ATM deficiency in WD-induced alterations in function and biochemical parameters of the heart may provide clinically applicable information on treatment and/or nutritional counseling for patients with ATM deficiency.

Introduction

Chronic consumption of Western-type diet (WD) induces inflammation, oxidative stress, and mitochondrial dysfunction in skeletal as well as cardiac muscle (39, 40, 42). WD associates with hemodynamic and structural alterations of the heart leading to a clinical syndrome called obesity cardiomyopathy (2, 6). As metabolic demand increases with continually expanding adipose tissue, hyperdynamic circulation with increased blood volume and hemodynamic overload occurs (18, 32). Preload and afterload cardiac dysfunction can occur due to continual adaptive compensation for changes in blood volume (55, 74). In animal models, left ventricular (LV) hypertrophy, increased arrhythmic events, pump failure, atherosclerosis, biventricular stiffness and increased fibrosis have been noted with diet-induced obesity (1, 6, 9, 24).

Ataxia telangiectasia mutated kinase (ATM) is generally activated in response to double-strand DNA (dsDNA) breaks, oxidative damage, and other genotoxic stressors (8, 26, 68, 79). While the main function of ATM is to maintain genomic stability through redox sensing, coordination of DNA damage repair, and facilitation of cell cycle progression in response to dsDNA breaks, ATM is also suggested to play a pivotal role in metabolism, vesicle transport and mitochondrial function (8, 26, 68, 79). Disruption of the ATM gene results in a complex multisystem disorder called ataxia-telangiectasia (A-T) which associates with neurological, immunological, endocrinological and cardiovascular abnormalities (48, 53, 60, 68). Individuals with an ATM mutation in one allele (A-T carriers), constitute 1.4-2% of the general population, exhibit enhanced susceptibility to cancer and ischemic heart disease (79). Previously, we provided evidence that ATM deficiency in mice associates with increased cardiac fibrosis, myocyte hypertrophy, and exacerbation of heart dysfunction 28 days following myocardial infarction (MI) (16). However, there are no reports investigating the role of ATM deficiency in WD-induced

cardiac remodeling. Here, we hypothesized that ATM deficiency modulates WD-induced cardiac remodeling with a focus on functional and biochemical parameters of the heart. The data presented here suggest that ATM deficiency associates with rapid weight gain, systolic dysfunction with increased preload, and exacerbated cardiac remodeling in terms of hypertrophy and apoptosis in response to WD. It also affects WD-induced circulating levels of cytokines/chemokines, and expression/activation of protein associated with fibrosis, metabolism and inflammation.

Materials and Methods

Vertebrate animals and diets.

This study conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). East Tennessee State University Committee on Animal Care approved all protocols utilized in this study. Breeding pairs of ATM deficient mice (129S6/SvEvTac) were purchased from Jackson Laboratory (stock #002753). The homozygous KO mice are infertile and die ~2 months of age. Therefore, ATM heterozygous knockout (hKO; deficient) mice were used for breeding providing us with littermate WT and hKO mice. Primers suggested by the Jackson Laboratory were used to genotype the mice using PCR. Six-week-old male WT and hKO mice were placed on a normal chow (NC; Envigo 8604) or Western-type diet (WD; Envigo TD 88137) for 14 weeks. The energy composition of NC is - 32% kcal protein, 14% kcal fat, 54% kcal carbohydrate and 4% sugar (by weight). The energy composition of WD is - 15.2% kcal protein, 42.0% kcal fat, 42.7% kcal carbohydrate and 34% sugar (by weight). All mice were kept on a 12 hour-dark/light cycle with food and water available *ad libitum*.

Fasting glucose levels.

NC and WD mice from both genotypes were fasted for 12 hours. The tail was then gently nicked using a 5mm Goldenrod lancet. Approximately 1µl of blood sample was collected from the tail vein and blood glucose was measured using a ReliOn monitoring system.

Echocardiography.

Structural and functional parameters of the heart were measured 14 weeks post-WD using a Vevo 1100 imaging system (VisualSonics, Fujifilm) equipped with a 22- to 55-MHz MS550D transducer (14, 59). For this, mice were anesthetized using a mixture of isoflurane (2%) and oxygen (0.6 L/min). Heating pad was used to maintain body temperature ~37˚C. M-mode recordings, obtained using transthoracic short axis view at mid-papillary level, were used to measure/calculate heart rate, % ejection fraction (%EF), % fractional shortening (%FS), LV end systolic diameter (LVESD), LV end diastolic diameter (LVEDD), LV end systolic volume (LVESV), LV end diastolic volume (LVEDV), stroke volume and cardiac output. Doppler tracings, acquired from apical four-chamber view, were used to measure peak velocity of early ventricular filling (E wave), aortic ejection time (AET), isovolumetric relaxation time (IVRT; measured from the aortic valve closure to the mitral valve opening) and isovolumetric contraction time (IVCT; measured from the closing of the mitral valve to the opening of the aortic valve) (59). Tei index, also known as myocardial performance index, was calculated as: IVRT+IVCT/AET.

Morphometric analysis.

Mice were weighed on a weekly basis until 14-week diet completion. Mice were anesthetized using a mixture of isoflurane (2%) and oxygen (0.6 L/min). The heart was excised through an opening of the diaphragm region. The heart was perfused with Krebs-Henseleit buffer to ensure blood clearance, arrested in diastole using 16 mM KCl and weighed. Epidermal skin layer was removed post-mortem, subcutaneous and visceral fat were collected and weighed. Tibia length was measured using Vernier calipers (Monostat). The heart was then divided into two transverse

sections (base/mid and apex) and embedded in paraffin. Mid-cardiac transverse sections (5μm thick) were stained with Masson's trichrome staining to measure fibrosis and septal wall hypertrophy. For fibrosis, ten separate septal images from each heart were analyzed using Nikon NIS software as previously described (16). Percent fibrosis was calculated by dividing the total fibrosis area by the total tissue area of each image and multiplying by 100. To measure septal wall hypertrophy, six separate transverse measurements were averaged from each heart. To examine lipid deposition, cryosections (10 μm thick) of the heart were stained with Oil red O. Ten separate septal images from each heart were analyzed using Nikon NIS software as described (16).

Western blot analysis.

Cardiac lysates were prepared in RIPA buffer [10 mM Tris·HCl (pH 7.2), 158 mM NaCl, 1 mM EGTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mM sodium orthovanadate, and 0.2 mM phenylmethylsulfonyl fluoride] supplemented with Halt protease inhibitor cocktail. Equal amounts of proteins (50 μg) were resolved by SDS-PAGE and transferred to PVDF membranes. All membranes were blocked with 5% nonfat dry milk and incubated overnight with primary antibodies against ATM (1:500, Cat# sc23921, Santa Cruz), MMP-9 (1:1000, Cat# AB19016, Millipore), MMP-2 (1:500, Cat# MAB3308, Millipore), BAX (1:1000, Cat# SC7480, Santa Cruz), PARP-1 (1:1000, Cat# 9542, Cell Signaling), p-Akt (ser473; 1:1000, Cat# 9271S, Cell Signaling), total Akt (1:1000, Cat# 9272S, Cell Signaling), p-NFκB (ser-536; 1:1000, Cat# 3033S, Cell Signaling), total NFκB (1:1000, Cat# 6956S, Cell Signaling), p-IKK α/β (ser180/Ser181; 1:500, Cat# 2681S, Cell Signaling), total IKK-α (1:1000, Cat# 61294S, Cell Signaling), Glut4 (1:500, Cat# sc7938, Santa Cruz), p-mTOR (ser-2448; 1:1000, Cat# 5536S, Cell Signaling), total mTOR (1:1000, Cat# 2983S, Cell Signaling), p-AMPK (thr-172; 1:1000, Cat# 2535S, Cell Signaling),

total AMPK (1:1000, Cat# 5832S, Cell Signaling) and Collagen-1α1 (1:500, Cat#72026, Cell Signaling). The immune complexes were detected using appropriate secondary antibodies and chemiluminescent reagents. There was no significant difference in GAPDH levels among the groups, therefore protein loading in each lane was normalized using GAPDH immunostaining (1:10,000; Cat# 32233, Santa Cruz). Band intensities were quantified using ImageQuant LAS 500 imaging system (GE Healthcare) (69). For all phosphoproteins, total protein expression was normalized using GAPDH immunostaining. This analysis showed no significant difference in the expression of total proteins (Figs 9 and 10). Therefore, phosphoprotein band intensities were normalized using GAPDH immunostaining. Western blot analysis for ATM was performed using 6% SDS-PAGE and Ponceau S staining was used to indicate protein loading in each lane.

Terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay.

Tunnel assay was performed according to the manufacturer's instructions (In Situ Cell Death Detection Kit, Roche) (15). Tissue sections (5μm thick) were also stained with rhodamine-conjugated wheat germ agglutinin (WGA, Rl-1022, Vector) to visualize myocytes and Hoechst 33258 (10 μM; Sigma) to visualize nuclei. Hoechst-positive stained nuclei served as an index to count the total number of nuclei. Apoptotic myocytes were identified by TUNEL-positive staining clearly seen within WGA-stained cells and Hoechst-positively stained nuclei. The index of myocyte apoptosis was calculated as the percentage myocyte apoptotic nuclei/total nuclei. Total cardiac cells apoptosis was measured by counting the TUNEL-positive and Hoechst-positive nuclei. The index of cardiac cell apoptosis was calculated as the percentage of total apoptotic nuclei/total nuclei.

Myocyte cross-sectional area.

WGA-stained cross-sections of the heart were used to measure myocyte cross-sectional area. For this, suitable myocytes utilized for analysis included myocytes with a circular border with centrally localized nuclei. Images were obtained with the EVOS M7000 imaging system and myocyte cross-sectional area was quantified with Nikon NIS software as previously described (16).

Cholesterol and triglyceride assay.

Total cholesterol and triglyceride levels from non-hemolyzed serum were measured according to manufacturer's instructions (Pointe Scientific). Sample to reagent ratio was diluted to 1:1000 and serum standards provided by the manufacturer were used to verify the validity of reaction. Samples were read at 500nm wavelength using a BioTek PowerWave XS2 microplate spectrophotometer. Cholesterol and triglyceride levels were determined as: absorbance (sample)/absorbance (standard) multiplied by the concentration of standard.

Serum cytokine and chemokine assay.

The Bio-Plex Pro Mouse Cytokine 23-Plex assay was used to measure circulating cytokines and chemokines concentrations according to the manufacturer's instructions (Bio-Rad). The following cytokines/chemokines were assessed: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 subunit p40 [IL-12(P40)], IL-12(p70), IL-13, IL-17, eotaxin, granulocyte colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, keratinocyte chemoattractant (KC), monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α,

macrophage inflammatory protein (MIP)-1β, regulated upon activation, normal T cell expressed, and secreted (RANTES) and TNF-α.

Statistical analysis.

Data are expressed as means ± SE. Data were analyzed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test or 2-tailed Student's t-test. Probability (p) values of <0.05 were considered to be significant.

Results

ATM Expression.

ATM expression increases in response to many different stimuli including oxidative stress, β-adrenergic receptor (β-AR) stimulation and MI (20, 68). Western blot analysis of heart lysates using anti-ATM antibodies showed that ATM protein levels (expression) are ~50% lower in hKO-NC group vs WT-NC. WD significantly increased ATM expression (>2.0-fold) in WT group vs WT-NC. However, no increase in ATM expression was observed in hKO-WD group, and ATM expression remained significantly lower in hKO-WD vs WT-WD (Fig 3.1).

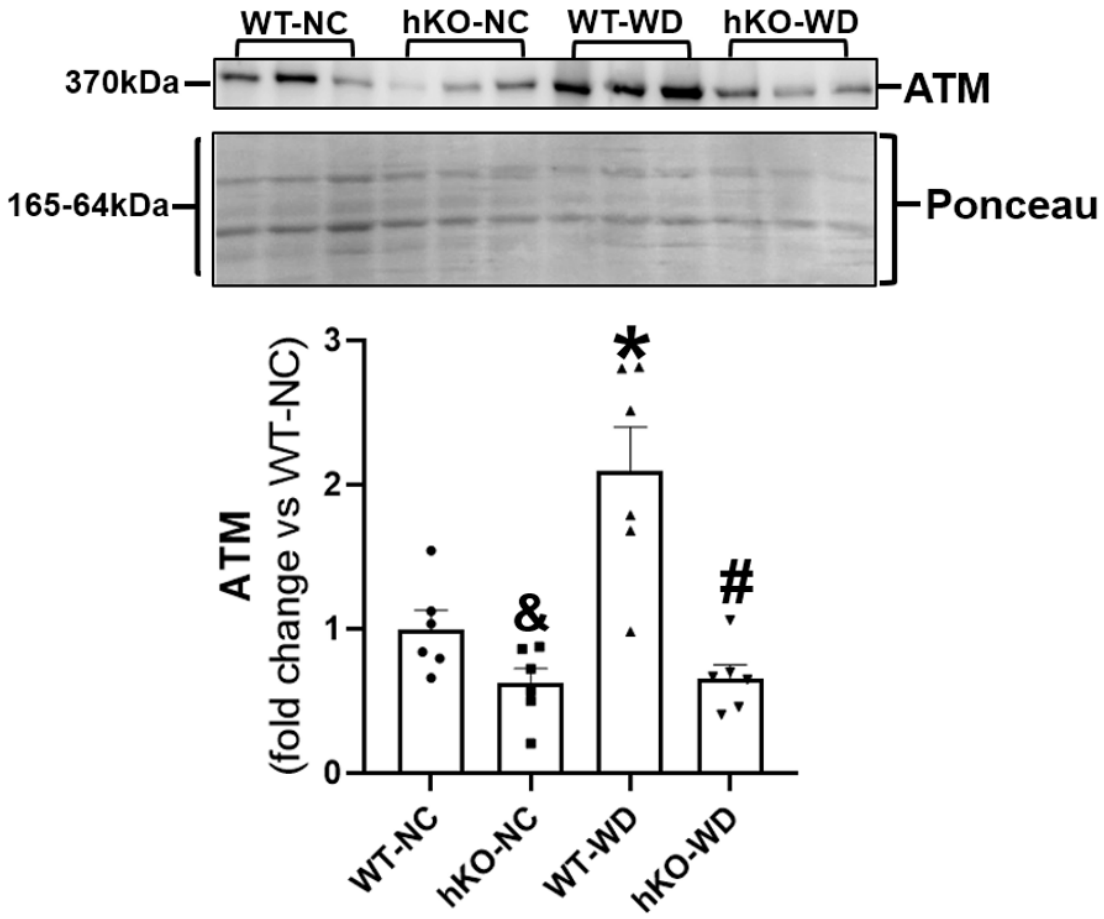


Figure 3.1 WD Increases ATM Expression in the Myocardium of WT Mice.

Total heart lysates were analyzed by western blot using anti-ATM antibodies. Ponceau-S staining indicates protein loading in each lane. $^{\&}p<0.05$ vs WT-NC, *$p<0.05$ vs WT-NC, $^{\#}p<0.05$ vs WT-WD, n=6.

Morphometric analyses.

Adiposity is commonly observed in mice fed with high fat diets (1). To assess differences in diet-induced weight gain, mice were weighed on weekly basis. The starting body weights (week 0) of six-weeks old mice were not significantly different between the two genotypes (WT, 21.2±0.6g; hKO, 20.1±0.7g; p=NS; n=6-8). Weekly measurement of weight gain vs week 0 showed a steady increase in weight gain in all 4 groups. However, weight gain was significantly greater in the WD groups vs their respective NC starting week 3 which continued until week 14 (Fig 3.2).

Interestingly, hKO-WD group exhibited rapid weight gain and weight gain was significantly greater in hKO-WD at weeks 5, 6, 7, 8 and 10 vs WT-WD. Although weight gain increased steadily from weeks 11-14 in both WD groups, there was no significant difference between the two WD groups. At weeks 12-14, there was no significant increase in weight gain in hKO-WD vs week 10 (p=NS; n=8).

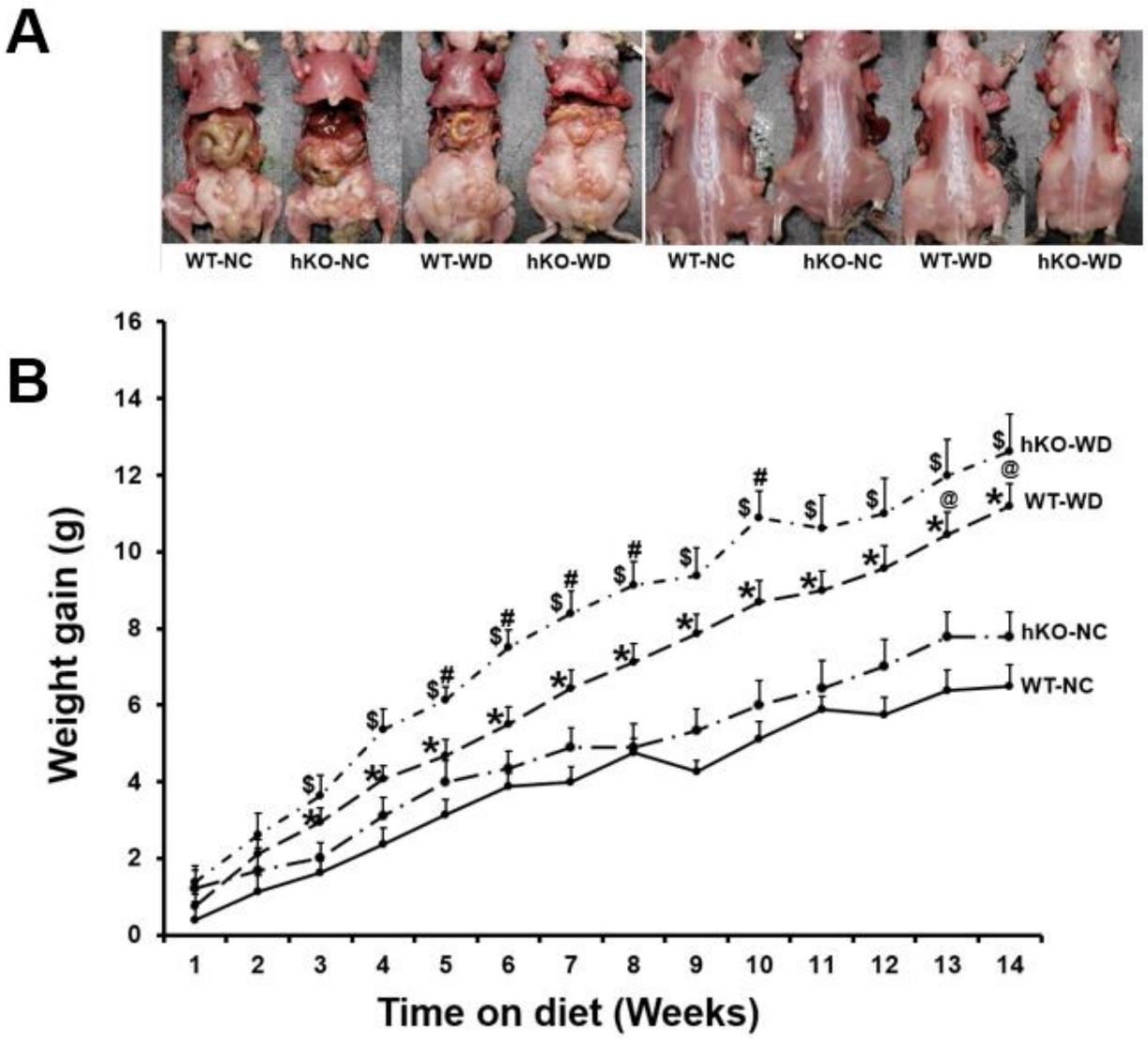


Figure 3.2 WD Induced Weight Gain with Time.

A. Visceral (left panel) and subcutaneous (right panel) adipose distribution after 14 weeks on NC or WD. **B.** Weight gain for normal chow (NC) and Western-type diet (WD) groups from week 0

to week 14. $^{\$}p<0.05$ vs hKO-NC, $^{*}p<0.05$ vs WT-NC, $^{\#}p<0.05$ vs WT-WD, $^{@}p<0.05$ vs week 10, n=8-16.

On the other hand, weight gain was significantly greater at weeks 13 and 14 vs week 10 in WT-WD group ($p<0.05$; n=16). At week 14, the increase in body weight, subcutaneous fat weight and visceral fat weight were not significantly different between the two NC or WD groups (Table 1). Heart weights remained unchanged among the four groups. Both WD groups exhibited significant decrease in heart weight to body weight (HW/BW) ratio vs their respective NC groups. However, no difference in HW/BW ratio was observed between the two WD groups. Heart weight to tibia length ratio is commonly used as a measure of hypertrophy (80). This ratio was found to be slightly, but significantly, higher in hKO-NC vs WT-NC. However, the ratio remained unchanged between the two WD groups and was not significantly different vs their NC counterparts. Total cholesterol levels were significantly increased in the two WD groups vs their respective NC groups with no significant difference between the two WD groups. Triglyceride levels trended higher in WD vs NC groups, however, the differences did not reach statistical significance. Oil red-O-staining showed no discernable deposition of lipids in the heart 14 weeks post-WD (data not shown). Fasting blood glucose tended to be higher WT-WD vs WT-NC ($p=0.05$). However, it was significantly higher in WT-WD group vs hKO-WD (mg/dL; WT-NC, 108.6±7.1; hKO-NC, 90.1±3.5; WT-WD, 136.4±10.5; hKO-WD, 97.9±5.98$^{\#}$; $^{\#}p<0.05$ vs WT-WD; n=7-10; Table 3.1).

Table 3.1 Morphometric and Biochemical Measurements

Parameters	WT-NC	hKO-NC	WT-WD	hKO-WD
Body weight (g)	27.1 ± 1.3	27.7 ± 0.7	31.4 ± 1.3*	31.9 ± 1.2$
Weight gain (g)	7.6 ± 1.0	7.2 ± 0.6	11.1 ± 0.9*	10.7 ± 0.9$
Heart weight (mg)	146 ± 3	152 ± 3	142 ± 5	141 ± 4
Heart weight/body weight (mg/g)	5.6 ± 0.2	5.5 ± 0.1	4.5 ± 0.2*	4.4 ± 0.2$
Heart weight/tibia length (mg/mm)	10.5 ± 0.2	11.3 ± 0.2&	10.3 ± 0.3	10.6 ± 0.3
Subcutaneous fat (mg)	550 ± 70	530 ± 60	1163 ± 120*	1190 ± 200$
Abdominal fat (mg)	840 ± 100	880 ± 90	2160 ± 170*	2390 ± 210$
Total cholesterol (mg/dL)	81.9 ± 8.7	66.9 ± 2.6	148.2 ± 3.9*	158.3 ± 5.2$
Triglycerides (mg/dL)	44.5 ± 6.5	54.8 ± 6.2	59.9 ± 10.4	68.1 ± 10.2
Fasting Glucose (mg/dL)	108.6 ± 7.1	90.1 ± 3.5	136.4 ± 10.5	97.9 ± 6.0#

Values are means ± SE, n=7-10. *p<0.05 vs WT-NC; &p<0.05 vs WT-NC ; $p<0.05 vs hKO-NC; #p<0.05 vs WT-WD.

Echocardiographic Measurements.

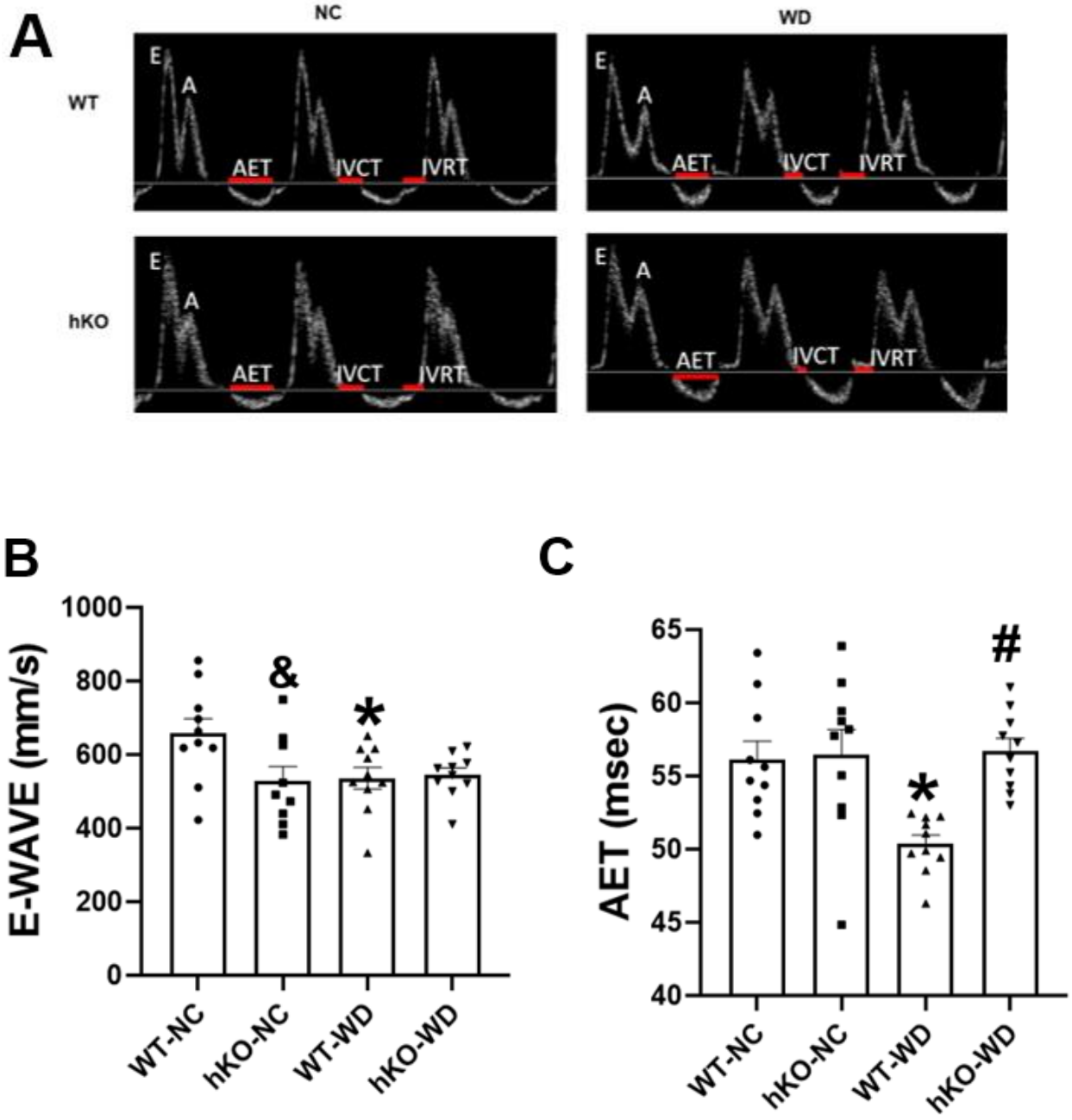
M-mode echocardiography revealed no difference in the parameters between the two NC groups. WD decreased %FS and %EF, and increased LVESD and LVESV to a similar extent in both genotypes vs their NC counterparts. Heart rate, LVEDD and LVEDV remained unchanged among the four groups. Interestingly, a significant decrease in stroke volume and cardiac output was observed in WT-WD vs WT-NC (Table 3.2).

Table 3.2 M-Mode Echocardiographic Parameters

Parameters	WT-NC	hKO-NC	WT-WD	hKO-WD
Heart Rate (BPM)	367 ± 9	344 ± 10	352 ± 14	370 ± 12
%EF	63.71 ± 1.77	66.18 ± 2.14	52.81 ± 1.44*	52.26 ± 1.53$
%FS	34.19 ± 1.39	36.13 ± 1.68	26.69 ± 0.90*	26.40 ± 0.95$
LVESD (mm)	2.47 ± 0.06	2.41 ± 0.11	2.77 ± 0.08*	2.84 ± 0.08$
LVEDD (mm)	3.76 ± 0.05	3.77 ± 0.11	3.78 ± 0.09	3.85 ± 0.10
LVESV (µL)	22.05 ± 1.28	21.19 ± 2.4	29.44 ± 2.19*	31.00 ± 2.14$
LVEDV (µL)	60.85 ± 1.94	61.78 ± 4.70	62.13 ± 3.66	64.97 ± 4.04
Stroke Volume (µL)	38.79 ± 1.67	40.59 ± 2.84	32.69 ± 1.95*	33.97 ± 2.35
Cardiac output (mL/min)	14.20 ± 0.55	13.81 ± 0.84	11.58 ± 0.89*	12.47 ± 0.78

Values are means ± SE, n=10. *p<0.05 vs WT-NC; $p<0.05 vs hKO-NC. BPM, beats per minute.

Pulsed wave doppler analysis revealed that E wave is significantly lower in hKO-NC vs WT-NC. WD led to a significant decrease in E wave in WT-WD, not in hKO-WD group (mm/sec; WT-NC, 656.8±41.1; hKO-NC, 526.9±40.8&; WT-WD, 536.1±29.5*; hKO-WD, 544.4±19.0; &p<0.05 vs WT-NC; *p<0.05 vs WT-NC; n=9-10; Fig 3.3 A and B). AET was significantly lower in WT-WD vs WT-NC. WD had no effect on AET in hKO group, and AET was significantly lower in WT-WD vs hKO-WD (Fig 3.3 C). IVCT and IVRT were not different between the two NC groups. However, IVCT was significantly lower in hKO-WD vs hKO-NC and WT-WD groups (Fig 3.3 D). IVRT was significantly increased in WT-WD vs WT-NC. Conversely, IVRT was significantly lower in hKO-WD vs hKO-NC, and was found to be significantly lower in hKO-WD vs WT-WD (Fig 3.3 E). Tei index (an indicator of global cardiac function) was significantly higher in WT-WD vs WT-NC, while Tei index was significantly lower in hKO-WD vs hKO-NC, and was found to be significantly lower in hKO-WD vs WT-WD (Fig 3.3 F).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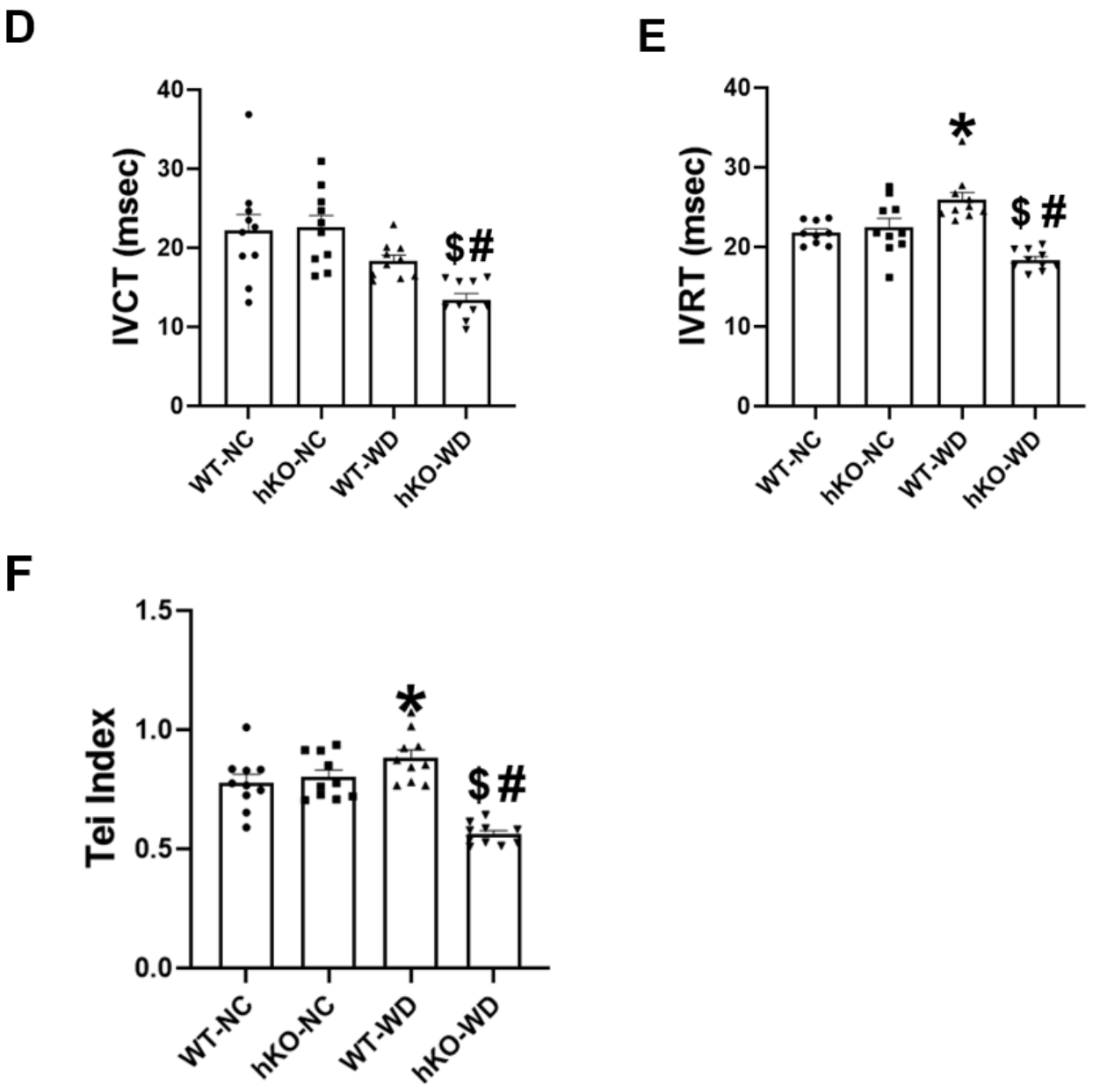


Figure 3.3 WD-Induced Changes in Doppler Flow Parameters of the Heart.

Indices of doppler flow parameters: E-wave, aortic ejection time (AET), isovolumetric contraction time (IVCT), isovolumetric relaxation time (IVRT), Tei index were measured/calculated using pulsed wave doppler echocardiographic images after 14 weeks on normal chow (NC) or western-type diet (WD). (A) Representative doppler tracings for each group; (B) E-wave; (C) AET; (D) IVCT; (E) IVRT; (F) Tei index. $^{\&}p<0.05$ vs WT-NC, $^{*}p<0.05$ vs WT-NC, $^{\$}p<0.05$ vs hKO-NC, $^{\#}p<0.05$ vs WT-WD, n=9-10.

Fibrosis, apoptosis and hypertrophy.

Increased cardiac fibrosis, myocyte apoptosis and myocardial dysfunction are often observed with high fat diets (1, 6, 36, 67). Percent fibrosis was significantly higher in hKO-NC vs WT-NC. WD significantly increased percent fibrosis in WT, not in hKO group (Fig 3.4 A and B). Myocyte and total cell apoptosis was significantly higher in hKO-NC vs WT-NC. WD increased myocyte apoptosis in both genotypes. However, the increase in myocyte and total cell apoptosis was significantly greater in hKO-WD vs WT-WD (Fig 3.5 A-C). Cardiac hypertrophy as measured by increased myocyte cross-sectional area and septal wall width was higher in hKO-NC vs WT-NC. Myocyte cross-sectional area and septal wall width were significantly increased in both WD groups vs their respective NC groups. However, myocyte cross-sectional area and septal wall width were significantly greater in hKO-WD vs WT-WD (Fig 3.6 A-C).

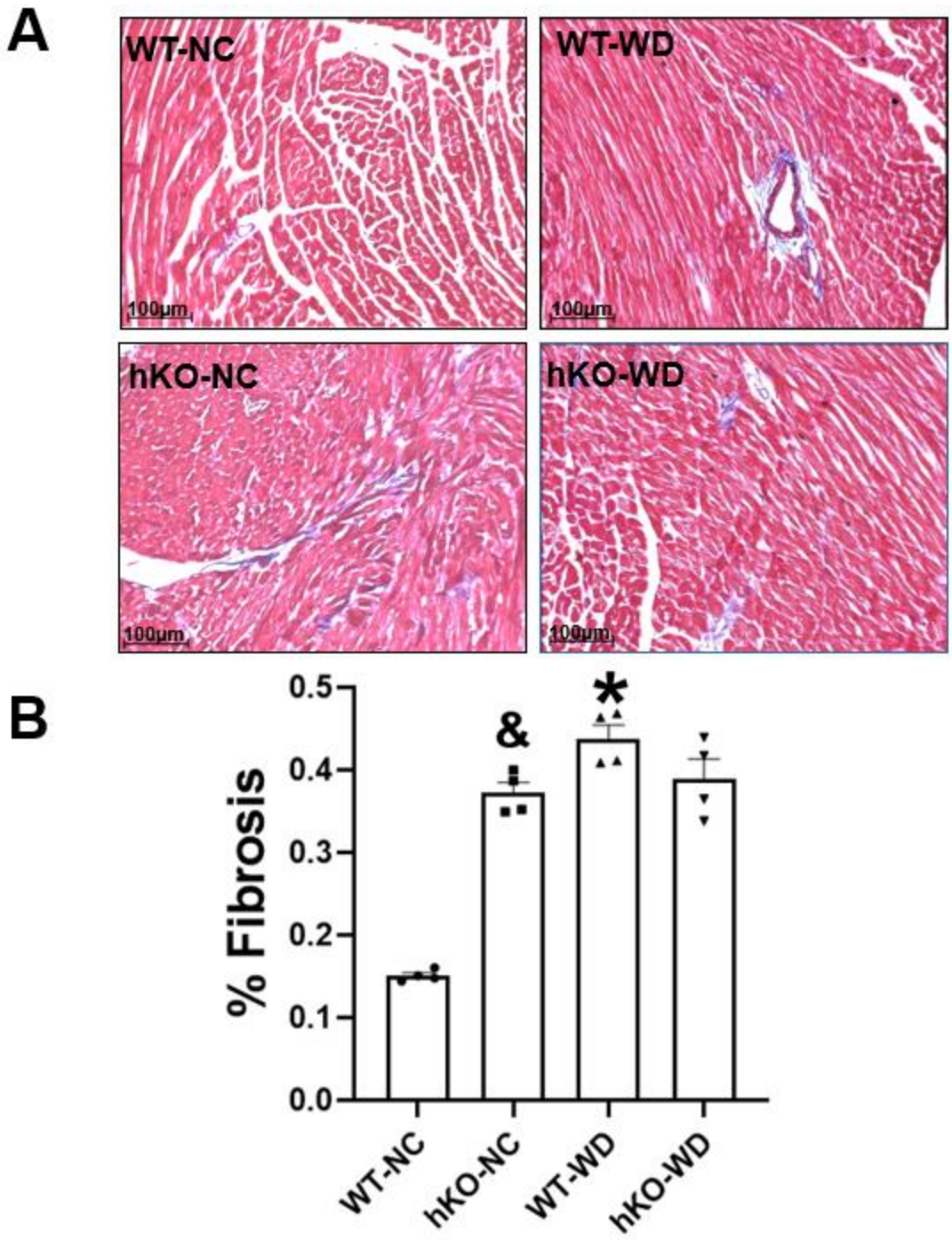


Figure 3.4 Analysis of Fibrosis in the Heart.

A. Representative images obtained from Masson's trichrome-stained sections of the heart. Blue staining indicates fibrosis, while red staining indicates live tissue. B. Quantitative measurements of fibrosis. $^{\&}p<0.05$ vs WT-NC, $^{*}p<0.05$ vs WT-NC, n=4.

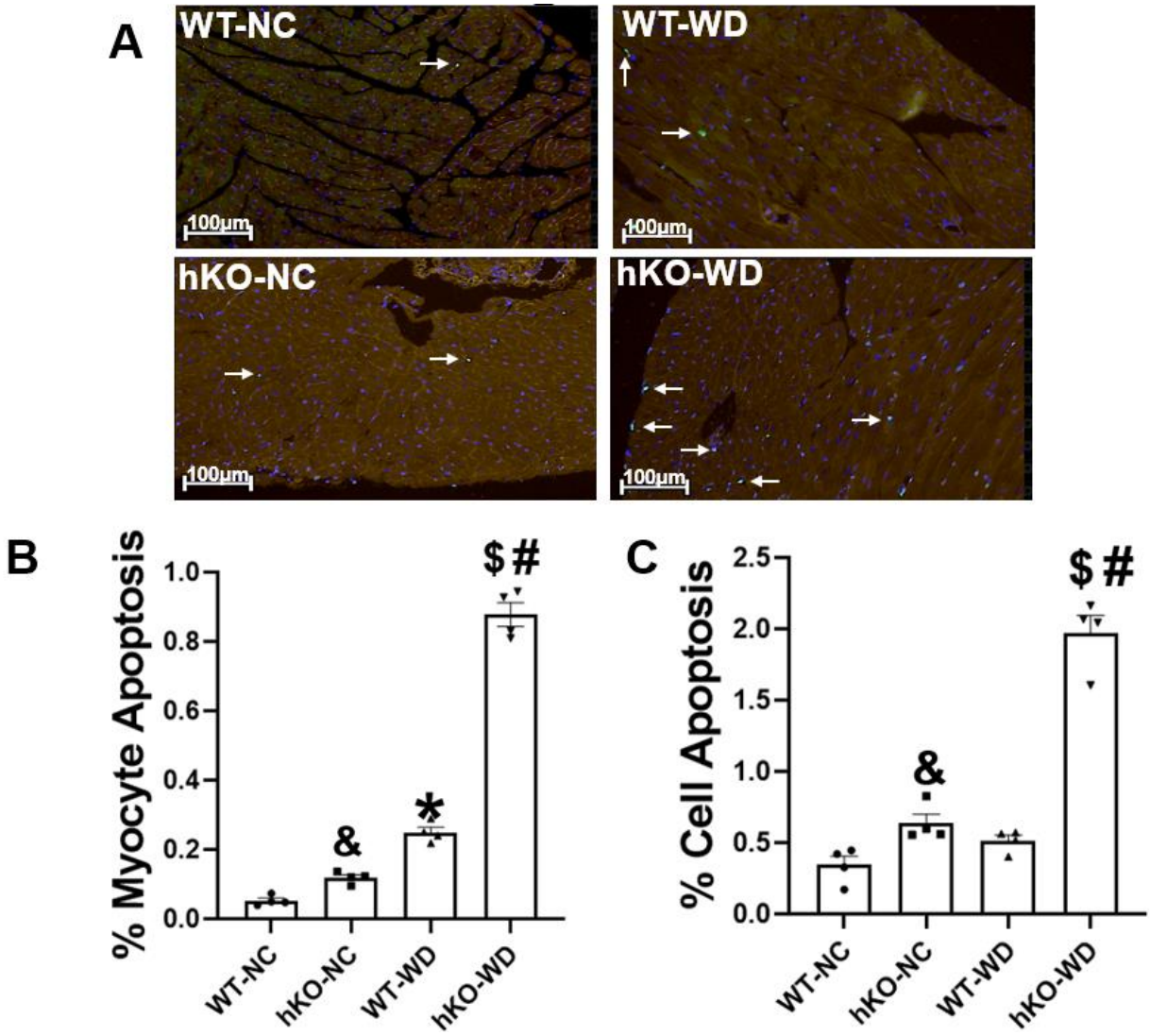


Figure 3.5 ATM Deficiency Exacerbates Apoptosis in the Heart in Response to Western-Type Diet.

A. Representative images of TUNEL (green), WGA (red) and Hoechst (blue) stained hearts. B. Quantitative analysis of myocyte apoptosis. C. Quantitative analysis of cardiac cell apoptosis. $^{\&}p<0.05$ vs WT-NC, $^{*}p<0.05$ vs WT-NC, $^{\$}p<0.05$ vs hKO-NC, $^{\#}p<0.05$ vs WT-WD, n=4.

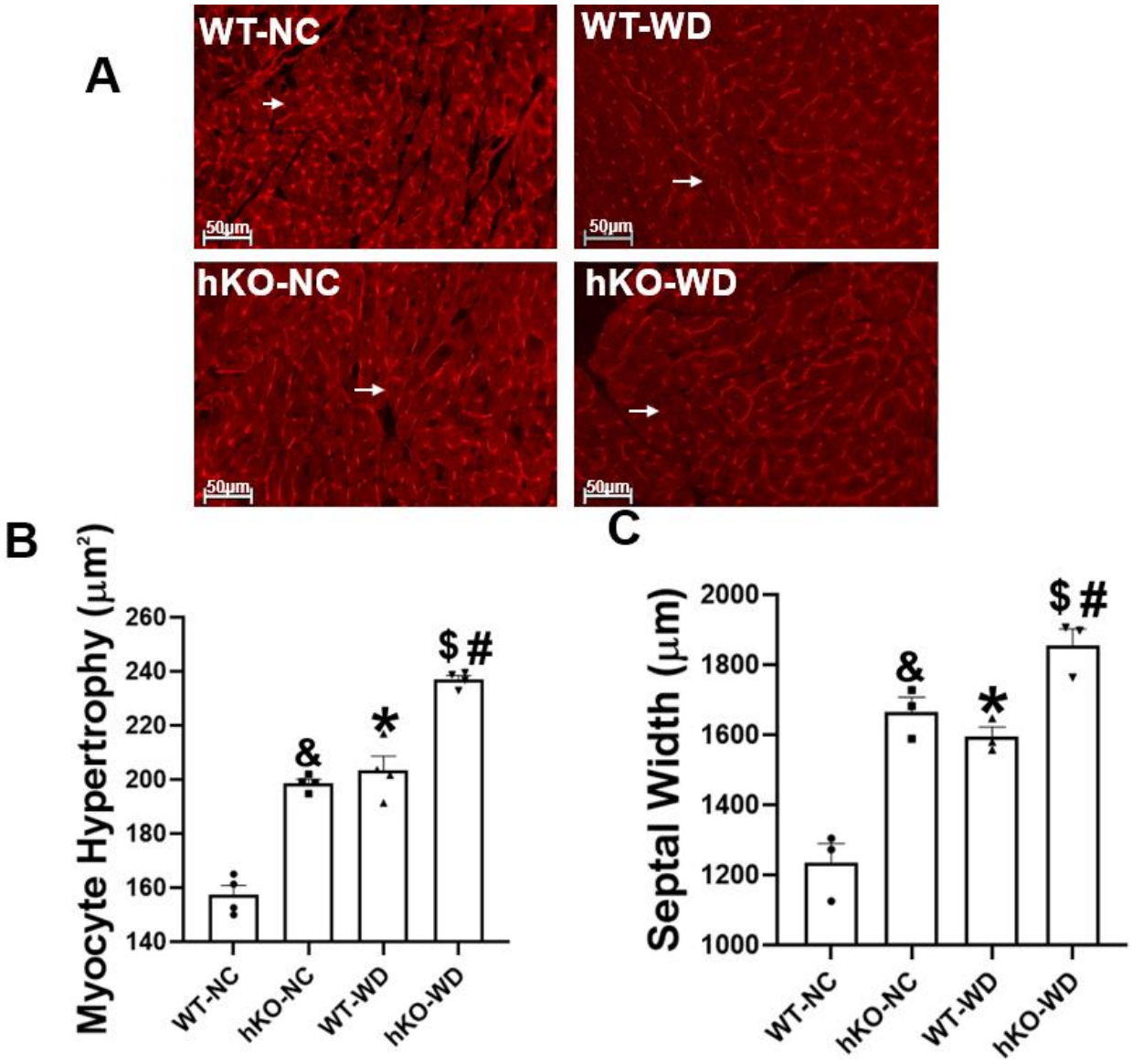
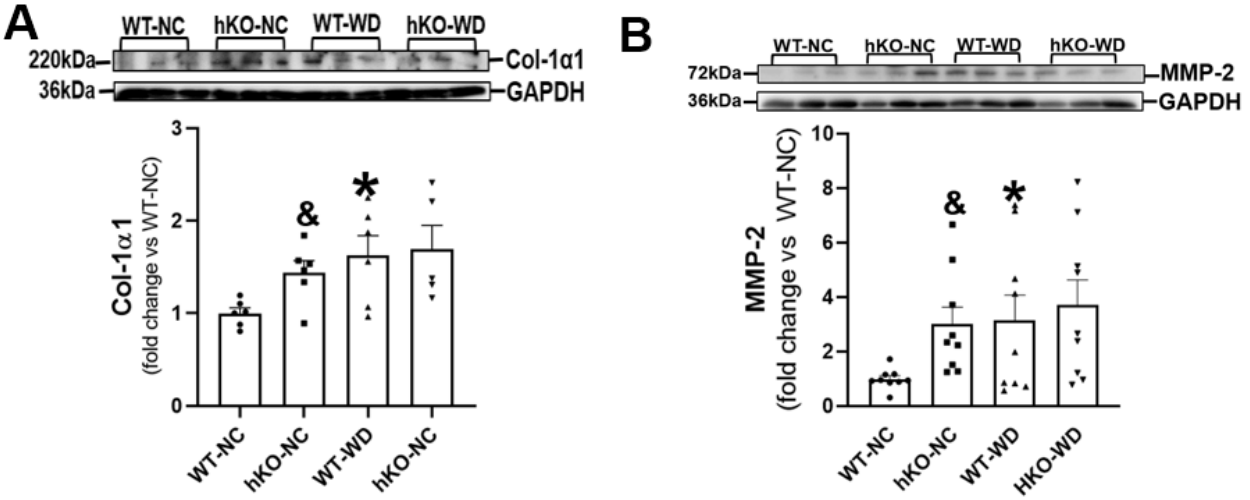


Figure 3.6 ATM Deficiency Exacerbates Myocyte and Septal Hypertrophy in Response to Western-Type Diet.

A. Representative images of wheat germ agglutinin (WGA)-stained cross-sections of the heart depicting myocytes. B. Quantitative analysis of myocyte cross-sectional area. C. Quantitative analysis of septal hypertrophy using Masson's trichrome-stained hearts. $^{\&}p<0.05$ vs WT-NC, $^{*}p<0.05$ vs WT-NC, $^{\$}p<0.05$ vs hKO-NC, $^{\#}p<0.05$ vs WT-WD, n=4.

Expression of Collagen-1α1, MMP-2, MMP-9.

WD and ATM deficiency independently associate with increased cardiac fibrosis (15, 16, 21, 24). Excessive extracellular matrix deposition causes cardiac scar formation and stiffness. Chronic high fat diets associate with increased collagen-1 production and exacerbated cardiac fibrosis (56)**.** Western blot analysis of heart lysates showed that the expression of collagen-1α1 is significantly higher in hKO-NC vs WT-NC group. WD increased collagen-1α1 expression in WT-WD vs WT-NC (Fig 3.7 A). However, no significant increase in collagen-1α1 expression was observed in hKO-WD vs hKO-NC. Matrix metalloproteinase (MMP)-2 and MMP-9 play a critical role in extracellular matrix turnover and remodeling (63). Protein levels of MMP-2 and MMP-9 were higher in hKO-NC vs WT-NC group. MMP-2 and MMP-9 protein levels remained unchanged in hKO-WD vs hKO-NC. Conversely, a significant increase in MMP-2 and MMP-9 protein levels was observed in WT-WD vs WT-NC (Fig 3.7 B and C).



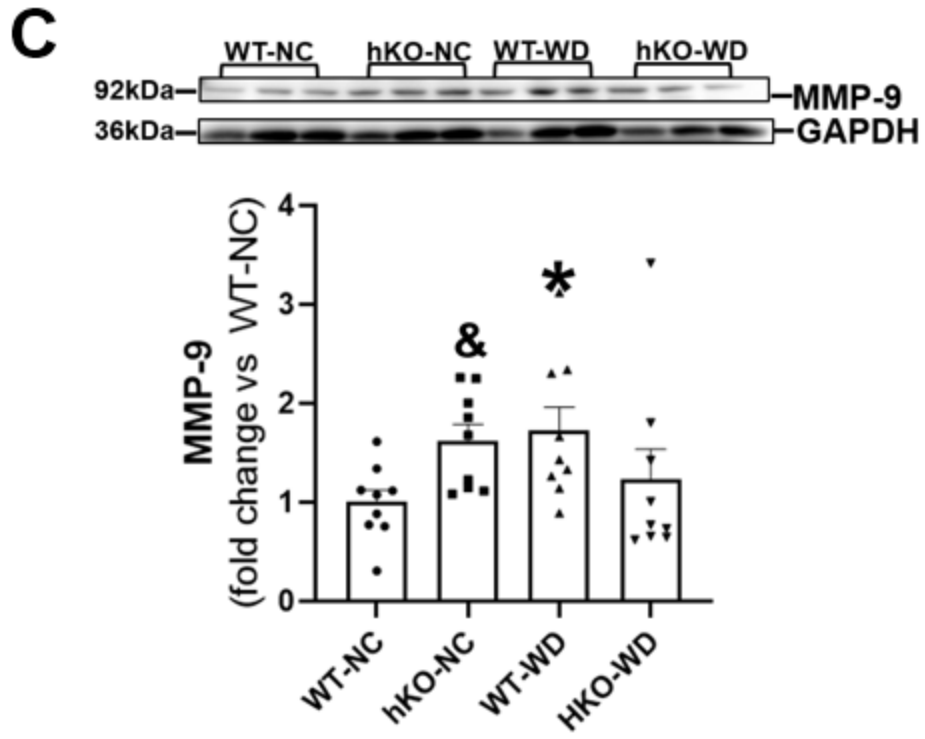


Figure 3.7 Expression of Collagen-1α1 (Col-1α1), MMP-2 and MMP-9 in the Heart.

Heart lysates were analyzed by western blots using anti-Col-1α1 (A), MMP-2 (B), and MMP-9 (C) antibodies. Upper panels exhibit immunostaining for Col-1α1, MMP-2 and MMP-9. Lower panels exhibit quantitative analyses normalized to GAPDH. $^{\&}p<0.05$ vs WT-NC, *$p<0.05$ vs WT-NC, $^{\#}p<0.05$ vs WT-WD, n=6-9.

Activation of proteins related to apoptosis and energy metabolism.

Bax is a pro-apoptotic protein that modulates mitochondrial membrane potential to increase the release of cytochrome C (15). Bax protein levels were higher in hKO-NC vs WT-NC. WD increased Bax expression in WT-WD vs WT-NC. However, no significant increase in Bax expression was observed in hKO-WD vs hKO-NC (Fig 3.8 A). Full length PARP-1 (117 kDa) plays an important role in DNA repair (51). WD increased PARP-1 expression in WT group. Interestingly, no increase in PARP-1 protein levels was observed hKO-WD vs hKO-sham, and PARP-1 protein levels were significantly lower in hKO-WD vs WT-WD (Fig 3.8 B).

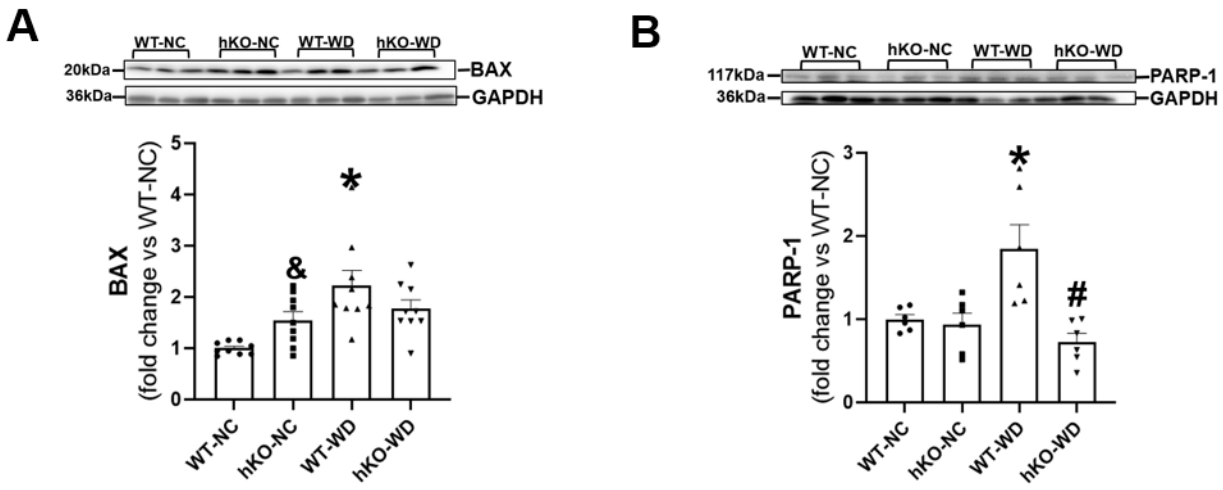
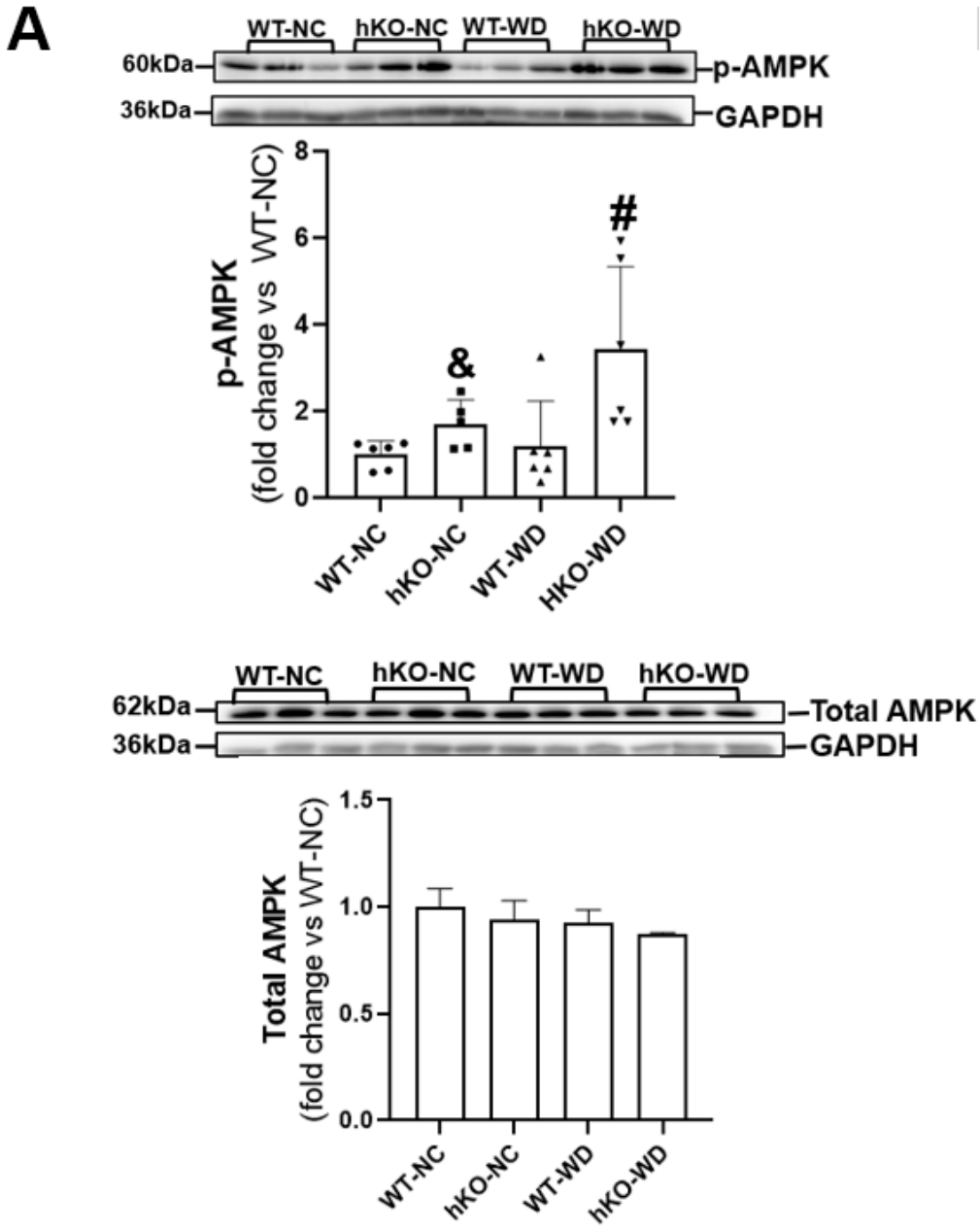


Figure 3.8 Expression of Bax and PARP-1 in the Heart.

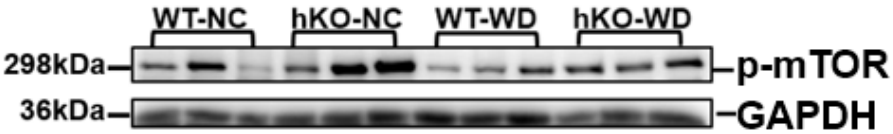
Heart lysates were analyzed by western blots using anti-BAX (A) and PARP-1 (B) antibodies. Upper panels exhibit immunostaining for BAX and PARP-1. Lower panels exhibit quantitative analyses normalized to GAPDH. $^{\&}p<0.05$ vs WT-NC, $^{*}p<0.05$ vs WT-NC, $^{\#}p<0.05$ vs WT-WD, n=6-9. Western blot membrane used in Fig 8B was also probed for total Akt in Fig 9C. GAPDH in Fig 8B was also used to normalize total Akt signal in Fig 9C as the same membrane was probed for PARP-1, total Akt and GAPDH.

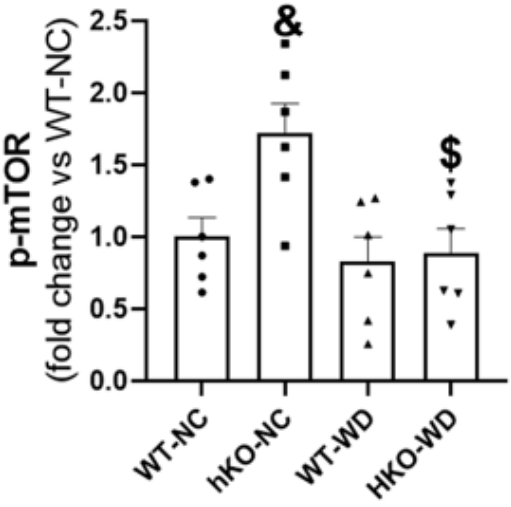
AMPK is a sensor for alterations in energy metabolism (37). AMPK activation was significantly higher in hKO-NC vs WT-NC. WD had no effect on AMPK activation in both genotypes vs their NC groups. However, AMPK activation remained higher in hKO-WD vs WT-WD (Fig 3.9 A). mTOR is a critical regulator of cell growth, metabolism, and cell survival. Dysregulation of mTOR signaling associates with obesity (45). mTOR activation was higher in hKO-NC vs WT-NC group. mTOR activation remained unchanged in WT-WD vs WT-NC. However, mTOR activation was significantly lower in hKO-WD vs hKO-NC (Fig 3.9 B). Activation of Akt, a master regulator of apoptosis and cell metabolism (12), was significantly higher in hKO-NC vs WT-NC group (Fig 3.9 C). Akt activation was significantly higher in WT-WD mice vs WT-NC, while Akt activation remained unchanged in hKO-WD vs hKO-NC. Akt activation upregulates Glut-4, which facilitates

glucose uptake (79). Similar to Akt activation, Glut-4 protein levels were significantly higher in hKO-NC vs WT-NC. WD increased Glut-4 expression in WT group. Conversely, a decrease in Glut-4 expression was observed in hKO-WD vs hKO-NC. In addition, Glut-4 expression was significantly lower in hKO-WD vs WT-WD (Fig 3.9 D).

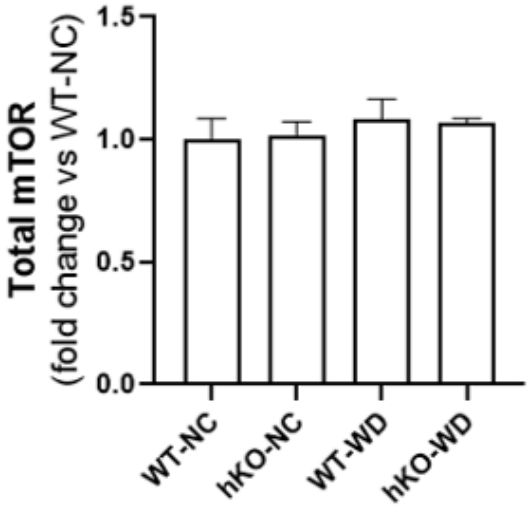


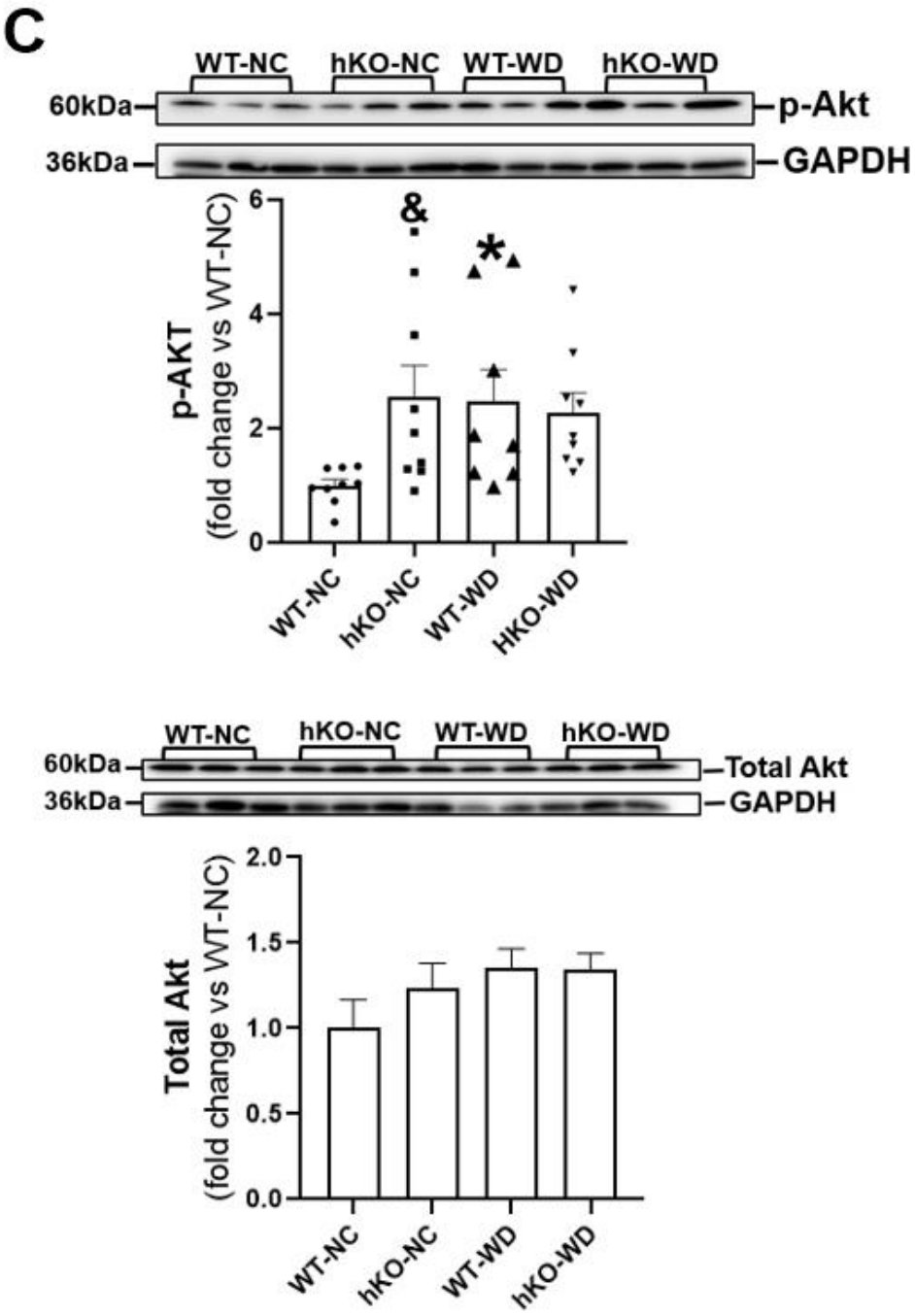
B


WT-NC
hKO-NC
WT-WD
hKO-WD
298kDa
p-mTOR
36kDa
GAPDH


p-mTOR
(fold change vs WT-NC)
2.5
2.0
1.5
1.0
0.5
0.0
&
$
WT-NC
hKO-NC
WT-WD
HKO-WD


WT-NC
hKO-NC
WT-WD
hKO-WD
298kDa
Total mTOR
36kDa
GAPDH


Total mTOR
(fold change vs WT-NC)
1.5
1.0
0.5
0.0
WT-NC
hKO-NC
WT-WD
hKO-WD


C
WT-NC
hKO-NC
WT-WD
hKO-WD
60kDa
p-Akt
36kDa
GAPDH
p-AKT
(fold change vs WT-NC)
&
*
Total Akt
Total Akt
(fold change vs WT-NC)

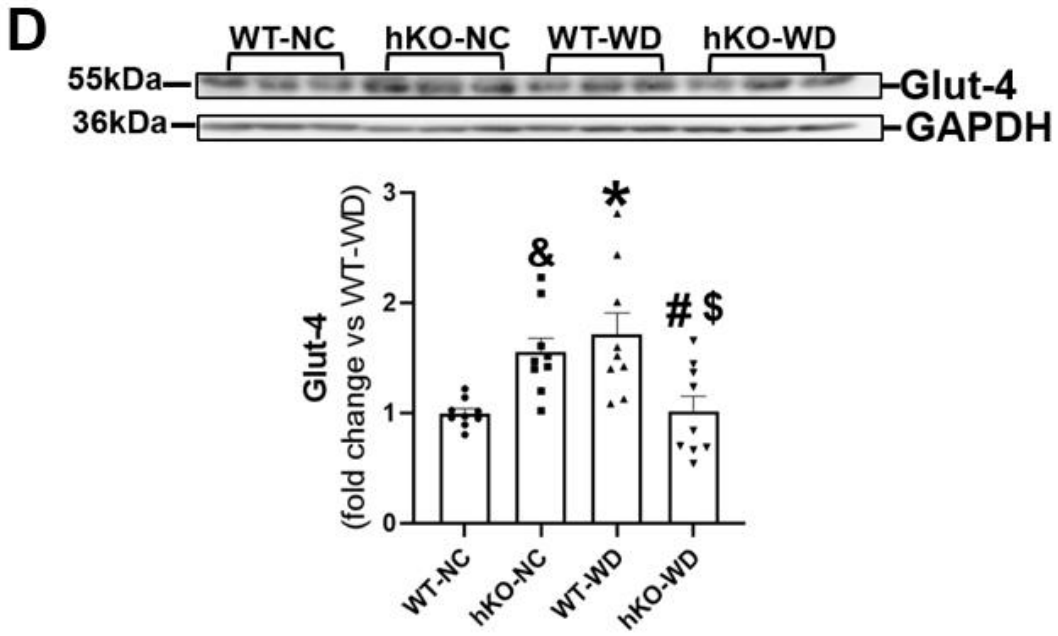
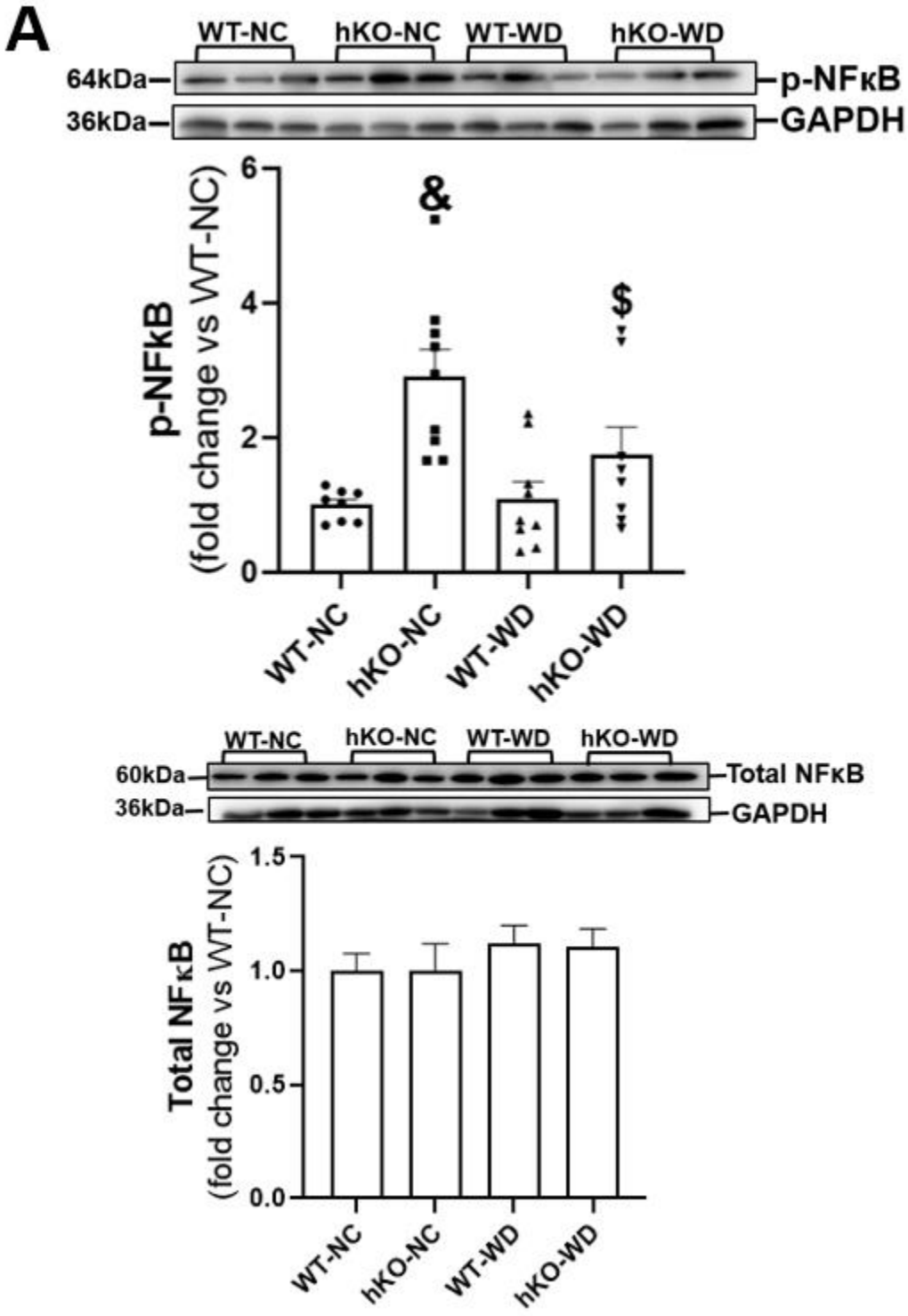


Figure 3.9 Phosphorylation of Akt, AMPK and mTOR, and Expression of GLUT-4.

Heart lysates were analyzed by western blots using anti-p-AMPK and total AMPK (A), p-mTOR and total mTOR (B), p-Akt and total Akt (C), Glut-4 (D) antibodies. Bar graphs exhibit quantitative analyses normalized to GAPDH. $^{\&}p<0.05$ vs WT-NC, $^{*}p<0.05$ vs WT-NC, $^{\#}p<0.05$ vs WT-WD, n=9 for phosphoproteins and Glut-4; n=3 for total proteins.

Inflammatory mediators.

ATM deficiency is shown to associate with increased inflammation (81). When activated, IKK and NFκB are primary regulators of the inflammatory response (41). Activation of NFκB and IKK α/β (upstream regulator of NFκB) was significantly higher in hKO-NC vs WT-NC. NFκB activation remained unchanged in WT-WD vs WT-NC, while a significant decrease in NFκB activation was observed in hKO-WD vs hKO-NC (Fig 3.10 A). WD induced a significant increase in IKK α/β activation in WT group (vs WT-NC). However, IKK α/β activation remained unchanged in hKO-WD vs hKO-NC (Fig 3.10 B).


A
WT-NC
hKO-NC
WT-WD
hKO-WD
64kDa
p-NFκB
36kDa
GAPDH
p-NFkB
(fold change vs WT-NC)
6
4
2
0
&
$
WT-NC
hKO-NC
WT-WD
hKO-WD
WT-NC
hKO-NC
WT-WD
hKO-WD
60kDa
Total NFκB
36kDa
GAPDH
Total NFκB
(fold change vs WT-NC)
1.5
1.0
0.5
0.0
WT-NC
hKO-NC
WT-WD
hKO-WD

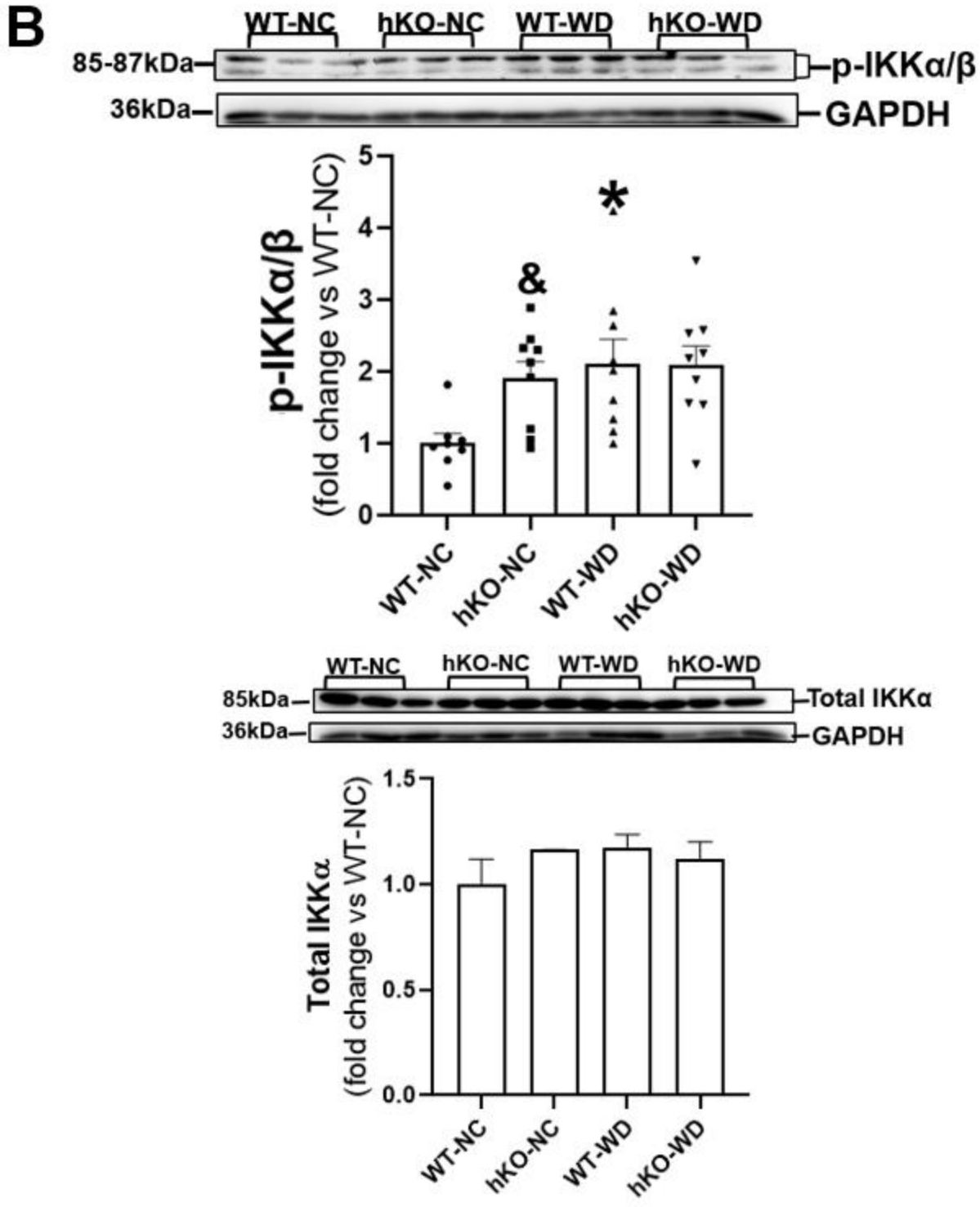


Figure 3.10 Phosphorylation of NFκB and IKK α/β.

Heart lysates were analyzed by western blots using anti-p-NFκB and total NFκB (A), and p-IKK α/β and total IKK α (B) antibodies. Bar graphs exhibit quantitative analyses normalized to

GAPDH. $^{\&}p<0.05$ vs WT-NC, $^{*}p<0.05$ vs WT-NC, n=8-9 for phosphoproteins; n=3 for total proteins.

Serum cytokine/chemokine levels.

Chronic inflammation commonly associates with prolonged high fat diets and obesity (17, 47). Interestingly, A-T patients exhibit elevated circulating concentrations of cytokines (28). Circulating levels of 23 cytokines/chemokines were not significantly different between the two NC groups, and remained unchanged in WT-WD vs WT-NC. However, serum concentrations of IL-12(p70), MIP-1α and MIP-1β were significantly higher in hKO-WD vs hKO-NC. In addition, serum concentrations of IL-12(p70), eotaxin, IFN-γ, MIP-1α and MIP-1β were significantly higher in hKO-WD vs WT-WD (Table 3.3).

Table 3.3 Serum Cytokine/Chemokine Concentrations

Cytokine/Chemokine	WT-NC	hKO-NC	WT-WD	hKO-WD
IL-1α	6.1 ± 0.3	7.3 ± 1.4	6.1 ± 1.3	8.0 ± 1.1
IL-1β	5.3 ± 0.6	5.2 ± 0.2	5.6 ± 0.2	5.4 ±0.7
IL-2	1.8 ± 0.2	2.0 ± 0.2	2.0 ± 0.1	2.4 ± 0.3
IL-3	1.9 ± 0.0	3.0 ± 1.2	1.4 ± 0.3	3.3 ± 1.1
IL-4	1.7 ± 0.1	1.6 ± 0.1	1.6 ± 0.2	1.8 ± 0.3
IL-5	4.8 ± 0.6	6.1 ± 1.8	4.5 ± 0.1	4.9 ± 0.2
IL-6	2.7 ± 0.4	5.8 ± 4.1	3.3 ± 0.3	3.2 ± 0.2
IL-9	11.6 ± 1.0	12.1 ± 0.7	11.2 ± 1.1	18.4 ± 3.0
IL-10	23.5 ± 5.4	16.2 ± 1.2	19.5 ± 0.6	20.3 ± 2.0
IL-12 (p40)	174.5 ± 21.6	268.4 ± 71.8	147.1 ± 32.5	171.5 ± 35.1
IL-12 (p70)	107.9 ± 20.5	88.6 ± 6.5	81.0 ± 9.4	111.9 ± 4.3$^{\$\#}$
IL-13	48.1 ± 6.0	51.4 ± 2.4	85.4 ± 21.6	64.7 ± 6.3
IL-17A	42.7 ± 9.0	102.0 ± 50.0	23.4 ± 3.5	46.0 ± 14.0
Eotaxin	322.2 ± 51.5	369.4 ± 66.4	206.8 ± 14.7	318.7 ± 26.9$^{\#}$
G-CSF	97.3 ± 25.2	119.0 ± 34.1	112.0 ± 52.3	85.3 ± 12.3
GM-CSF	27.5 ± 2.3	26.6 ± 0.7	27.2 ± 0.2	28.8 ± 1.4
IFN-γ	21.4 ± 2.6	20.9 ± 1.7	20.1 ± 1.7	33.5 ± 4.6$^{\#}$
KC	19.3 ± 1.0	19.6 ± 1.2	25.9 ± 6.3	21.3 ± 1.9
MCP-1	73.3 ± 5.5	79.1 ± 8.5	73.6 ± 3.5	76.5 ± 4.5
MIP-1α	2.3 ± 0.3	2.3 ± 0.1	2.3 ± 0.1	2.66 ± 0.1$^{\$\#}$
MIP-1β	33.0 ± 2.2	33.8 ± 1.8	32.6 ± 2.6	44.8 ± 3.1$^{\$\#}$
RANTES	35.0 ± 3.5	41.2 ± 5.7	59.7 ± 14.8	39.5 ± 5.0
TNF-α	44.2 ± 7.4	88.5 ± 53.7	35.5 ± 2.6	48.7 ± 7.0

Values are means ± SE (in pg/ml), n=4-5. *$p<0.05$ vs WT-NC; $^{\$}p<0.05$ vs hKO-NC, $^{\#}p<0.05$ vs WT-WD.

Discussion

Approximately 60-70% of US population consumes WD (27, 50). A-T carriers, make up a substantial portion of the general population (~1.4-2.0%), are more susceptible to ischemic heart

disease (79). Thus, ~2-4 million A-T carriers consume WD. This study investigated the cardiac impact of WD during ATM deficiency using ATM deficient mice. The main findings of the study are that ATM deficiency - 1) associates with initial rapid weight gain; 2) induced systolic dysfunction with increased preload; 3) increases myocyte and cardiac cell apoptosis; 4) induces myocyte hypertrophy; 5) induces alterations in the expression and activation of signaling molecules associated with remodeling, metabolism and inflammation; and 6) increases circulating levels of cytokines/chemokines.

ATM expression is shown to increase in response to a multitude of stimuli including genotoxic stress, cardiac injury, and oxidative stress (79). Our previous work has shown increased ATM expression in the heart following β-AR receptor stimulation (20). Increased ATM expression in the heart was also observed in the non-infarct and infarct LV regions of the heart 1- and 3-days post-MI (15). Here, ATM protein levels were ~50% lower in the myocardium of ATM deficient hearts when compared to their normal counterparts, confirming ATM deficiency in hKO group. WD increased ATM expression (by >2-fold) in the myocardium of WT, but not hKO mice. This WD-mediated increase in ATM expression in WT-WD heart may relate to changes in oxidative stress, inflammation and/or sympathetic nerve activity (9, 57, 66).

Increased adiposity is often observed with WD-feeding (24, 76). Lack of ATM is shown to decrease adipocyte maturation, and diet-induced fat accumulation (65). Diet-induced body weight gain and fat accumulation in A-T carriers in a time-dependent manner has not yet been established. Using a mouse model, this study provides evidence that ATM deficiency associates with rapid weight gain in response to WD vs their normal counterparts. The increase in body weight gain became apparent at week 5 on WD and stayed higher until 10 weeks on WD.

Thereafter, body weight gain tended to be higher in ATM deficient group, however, no significant differences in body weight gain were observed at weeks 11-14 between the two WD groups. At week 14 post-WD, there was no difference in fat (subcutaneous and visceral) accumulation, cardiac lipid deposition, and serum levels of cholesterol and triglycerides between the two WD groups. Based on the observation of initial rapid weight gain, it is speculated that ATM deficiency may initially augment adipocyte maturation leading to accelerated weight gain. However, persistent deficiency of ATM may interfere with adipocyte maturation and function. Obesity and insulin resistance independently associate with heart failure (4, 18). Interestingly, fasting blood glucose levels were significantly higher in WT-WD vs hKO-WD. This observation points towards the possibility that WT mice may have developed a level of insulin resistance 14 week post-WD.

Increased fat accumulation and body mass index associates with adaptations in cardiovascular structure and function (1, 6, 24). The severity of cardiac dysfunction is often proportional to the duration of WD-feeding and the degree of obesity (1, 18). A diet high in fat and sugar for 8 weeks is shown to impair systolic and diastolic parameters of the heart in mice (10). Tei index is considered as an index of global heart function capable of estimating combined systolic and diastolic performance. Volume overload, reduction in arterial compliance and ventricular diastolic stiffening are predicted to decrease Tei index, while increased afterload due to higher systemic resistance, reduction in end-systolic elastance and impairment of LV relaxation are predicted to increase Tei index (30). Alterations in LV dimensions is another common consequence of adiposity (32, 61). Cardiac structural alterations can alter the filling, relaxation and distensibility of the left ventricle (22, 55). Consequently, diastolic dysfunction is a common feature in obese patients and significantly contributes to the development of heart failure and independently increases the risk of mortality (31). The degree of LV dysfunction is linked to the

duration of obesity and associated changes in preload or afterload (3). Continual increase in systemic resistance can cause afterload dysfunction with alterations in doppler parameters such as decreased E wave and AET, and prolongation of IVRT (30). Obesity associated hyperdynamic circulation causes change in the filling and relaxation in preload (74). Consequently, as the heart continually compensates for changes in volume, preload and afterload dysfunction occurs (3, 55, 74). The data presented here demonstrate that WD induces systolic dysfunction as observed by decreased %FS and %EF, and increased LVESD and LVESV in WT mice. However, these parameters are equally affected by ATM deficiency. On the other hand, decreased E wave, AET, stroke volume and cardiac output, and increased IVRT and Tei index were only observed in WT-WD, suggesting that WD induces systolic dysfunction with concomitant increase in afterload in WT mice. In contrast, AET was significantly higher, while IVCT, IVRT and Tei index were significantly lower in hKO-WD when compared to the WT-WD group, suggesting that ATM deficiency induces systolic dysfunction with concomitant increase in preload in response to WD. It is interesting to note that the E wave is significantly smaller in hKO-NC vs WT-NC. WD did not significantly alter E wave in ATM deficient hearts. However, it decreased IVCT and IVRT which may suggest increased cardiac contractility during ATM deficiency. AT patients, with mutations in both alleles, exhibit severe dysfunction in autonomic nervous system (71). Therefore, increased contractility in hKO and preload during ATM deficiency in response to WD may relate to the changes in autonomic nervous system. Future investigations related to the measurement of arterial and LV blood pressures in a time dependent manner is needed to confirm the afterload and preload associated changes in the heart in response to WD.

Consumption of high fat diet associates with increased cardiac fibrosis, myocyte apoptosis and myocardial dysfunction (1, 6, 36, 67). Afterload associated dysfunction is suggested to

correlate with increased fibrosis in the heart (70). Further, increased levels of toxic lipid byproducts can induce cardiac myocyte death due to apoptosis (1, 78). Compensative hypertrophy can also occur in obesity models due to volume overload (1, 6). While hypertrophy is beneficial during the early stages of cardiac remodeling, it can lead to increased myocyte apoptosis and cardiac dysfunction (25). ATM deficiency independently associates with increased cardiac fibrosis and hypertrophy at basal levels, and following β-AR stimulation and MI (15, 16, 21). ATM deficiency also associates with increased apoptosis in the infarct LV region 1 and 3 days post-MI (15). Here we observed that WD increases myocardial fibrosis, and myocyte hypertrophy and apoptosis in both genotypes. However, the increase in myocyte hypertrophy and apoptosis was significantly greater in ATM deficient hearts in response to WD when compared to their WT counterpoints. Akt plays a significant role in regulation of cardiac cell apoptosis and hypertrophy. Transgenic mice studies provide evidence that a short-term activation of Akt associates with modest cardiac growth, while a chronic activation of Akt associates with excessive hypertrophy (75). Therefore, increased activation of Akt in WT-WD (vs WT-NC) may help explain the observed increase in hypertrophic response in this group. Conversely, Akt activation was higher in hKO-NC vs WT-NC with no significant change in Akt activation in response to WD in hKO-WD (Fig 9C), suggesting that a chronic activation of Akt may play a role in exaggerated hypertrophic response in ATM deficient hearts at basal levels and in response to WD.

Although ATM deficiency associated with increased basal fibrosis and collagen-1α1 expression, however, no significant increase in fibrosis or collagen-1α1 was observed in ATM deficient hearts in response to WD. Thus, increased myocyte apoptosis and hypertrophy may help explain systolic dysfunction in the two WD groups, while increased fibrosis in WT-WD group (vs WT-NC) may relate to increased afterload in WT-WD group. It was interesting to observe that

increase in fibrosis in WT-WD group associated with increased expression of MMP-2 and MMP-9. MMP-2 and MMP-9 plays a significant role in myocardial remodeling process by affecting the structure as well function in response to cardiac injury and increased hemodynamic load (63). Generally, increased expression of MMPs is anticipated to associate with decreased fibrosis. However, deposition of fibrosis involves an intricate relationship between collagen synthesis, degradation and deposition. This is shown by the observation that myocyte-specific expression of MMP-2 associates with hypertrophy, extensive fibrosis and systolic dysfunction (5). In ageing mice, deletion of MMP-9 attenuates myocardial fibrosis and diastolic dysfunction (13). Future investigations involving the analysis of other components of fibrosis may help understand the role of WD and ATM deficiency in myocardial fibrosis.

Bax, a pro-apoptotic protein, modulates mitochondrial membrane potential to increase the release of cytochrome C (15). Ablation of Bax plays a cardioprotective role in myocardial ischemia/reperfusion injury (29). Here, ATM deficient heart exhibited enhanced basal apoptosis and Bax expression. WD augmented apoptosis and Bax expression in WT group, suggesting that increased Bax expression may play a role in increased apoptosis in WT-WD group. However, WD did not induce a significant increase Bax expression in hKO-WD, suggesting involvement of Bax-independent mechanism in this group. PARP-1 serves as a DNA damage sensor and critical DNA repair regulator. Apoptotic stimuli induce PARP-1 cleavage via the activation of caspases (51). WD increased protein levels of intact PARP-1 (117 kDa) only in WT group. However, there was no increase in intact PARP-1 expression in hKO-WD group. No increase in PARP-1 expression in hKO-WD suggests interference in DNA damage response and/or enhanced PARP-1 cleavage, leading to enhanced apoptosis in hKO-WD vs WT-WD. However, further investigations are needed to clarify the role of PARP-1 in ATM-mediated cardiac cell apoptosis.

Akt, AMPK, mTOR are critical regulators of cell growth, metabolism and survival, and are commonly dysregulated in animal models of obesity (43, 45, 46). Acute activation of Akt associates with enhanced activation of Glut-4, while a chronic activation decreases Glut-4 expression in the heart (46). Diets high in fat decrease activation of AMPK and promote mTOR activation in the heart (38, 43, 72). Transgenic mice overexpressing a kinase dead isoform of AMPK exhibit exaggerated high fat diet-induced cardiac hypertrophy and contractile dysfunction with concurrent decrease in Akt activation and Glut-4 expression, and enhanced mTOR activation (72). Here we observed greater activation of Akt, AMPK and mTOR, and expression of Glut-4 in hKO-NC vs WT-NC. In our previous report using MI as a model, hKO deficient sham animals did not exhibit activation of Akt or AMPK, while mTOR activation was lower (69). The reasons for these discrepant findings may include the age of the mice and/or performance of sham surgeries in the MI model. The age of mice in this study is ~5 months, while the previous study used mice aged ~4 months. In addition, the sham mice in MI model underwent surgical preparation and sham ligation of the coronary artery. The observed increase in Akt activation and Glut-4 in hKO-NC (vs WT-NC) suggests the possibility of a metabolic shift towards glucose utilization in ATM deficient hearts. WD significantly increased Akt activation and Glut-4 expression in WT group. In ATM deficient hearts, WD had no effect on Akt activation, while Glut-4 expression was lower when compared to hKO-NC and WT-WD groups. These changes suggest that ATM deficient hearts may be experiencing decreased insulin production/sensitivity and reduced metabolic flexibility. In response to WD, ATM deficient hearts exhibited enhanced AMPK activation and decreased mTOR activation. Since AMPK deficiency exacerbates contractile function (72) and inhibition of mTOR (C1) improves ventricular function and reduces volume overload-induced hypertrophy

(58), alterations in the activation of AMPK and mTOR may play a crucial role in genotype-specific differences in diastolic dysfunction in response to WD.

Approximately two-thirds of A-T patients have immunological abnormalities. This includes a reduction in the release of B and T cells, low levels of immunoglobulin, and poor antibody production (33, 48, 73, 77). Elevated circulating concentrations of cytokines have also been reported with A-T patients (28). The inflammatory response is an essential component of host defense and tissue repair. While acute inflammation is beneficial, chronic inflammation has been linked to cardiovascular disease (17). High fat diet and obesity associate with chronic inflammation (17, 47). NFκB and IKK are central inflammatory regulators, and increased NFκB activation has been observed with high fat diet (11, 41). Here, activation of NFκB and IKK α/β was significantly higher in hKO-NC group vs WT-NC, suggesting that ATM deficiency associates with increased myocardial inflammation under basal conditions. WD induced a significant increase in IKK α/β activation in WT-WD vs WT-NC. Conversely, WD failed to activate IKK α/β in hKO-WD vs hKO-NC, while decreasing NFκB activation. In addition, ATM deficiency associated with increased circulating levels of inflammatory mediators such as IL-12(p70), eotaxin, IFN-γ, MIP-1α and MIP-1β in response to WD. Increase in the pro-inflammatory IL-12(p70) has been observed during heart failure, and has the potential to enhance production of IFN-γ in T-cells (44, 54). An increase in IFN-γ is suggested to play a role in inflammation, insulin sensitivity, apoptosis and cardiac remodeling (35, 49, 62). Further, MIP-1α and MIP-1β are important chemotactic chemokines, which play a role in the inflammatory response (7). Increase in MIP-1β has also been linked to the development of adiposity, and IFN-γ can upregulate MIP-1α in the heart (35, 64). Eotaxin, an eosinophil-specific chemokine, is an important inflammatory mediator. Circulating levels of eotaxin are shown to be higher in patients with ischemic heart

disease(19). In MI model, ATM deficiency associated with delayed inflammatory response and decreased dilative remodeling in the heart 1 day post-MI (15). Collectively, these data suggest that ATM deficiency plays a critical role in modulation of inflammatory response. However, future investigations are warranted to clarify the mechanism by which ATM deficiency modulates WD-induced inflammation and heart function.

In summary, the data presented here provide evidence that WD induces systolic dysfunction with increased afterload in WT group as observed by an increase in Tei index, prolongation of IVRT, and decrease in AET, E wave, stroke volume and cardiac output. On the other hand, ATM deficiency induces systolic dysfunction with increased preload as observed by decreased Tei index, IVCT and IVRT. Further, ATM deficiency plays an important role in alterations in WD-induced fibrosis, apoptosis, hypertrophy, and metabolic and inflammatory signaling proteins. It should be emphasized that cardiac structural and functional changes may vary with the duration of WD feeding/obesity (3, 78). Here, all the observations, except body weights, were made 14 weeks post-WD. WD-feeding time of 14 weeks was chosen based on the observations that WD-induced obesity in mice occurs within 12-16 weeks (34, 52, 82). Additional time points are needed to elucidate the progression of cardiac dysfunction during ATM deficiency in response to WD. In addition, sex-specific differences are known to influence glucose and lipid metabolism, and cardiac energy metabolism and function (23). Therefore, complementary investigations should be performed using age-matched female mice.

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CHAPTER 4. DEFICIECNY OF ATAXIA-TELANGIECTASIA MUTATED KINASE ATTENUATES CARDIAC DYSFUNCTION IN FEMALE MICE IN REPONSE TO WESTERN-TYPE DIET

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Abstract

Ataxia telangiectasia mutated kinase (ATM) is a DNA damage response protein, which aids in maintaining genomic stability. Previously, we have shown that Western-type diet (WD) during ATM deficiency in male mice associates with accelerated weight gain, systolic dysfunction with increased preload, exacerbation of hypertrophy, apoptosis, and inflammation. The objective of this study was to investigate the role of ATM deficiency in WD-induced changes in functional and biochemical parameters of the heart using female mice. For this, 6-week-old female wild-type (WT) and ATM heterozygous knockout (hKO) mice were placed on WD for 14 weeks. Weight gain was measured on a weekly basis, while cardiac biochemical and functional outcomes were measured 14 weeks post-WD. ATM deficiency attenuated WD-induced weight gain and fat accumulation compared to WT-WD throughout the 14-week WD duration. Measurement of heart function using echocardiography showed that WD induced a decrease in percent fractional shortening, ejection fraction, cardiac output, systolic posterior wall and diastolic anterior wall thickness and increased systolic volume and diameter in WT-WD vs WT-NC (normal chow). However, these functional parameters of the heart remained unchanged in hKO-WD vs hKO-NC. Myocardial fibrosis, hypertrophy and apoptosis were higher in WT-WD vs WT-NC. While WD did significantly increase hypertrophy and apoptosis in the myocardium of hKO mice, apoptosis was significantly lower in hKO-WD vs WT-WD. MMP-2 and MMP-9 expression were increased, and mTOR activation was decreased only in WT-WD vs WT-NC. Protein levels of Collagen-1α1 and PARP-1 were higher, while Bax expression was lower in hKO-WD vs WT-WD and hKO-NC. Thus, ATM deficiency in female mice attenuates weight gain and preserves heart function with decreased apoptosis in response to WD.

New & Noteworthy

Ataxia telangiectasia mutated kinase (ATM) deficiency in humans exponentially increases the risk of ischemic heart disease. Previously, we provided evidence that Western-type diet (WD) is deleterious in male ATM deficient mice causing systolic dysfunction, increased preload, apoptosis, hypertrophy and inflammation. Here, we provide evidence that ATM deficiency in female mice attenuates body weight gain and plays a cardioprotective role with preservation of heart function with decreased cardiac cell apoptosis 14 weeks post-WD. Future investigations of sex-specific effects on WD-induced cardiac functional and structural changes during ATM deficiency may help to elucidate the appropriate sex-specific treatment and nutritional counseling of patients with ATM deficiency.

Introduction

Western-type diet (WD) and adiposity have been associated with changes in cardiac structural and mechanical function as well as low-grade systemic inflammation, increased oxidative stress and metabolic dysfunction (34, 37, 38). Obesity cardiomyopathy, which often occurs following extended consumption of WD and/or obesity, is characterized by functional and structural derangements, hemodynamic changes and altered cardiac remodeling (3, 5, 11, 26). The severity of obesity directly influences the change in blood volume, degree of hyperdynamic circulation, and associated preload and afterload dysfunction (4, 11). Therefore, deleterious changes in cardiac structure and function are often observed with obesity cardiomyopathy, which may include right and left ventricular eccentric/concentric hypertrophy, chamber dilation, increased fibrosis, systolic and diastolic dysfunction (4, 11). Further, studies show that animal models of obesity display diet-induced cardiac dysfunction similar to humans such as biventricular stiffness, exacerbation of fibrosis, hypertrophy, plaque accumulation, and ischemic events (1, 11, 13, 26).

Ataxia telangiectasia mutated kinase (ATM) becomes activated in response to DNA double-stranded breaks, oxidative damage, ionizing radiation and other genotoxic mediators (58, 65). ATM is located in the nucleus, cytoplasm and mitochondria, and therefore has a multitude of important functions (22, 58, 65). Cell cycle regulation and DNA damage repair are important functions of ATM. However, ATM is actively involved in many other processes such as regulation of glucose metabolism, angiogenesis, redox sensing, vesicle transport, autophagy, and peroxisome and mitochondrial function (6, 12, 17, 29, 33). Therefore, mutations which impair the ATM kinase function result in a multisystem disorder called ataxia-telangiectasia (A-T) (22). While the severity of the A-T phenotype is based on the type and frequency of mutation occurrence, neurological,

endocrine and cardiovascular dysfunction are commonly observed (46, 51, 55, 60). A-T carriers, patients with mutations in one allele, account for approximately 2% of the population (65). A-T carriers have a less severe phenotype than A-T patients. However, these patients still have an increased risk of metabolic dysfunction, cancer and ischemic heart disease (65).

While the cardiovascular effects of WD during ATM deficiency is not fully elucidated, we have previously provided evidence that WD during ATM deficiency in male mice associate with systolic dysfunction with increased preload, exacerbation of cardiac remodeling including increased hypertrophy and apoptosis, and alterations in inflammatory and metabolic signaling (64). The objective of this study was to investigate the role of ATM deficiency in WD-induced changes in functional and biochemical parameters of the heart using female mice. The major finding of the study is that ATM deficiency in female mice associates with lower body weight and fat gain, preserved cardiac function and attenuation of cardiac apoptosis.

Materials and Methods

Vertebrate animals and diets.

This investigation follows the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experiments were executed following protocols approved by the East Tennessee State University Committee on Animal Care. ATM deficient mice (129S6/SvEvTac) were acquired from Jackson Laboratory for breeding (stock #002753). ATM heterozygous knockout (hKO) mice are used for breeding as ATM KO mice have a limited lifespan (~2 months). All mice are genotyped prior to diet assignment and following exsanguination by PCR using primers suggested by the Jackson Laboratory. Age matched (~6 weeks old) WT and hKO female mice were placed on a normal chow

(NC; Envigo 8604) or Western-type diet (WD; Envigo TD 88137) for a 14-week duration. The normal chow energy composition is - 32% kcal protein, 14% kcal fat, 54% kcal carbohydrate and 4% sugar (by weight). The high fat/high sugar WD energy composition is - 15.2% kcal protein, 42.0% kcal fat, 42.7% kcal carbohydrate and 34% sugar (by weight). All mice had food and water available *ad libitum*, except when fasted for glucose level assessment and were kept on a 12 hour-dark/light cycle.

Fasting glucose levels.

When all groups reached the 14^{th} week NC or WD feeding, age matched mice from both genotypes were fasted for 4 hours. A 5mm Goldenrod lancet was used to gently nick the tail, which allowed collection of approximately 1µl of blood sample. Blood glucose was then measured using a ReliOn monitoring system.

Echocardiography.

Following the 14-week duration of WD or NC, mice were gently anesthetized using a mixture of isoflurane (2%) and oxygen (0.6 L/min). After which, Vevo 1100 imaging system (VisualSonics, Fujifilm) equipped with a 22- to 55-MHz MS550D transducer was used in order to assess the structural and functional parameters of the heart (18, 54). Transthoracic short axis view at mid-papillary level view was used to obtain M-mode recordings while doppler tracings were acquired from the apical four chamber view. These recordings were used to measure and/or calculate the structural and functional parameters of the heart.

Morphometric analysis.

Female mice assigned to NC or WD were weighed on a weekly basis during the 14-weeks of diet duration. During exsanguination mice were anesthetized using a mixture of isoflurane (2%) and oxygen (0.6 L/min). Each animal was checked for adequate sedation, after which the heart was quickly excised through the abdominal/diaphragm region. Heart perfusion with Krebs-Henseleit buffer was used to provide adequate blood clearance. KCL (30 mM) was used to arrest the heart in diastole. The heart was then blotted to remove excess fluid and weighed. Collection and weighing of adipose tissue (subcutaneous and visceral fat) was performed via the removal of the epidermal skin layer post-mortem. Shortly after exsanguination, the left leg was fully extended and Vernier calipers (Monostat) were used to measure tibia length. Isolated heart was subdivided into two transverse sections (base/mid and apex) and paraffin embedded. Mid-cardiac transverse sections (5μm thick) were used for fibrosis and septal wall measurements using Masson's trichrome staining. WD-induced fibrosis was assessed and analyzed using ten separate septal images using Nikon NIS software as previously described (20). Percent fibrosis was calculated by division of total fibrosis by total area multiplied by 100. To determine septal wall hypertrophy, six separate transverse measurements were acquired for each heart and averaged. To examine WD-induced lipid accumulation, mid-cardiac cryosections (10 μm thick) were stained with Oil red O. Nikon NIS software was then used to visualize the staining.

Western blot analysis.

Cardiac lysates were prepared in RIPA buffer and supplemented with Halt protease inhibitor cocktail as described (64). Equal amounts of proteins (50 μg) were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% nonfat dry milk for 1

hour and incubated overnight with primary antibodies against ATM (1:500, Cat# sc23921, Santa Cruz), MMP-9 (1:1000, Cat# AB19016, Millipore), MMP-2 (1:500, Cat# MAB3308, Millipore), BAX (1:1000, Cat# SC7480, Santa Cruz), PARP-1 (1:1000, Cat# 9542, Cell Signaling), p-Akt (ser473; 1:1000, Cat# 9271S, Cell Signaling), total Akt (1:1000, Cat# 9272S, Cell Signaling), p-mTOR (ser-2448; 1:1000, Cat# 5536S, Cell Signaling), total mTOR (1:1000, Cat# 2983S, Cell Signaling), Glut-4 and Collagen-1α1 (1:500, Cat#72026, Cell Signaling). The immune complexes were detected using appropriate secondary antibodies and chemiluminescent reagents (64). There was no significant difference in GAPDH levels among the groups, therefore protein loading in each lane was normalized using GAPDH immunostaining (1:10,000; Cat# 32233, Santa Cruz). Band intensities were quantified using ImageQuant LAS 500 imaging system (GE Healthcare). For phosphoproteins, total protein levels were normalized using GAPDH immunostaining. This analysis showed no significant difference in the expression of total proteins (Fig 8). Therefore, phosphoprotein band intensities were normalized using GAPDH immunostaining.

Terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay.

Tunnel assay was performed according to the manufacturer's instructions (In Situ Cell Death Detection Kit, Roche) (19). Mid-cardiac tissue sections (5μm thick) were counterstained with rhodamine-conjugated wheat germ agglutinin (WGA, Rl-1022, Vector) to visualize myocytes and Hoechst 33258 (10 μM; Sigma) to visualize nuclei. To count the total number of nuclei, Hoechst-positive stained nuclei were examined and quantified. TUNEL-positive staining clearly allowed visualization and quantification of apoptotic myocytes within WGA-stained cells and Hoechst-positively stained nuclei. The index of myocyte and total cell apoptosis was calculated as the percentage myocyte apoptotic nuclei/total nuclei as described (64).

Myocyte cross-sectional area.

Mid-cardiac cross-sections (5μm thick) were stained with WGA to measure myocyte cross-sectional area. Images were obtained with the EVOS M7000 imaging system and myocyte cross-sectional area was quantified with Nikon NIS software as previously described (64). Suitability of myocytes for cross-sectional area was defined by circular cell body with a centrally located nucleus.

Cholesterol and triglyceride assay.

Non-hemolyzed serum was used for measurement of total cholesterol and triglyceride levels according to manufacturer's instructions (Pointe Scientific). Sample to reagent ratio was diluted to 1:1000 and serum standards provided by the manufacturer were used to verify the validity of reaction. Samples were read at 500nm wavelength using a BioTek PowerWave XS2 microplate spectrophotometer. Cholesterol and triglyceride levels were determined as: absorbance (sample)/absorbance (standard) multiplied by the concentration of standard.

Statistical analysis.

All the data shown is expressed as means ± SE. Data were analyzed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test or 2-tailed Student's t-test. Probability (p) values of <0.05 were considered to be significant.

Results

Morphometric analyses.

Weight gain with concomitant increases in visceral and subcutaneous adipose tissue is commonly observed with WD (27, 42). All groups were weighed weekly during the 14-week diet duration. Previously, male WT and ATM deficient mice displayed significant WD-induced weight gain compared to NC groups. However, hKO-WD group accelerated weight gain at weeks 5, 6, 7, 8 and 10 compared to WT-WD (64). Here, starting weights were not different between all four groups. However, WD significantly increased weight gain in WT-WD vs WT-NC at week 1, 3, 4, 6, 7, 9, 10, 11, 13 and 14 (Fig 4.1).

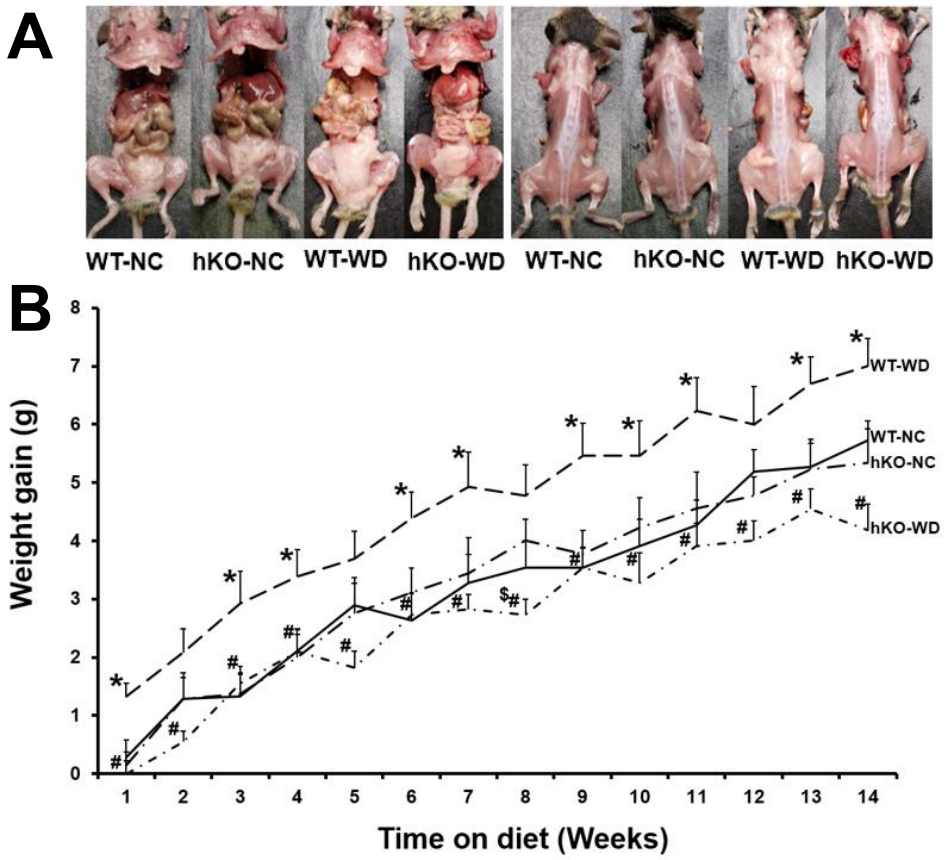


Figure 4.1 WD Induced Weight Gain with Time.

A. Visceral (left panel) and subcutaneous (right panel) adipose distribution after 14 weeks on NC or WD. **B.** Weight gain for normal chow (NC) and Western-type diet (WD) groups from week 0 to week 14. $^{\$}p<0.05$ vs hKO-NC, $^{*}p<0.05$ vs WT-NC, $^{\#}p<0.05$ vs WT-WD, n=9-13.

Interestingly, WD significantly increased weight gain in WT-WD vs hKO-WD throughout the entire duration of feeding (Weeks 1-14). WT-WD mice exhibited significantly increased total fat (visceral and subcutaneous) accumulation vs WT-NC. Conversely, hKO-WD displayed a significant decrease in weight gain and total fat compared to WT-WD, and WD failed to significantly increase weight gain and total fat accumulation vs hKO-NC (Table 4.1). Heart weight (HW) to body weight (HW/BW) ratio equally decreased in both WD groups compared to their respective NC counterpart. Weight to tibia length ratio is commonly used to assess cardiac hypertrophy (64, 66). Similar to male mice (64), the heart weight/tibia length ratio was found to

be significantly higher in hKO-NC vs WT-NC. However, the ratio was found to be significantly lower in both WD groups vs NC. WD significantly increased serum cholesterol levels in both WD groups. WD failed to increase triglyceride levels in WT-WD vs WT-NC (Table 4.1). However, triglyceride levels were significantly higher in hKO-WD vs WT-WD and hKO-NC. Oil red-O-staining displayed no visible lipid accumulation in the myocardium of WD groups (data not shown). Fasting glucose levels were significantly higher in hKO-NC vs WT-NC and remained higher in hKO-WD vs WT-WD (Table 4.1).

Table 4.1 Morphometric and Biochemical Measurements

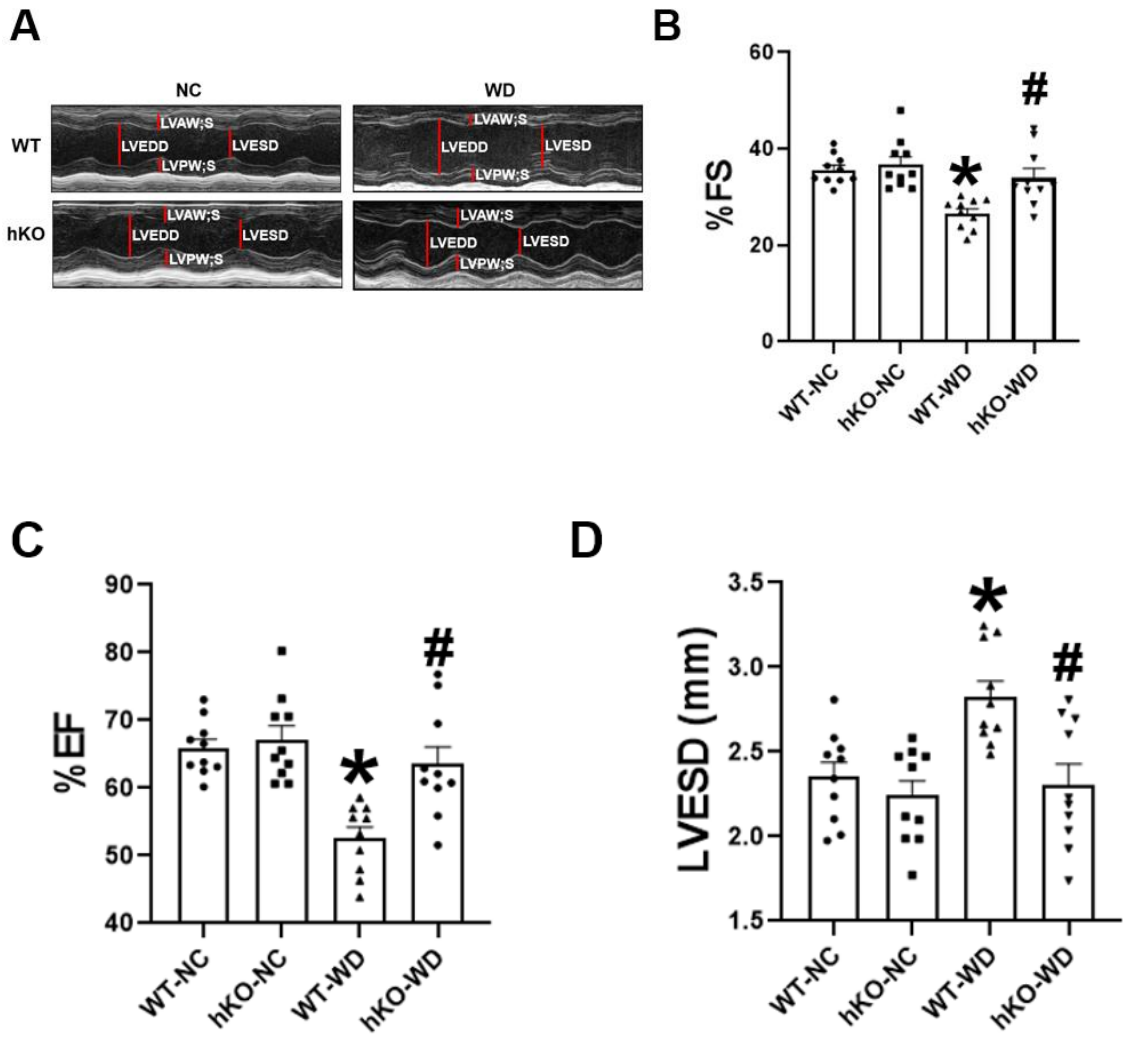
Parameters	**WT-NC**	**hKO-NC**	**WT-WD**	**hKO-WD**
Weight gain (g)	5.9 ± 0.39	4.8 ± 0.76	7.1 ± 0.38*****	4.5 ± 0.55$^{\#}$
Heart weight (mg)	126 ± 4	131 ± 5	110 ± 5*****	108 ± 4$^{\$}$
Heart weight/body weight (mg/g)	5.5 ± 0.1	5.6 ± 0.2	5.0 ± 0.2	4.9 ± 0.2$^{\$}$
Heart weight/tibia length (mg/mm)	15.1 ± 0.5	17.7 ± 0.7$^{\&}$	13.2 ± 0.6*****	14.6 ± 0.6$^{\$}$
Total fat (mg)	716 ± 75	666 ± 89	1838 ± 124*****	1054 ± 84$^{\#}$
Total cholesterol (mg/dL)	80 ± 2.6	91 ± 5.9	164 ± 6.8*****	214 ± 17.7$^{\$\#}$
Triglycerides (mg/dL)	40 ± 5.9	36 ± 6.5	59 ± 14.7	127 ± 7.9$^{\$\#}$
4 hr Fasting Glucose (mg/dL)	133 ± 2.4	146 ± 1.6$^{\&}$	130 ± 6.0	148 ± 6.3$^{\#}$

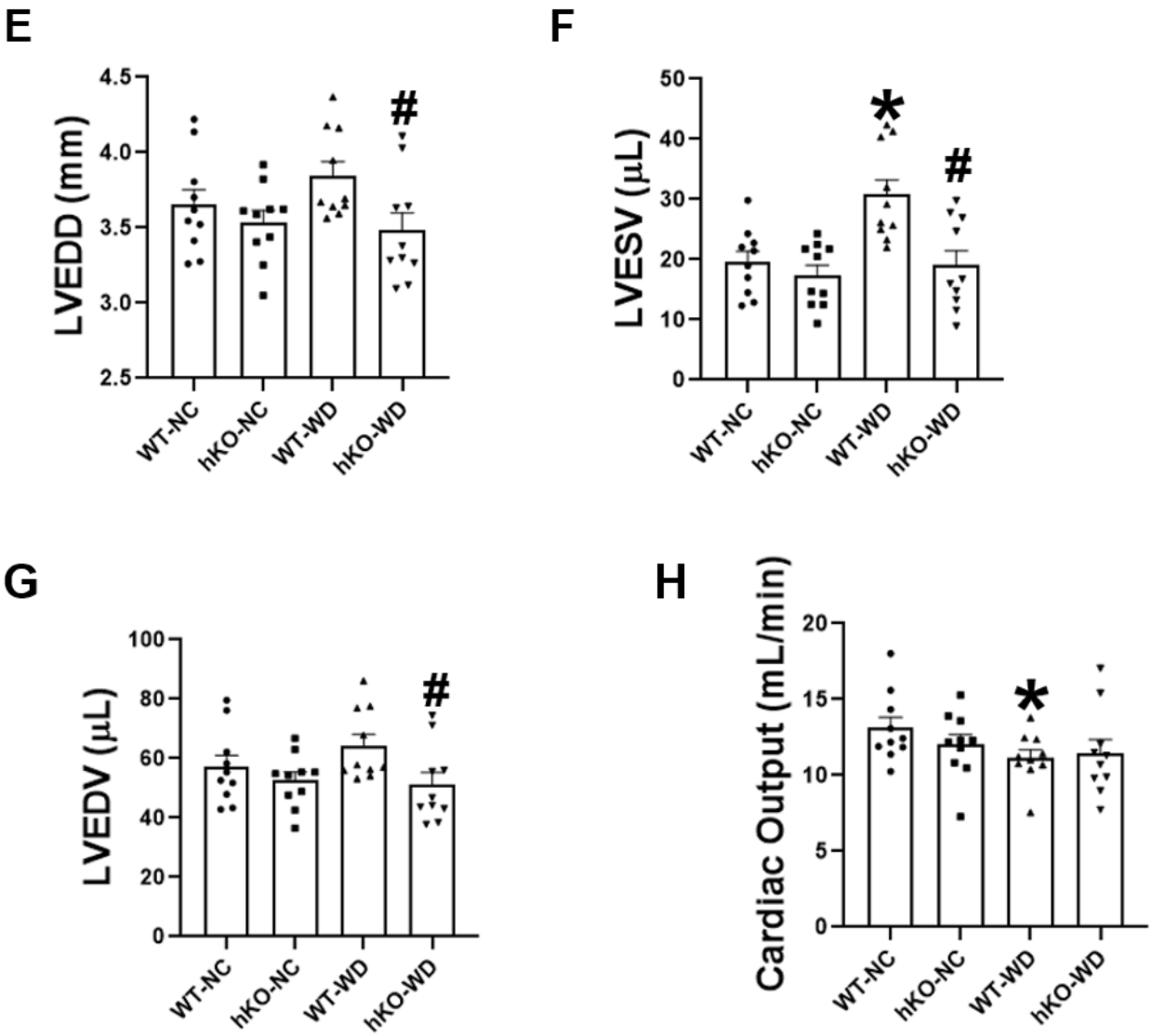
Values are means ± SE, n=8-13. *p<0.05 vs WT-NC; $^{\&}$p<0.05 vs WT-NC; $^{\$}$p<0.05 vs hKO-NC. $^{\#}$p<0.05 vs WT-WD. WT-NC, wild-type on normal chow; hKO-NC, heterozygous knockout on normal chow; WT-WD, wild-type on western-type diet; hKO-WD, heterozygous knockout on western diet$^{\$}$.

Echocardiographic measurements.

In male mice, WD decreased percent fractional shortening (%FS), ejection fraction (%EF) and increased LV end systolic diameter (LVESD) and volume (LVESV) to a similar extent in WT-WD and hKO-WD groups (64). Here, M-mode echocardiography revealed no functional or structural difference between the female NC groups. WD decreased %FS, %EF, cardiac output,

LV diastolic anterior wall and LV systolic posterior wall and increased LVESD and LVESV in WT-WD group when compared to NC counterpart (Fig 4.2 B; C; D; F; H; I; J; L). Interestingly, WD failed to induce any functional or structural changes in hKO-WD compared to normal chow counterpart. %FS, %EF, cardiac output, LVAW;D and LVPW;S were significantly higher and LVESD and LVESV were significantly lower in hKO-WD vs WT-WD (Fig 4.2). Pulsed-wave Doppler analysis previously showed no significant change in diastolic parameters such as AET, IVCTR, IVRT and Tei Index among the 4 groups (Table 4.2).

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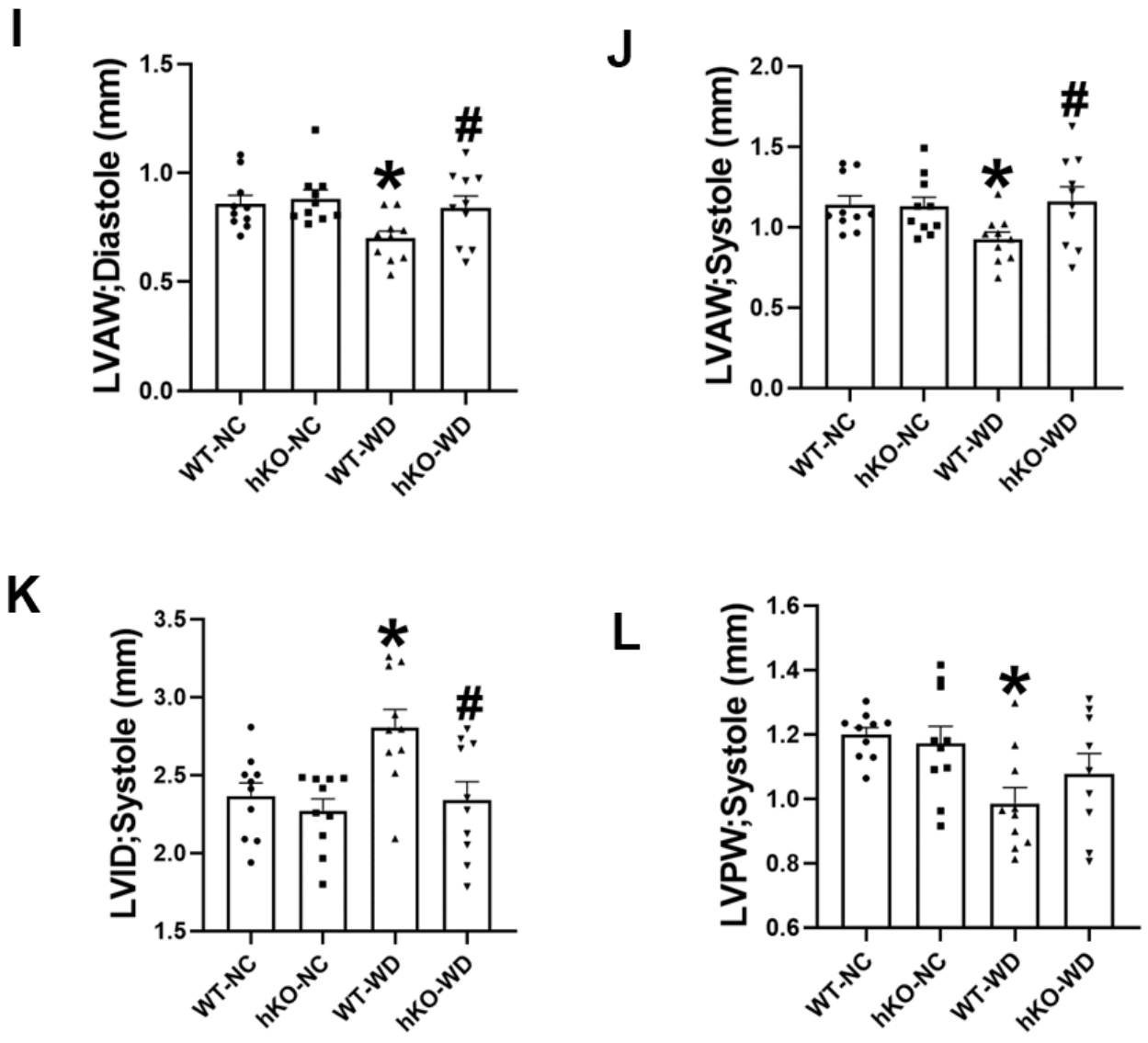


Figure 4.2 WD-Induced Changes in M-mode Parameters of the Heart.

Indices of m-mode parameters: % fractional shortening (%FS), % ejection fraction (%EF), LV end systolic diameter (LVESD), LV end diastolic diameter (LVEDD), LV end systolic volume (LVESV), LV end diastolic volume (LVEDV), cardiac output, Diastolic LV anterior wall thickness (LVAW;D,) Systole LV anterior wall thickness (LVAW;S) Systole LV internal diameter (LVID;S) and Systole LV posterior wall thickness (LVPW;S) were measured using M-mode echocardiographic images following 14-weeks of normal chow or Western-type diet (WD). (A) Representative M-mode tracings for each group; (B) %FS; (C) %EF; (D) LVESD; (E) LVEDD; (F) LVESV; (G) LVEDV; (H) cardiac output; (I) LVAW,D; (J) LVAW,S; (K) LVID,S; and (L) LVPW,S. *$p<0.05$ vs WT-NC, $^{\#}p<0.05$ vs WT-WD, n=10.

Table 4.2 PW Echocardiographic Parameters

Parameters	WT-NC	hKO-NC	WT-WD	hKO-WD
E-wave (mm/s)	531.6 ± 48.1	486.6 ± 29.8	421.91 ± 36.6	497.50 ± 28.5
A-wave (mm/s)	344.6 ± 48.4	258.95 ± 16.3	255.18 ± 19.5	301.95 ± 23.3
AET (msec)	59.06 ± 1.43	62.65 ± 1.59	58.76 ± 1.73	59.37 ± 1.93
IVCT (msec)	17.78 ± 1.10	19.12 ± 1.01	17.87 ± 0.86	17.97 ± 1.17
IVRT (msec)	23.45 ± 0.71	22.75 ± 0.67	24.40 ± 1.06	24.51 ± 1.06
Tei Index	0.69 ± 0.01	0.67 ± 0.02	0.72 ± 0.04	0.72 ± 0.04

Values are means ± SE, n=10. AET, aortic ejection time; IVCT, isovolumetric contraction time; and IVRT, isovolumetric relaxation time.

Fibrosis, apoptosis, and hypertrophy.

WD can induce a variety of pathological modifications, which include increases in cardiac fibrosis, hypertrophy and exacerbation of apoptosis (3, 26, 35, 44). Previously, male hKO-NC group exhibited increased fibrosis compared WT-NC (64). Consistent with these findings, we observed a significant increase in percent fibrosis in female hKO-NC group vs WT-NC. WD induced a significant increase WT group, while no increase in fibrosis was observed in hKO-WD vs hKO-NC (Fig 4.3 A and B). Percent of myocyte and total cell apoptosis were also significantly higher in hKO-NC vs WT-NC. WD induced a significant increase in myocyte and total cell apoptosis in both female genotypes. However, hKO-WD group exhibited significantly lower myocyte and total apoptosis vs WT-WD (Fig 4.4 A-C). WD and excessive adipose accumulation independently contribute to the development of cardiac hypertrophy (4, 27). Percent hypertrophy, as assessed by myocyte cross-sectional area and septal wall width was higher in female hKO-NC vs WT-NC. WD significantly increased myocyte cross-sectional area and septal wall width in WT-WD groups vs WT-NC (Fig 4.5 A-C). However, no significant increase in myocyte cross-sectional

area or septal wall width was observed in hKO-WD vs hKO-NC. Myocyte cross-sectional area, not septal width, was found to be significantly higher in hKO-WD vs WT-WD.

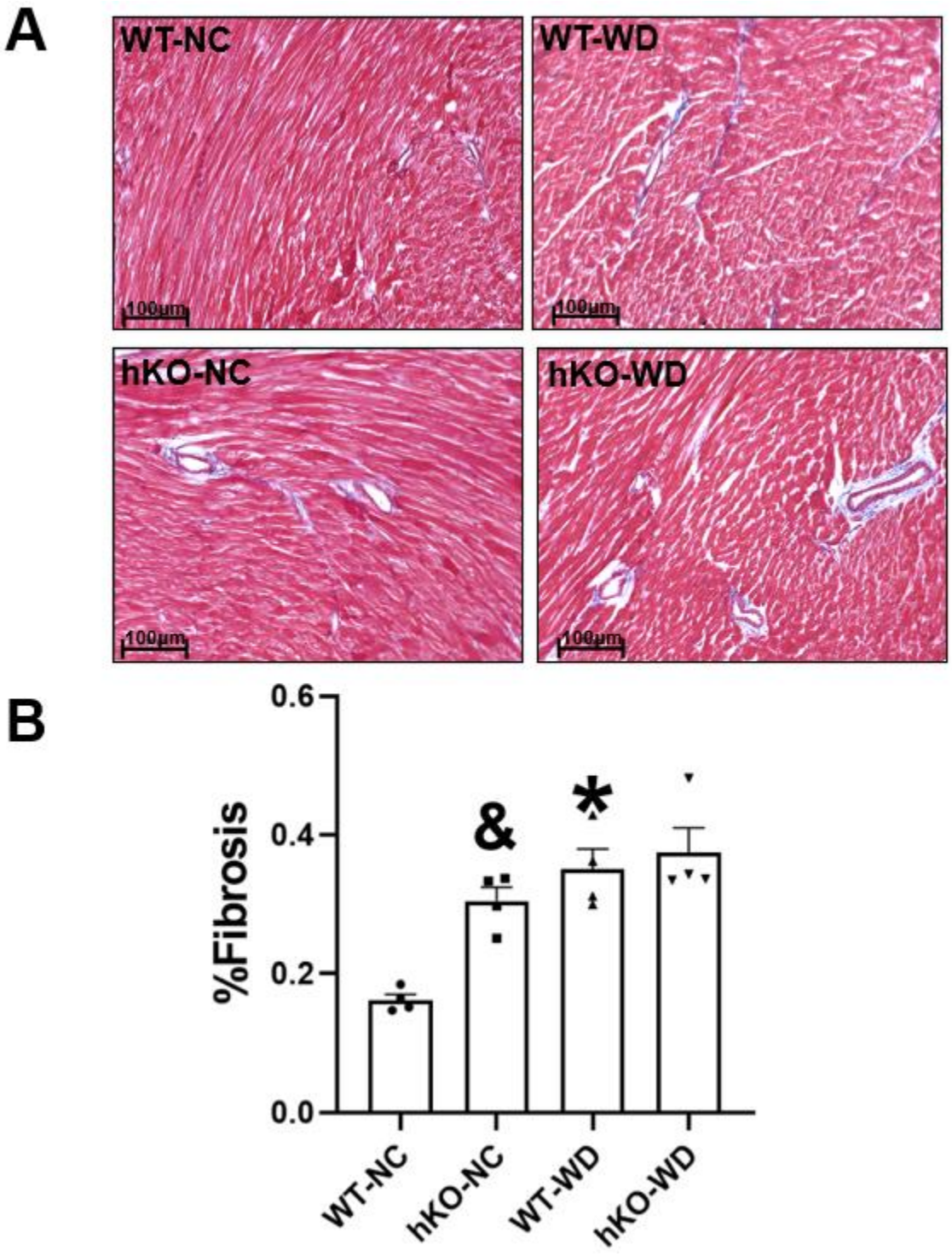
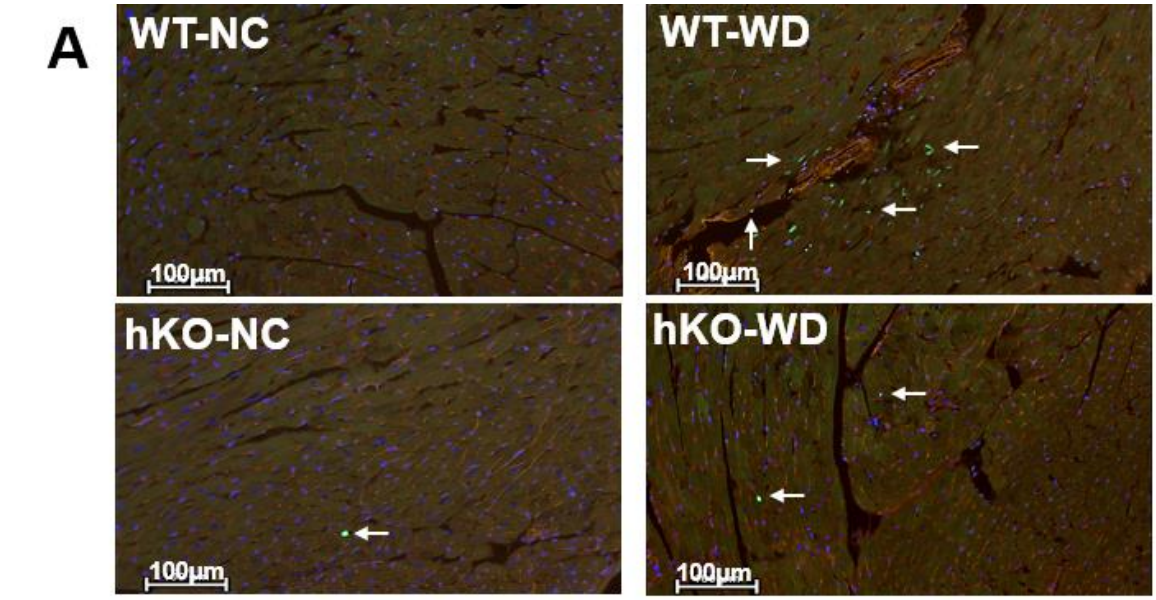


Figure 4.3 WD Exacerbates Fibrosis in Wild-Type Mice.

A. Masson's trichrome-stained sections of the heart for each group and associated diet. Blue staining indicates fibrosis, while red staining indicates live tissue. B. Quantitative measurements of fibrosis. $^{\&}p<0.05$ vs WT-NC, *$p<0.05$ vs WT-NC, n=4.



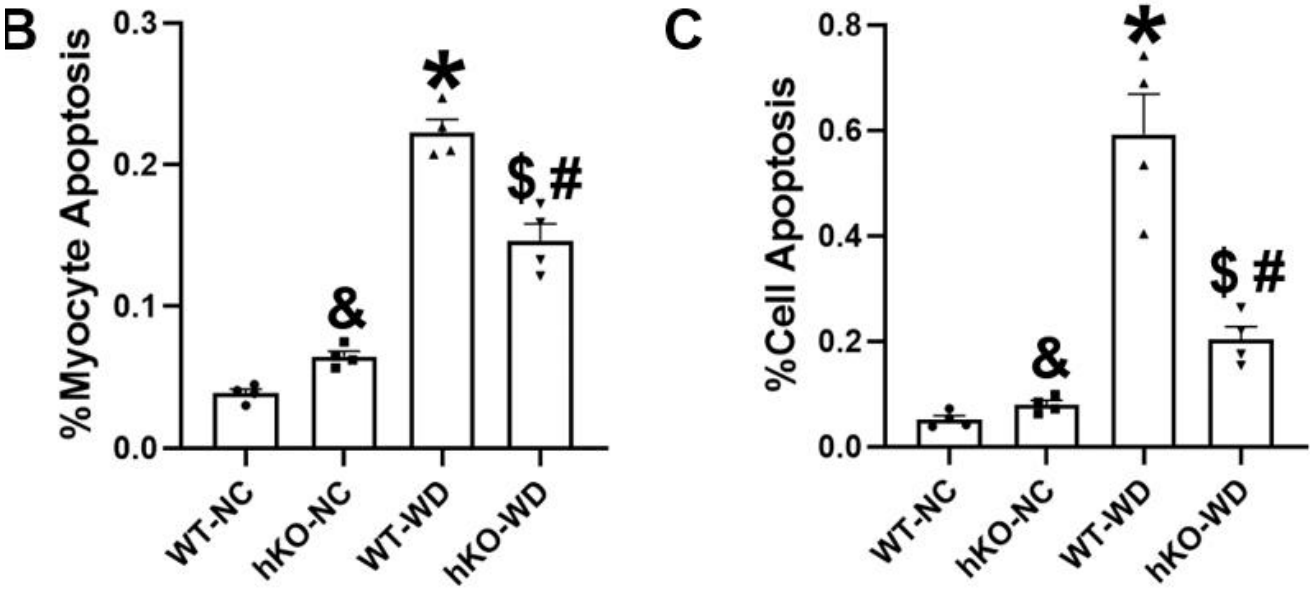


Figure 4.4 ATM Deficiency Attenuates Apoptosis in the Heart in Response to Western-Type Diet.

A. Representative images of TUNEL (green), WGA (red) and Hoechst (blue) stained hearts. B. Quantitative analysis of myocyte apoptosis. C. Quantitative analysis of cardiac cell apoptosis. $^{\&}p<0.05$ vs WT-NC, *$p<0.05$ vs WT-NC, $^{\$}p<0.05$ vs hKO-NC, $^{\#}p<0.05$ vs WT-WD, n=4.

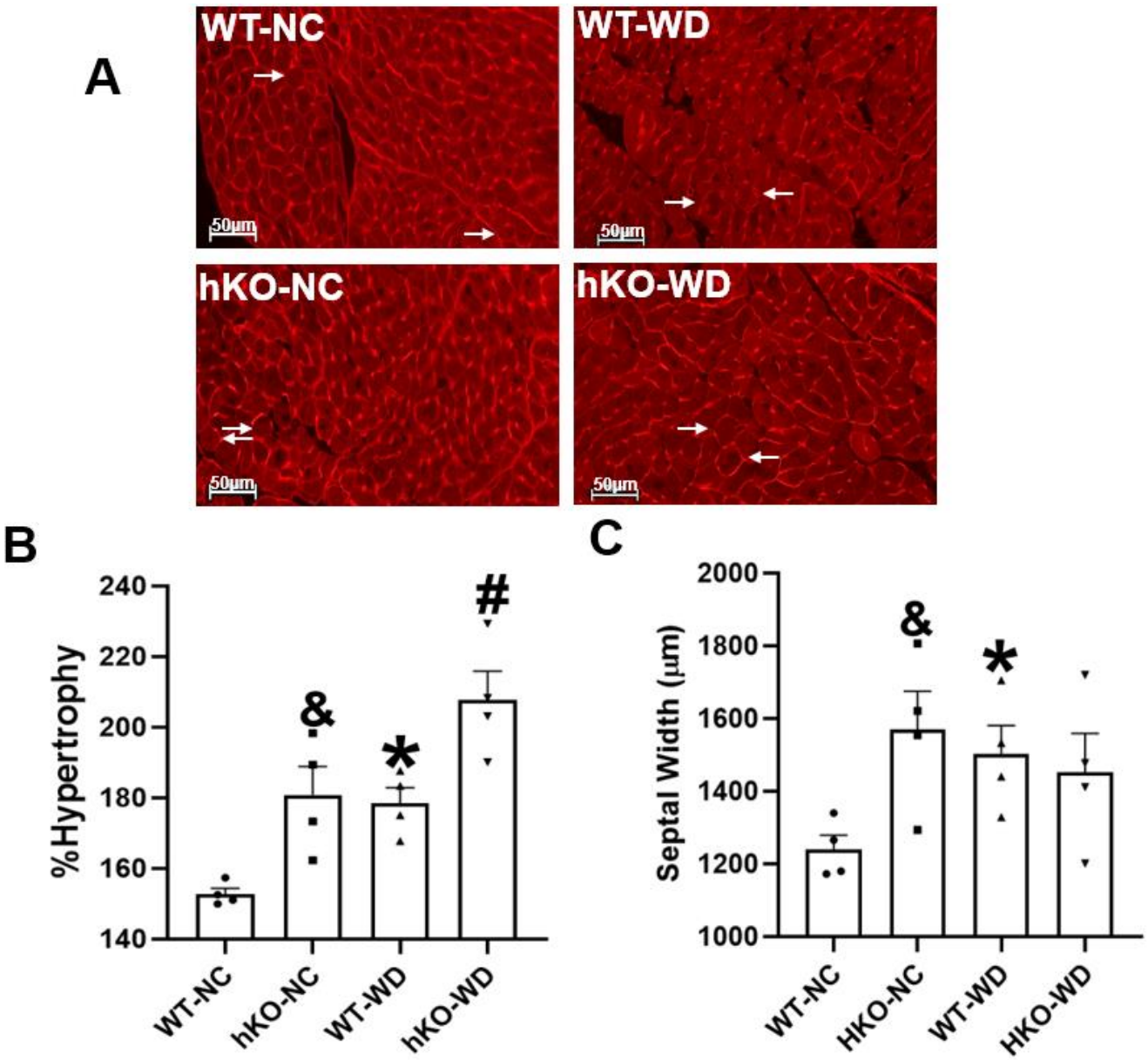


Figure 4.5 ATM Deficiency Exacerbates Myocyte and Septal Hypertrophy in Response to Western-Type Diet.

A. Representative images of wheat germ agglutinin (WGA)-stained cross-sections of the heart depicting myocytes. B. Quantitative analysis of myocyte cross-sectional area. C. Quantitative analysis of septal hypertrophy using Masson's trichrome-stained hearts. $^{\&}p<0.05$ vs WT-NC, $^{*}p<0.05$ vs WT-NC, $^{\#}p<0.05$ vs WT-WD, n=4.

Expression of collagen-1α1, MMP-2, MMP-9.

Cardiac fibrosis is commonly observed with WD and ATM deficiency (19, 27, 34, 37, 38). Excessive accumulation of extracellular matrix and collagen-1 deposition can increase tensile scar strength and reduce myocardial contractile ability (1, 25, 27, 41, 53). Previously, western blotting analysis revealed that expression of collagen-1α1 was significantly greater in male hKO-NC vs WT-NC group (64). Here, female hKO-NC group exhibited a significant increase in collagen-1α1 expression vs WT-NC group. WD significantly increased collagen-1α1 expression in hKO-WD VS hKO-NC (Fig 4.6 A). However, no significant increase in collagen-1α1 expression was observed in female WT-WD vs WT-NC. Collagen-1α1 expression was found to be significantly higher in hKO-WD vs WT-WD (Fig 4.6 A).

The dysregulation of matrix metalloproteinase (MMP)-2 and MMP-9 have been observed with obesity and contribute to the development of cardiovascular disease (8, 31, 59). Previously, male hKO-NC group exhibited increased expression of MMP-2 and MMP-9 vs WT-NC group and WD only increased MMP-2 and MMP-9 expression in WT groups (64). Here, MMP-2 and MMP-9 expression was significantly increased in female hKO-NC vs WT-NC. WD significantly increased expression of MMP-2 and MMP-9 in WT-WD vs WT-NC. However expression of MMP-2 and MMP-9 remained unchanged in hKO-WD vs hKO-NC and there was no significant change between hKO-WD vs WT-WD (Fig 4.6 B and C).

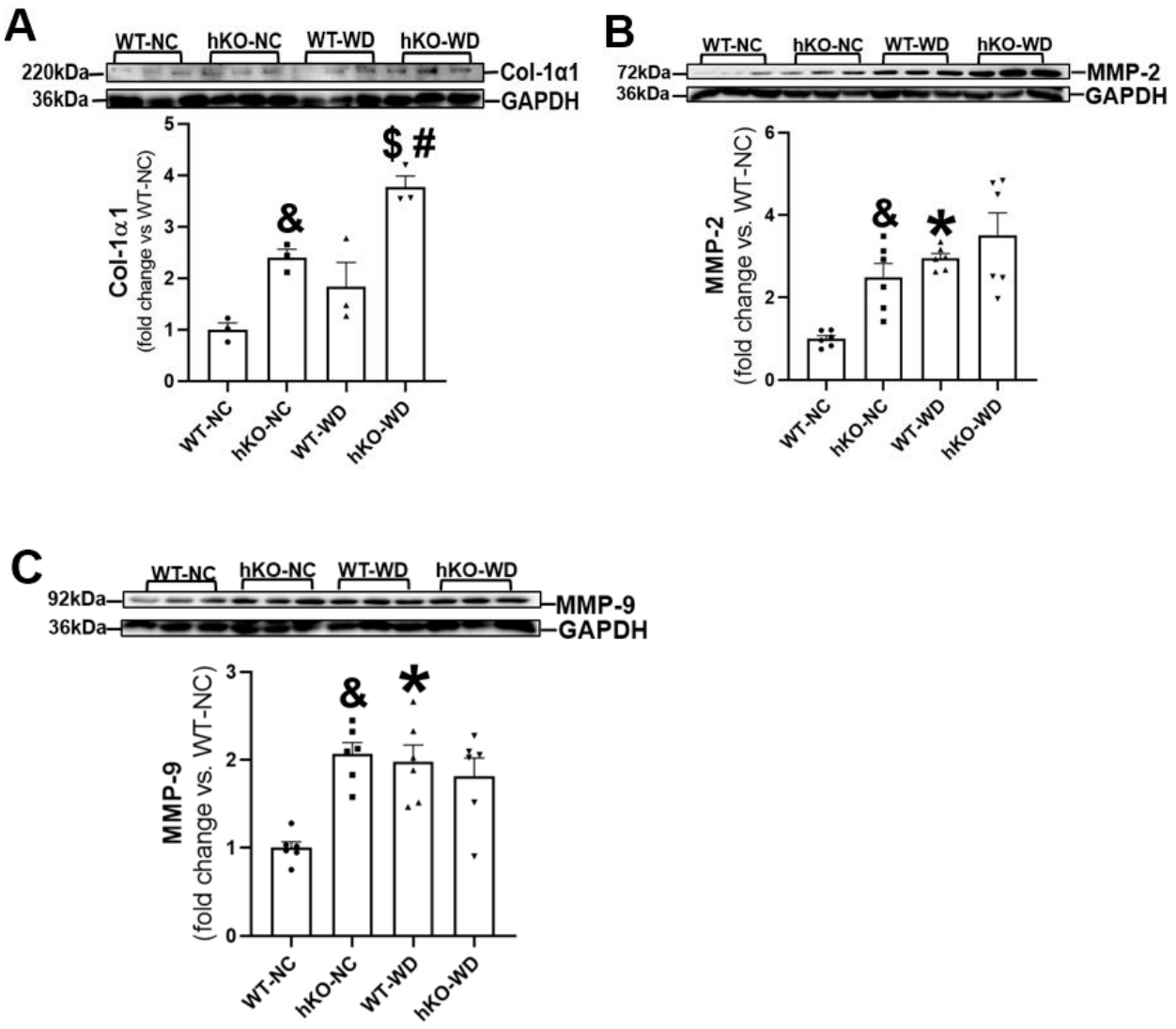


Figure 4.6 Expression of Collagen-1α1 (Col-1α1), MMP-2 and MMP-9 in the Heart.

Heart lysates were analyzed by western blots using anti-Col-1α1 (A), MMP-2 (B), and MMP-9 (C) antibodies. Upper panels exhibit immunostaining for Col-1α1, MMP-2 and MMP-9. Lower panels exhibit quantitative analyses normalized to GAPDH. $^{\&}p<0.05$ vs WT-NC, $^{*}p<0.05$ vs WT-NC, $^{\$}p<0.05$ vs hKO-NC, $^{\#}p<0.05$ vs WT-WD, n=3-6. Western blot membrane used in Fig 8B was also probed for Glut-4 in Fig 8C. GAPDH in Fig 8B was also used to normalize total Glut-4 signal in Fig 8C as the same membrane was probed for p-Akt, Glut-4 and GAPDH.

Activation of proteins related to apoptosis and energy metabolism.

Diets high in fat associate with increased expression of Bax, a pro-apoptotic protein, in the myocardium (9). Previously, male hKO-NC group associated with increased Bax expression compared to WT-NC and WD induced a significant increase in Bax expression in male hKO-WD group vs WT-WD (64). Here Bax expression was higher in hKO-NC vs WT-NC. WD significantly increased Bax expression in WT-WD vs WT-NC and was found to significantly lower in hKO-WD vs WT-WD and hKO-NC (Fig 4.7 A).

PARP-1 is an important DNA damage senor for single and double stranded DNA breaks (48). Previously, male WT-WD mice exhibited increased PARP-1 (full-length, 117kDa) protein levels vs hKO-WD and WT-NC counterpart, while no change was observed in hKO-WD vs hKO-NC (64). Here, PARP-1 (117kDa) protein levels were significantly lower in hKO-NC vs WT-NC. WD decreased PARP-1 protein levels in WT-WD vs WT-NC, while hKO-WD exhibited significantly higher levels of PARP-1 vs WT-WD and hKO-NC (Fig 4.7 B).

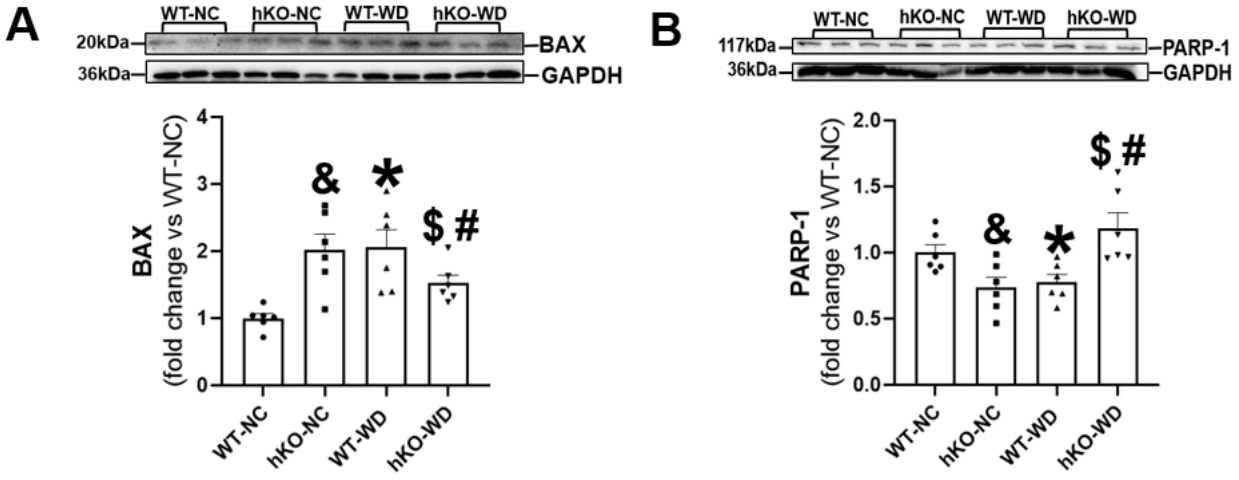
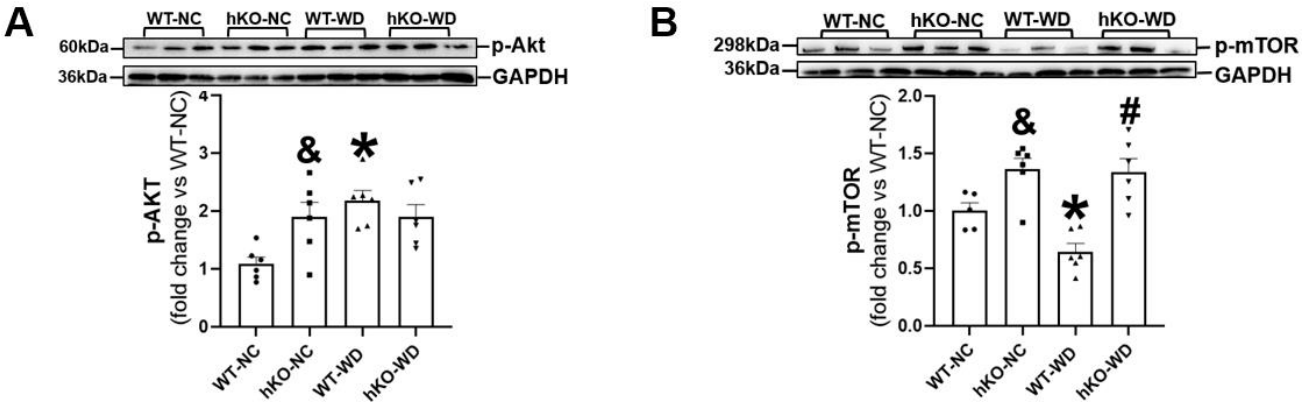


Figure 4.7 ATM Deficiency Attenuates Expression of Bax and Increases PARP-1 Expression Following WD.

Heart lysates were analyzed by western blots using anti-BAX (A) and PARP-1 (B) antibodies. Upper panels exhibit immunostaining for BAX and PARP-1. Lower panels exhibit quantitative analyses normalized to GAPDH. $^{\&}p<0.05$ vs WT-NC, $^{*}p<0.05$ vs WT-NC, $^{\$}p<0.05$ vs hKO-NC, $^{\#}p<0.05$ vs WT-WD, n=6.

Obesity and WD modulate mTOR signaling, which may exacerbate insulin resistance and other obesity-related diseases (32, 40). Studies have also shown increased Akt activation in the myocardium following WD (23). Similar to our previous findings in male mice (64), female hKO-NC group displayed significantly increased Akt activation vs WT-NC group. WD induced an increase in Akt activation in WT-WD vs WT-NC. Akt activation remained unchanged in hKO-WD vs hKO-NC with no significant difference between the two WD groups (Fig 4.8 A). Previously, mTOR activation was higher in male hKO-NC vs WT-NC group, and WD attenuated mTOR activation only in hKO-WD vs hKO-NC (64). Here, mTOR activation was significantly higher in hKO-NC vs WT-NC (Fig 8B). WD decreased activation of mTOR in WT-WD vs WT-NC. However, its activation remained hKO-WD vs hKO-NC, and mTOR activation was higher in hKO-WD vs WT-WD (Fig 4.8 B). While hKO mice exhibit higher glucose levels vs their WT counterparts, Glut-4 protein expression remained unchanged among the four groups (Fig 4.8 C).



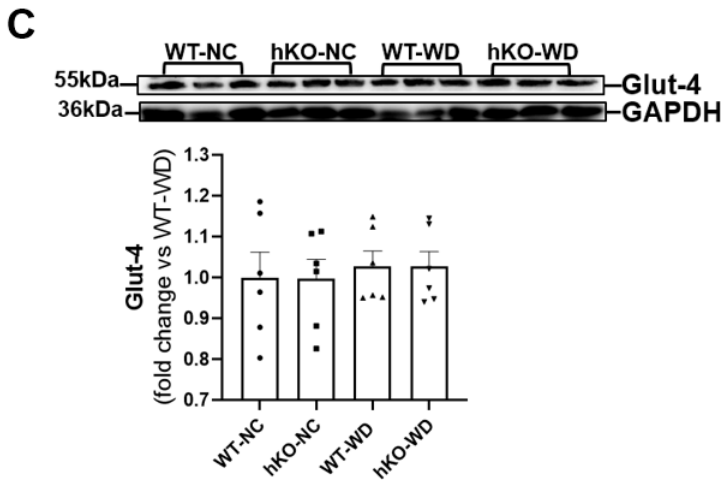


Figure 4.8 Phosphorylation of Akt, mTOR, and Expression of GLUT-4.

Heart lysates were analyzed by western blots using anti-p-Akt and total Akt (A), p-mTOR and total mTOR (B), Glut-4 (D) antibodies. Bar graphs exhibit quantitative analyses normalized to GAPDH. $^{\&}p<0.05$ vs WT-NC, $^{*}p<0.05$ vs WT-NC, $^{\#}p<0.05$ vs WT-WD, n=6 for phosphoproteins and Glut-4; n=3 for total proteins.

Discussion

The majority (~60%) of the American population consume a WD and are overweight or obese (15, 30, 45, 47). A-T carriers, make up ~1.4-2.0% of the general population, are more vulnerable to ischemic heart disease. Therefore, consumption of WD can further increase the risk of A-T carriers developing heart disease (28, 65). Previously, we have shown that WD during ATM deficiency associates with systolic dysfunction, increased preload and exacerbation of cardiac remodeling in male mice (64). This study explored the WD-induced structural and functional alterations in female ATM deficient mice. The major findings of the study are that ATM deficiency in female mice- 1) attenuates body weight and fat weight gain; 2) preserves cardiac function; 3) increases myocyte hypertrophy; 4) inhibits WD-induced cardiac cell apoptosis; 5) alters the expression and activation of signaling molecules associated with fibrosis, apoptosis, and metabolism; and 6) increases triglycerides, and cholesterol levels.

Increases in visceral and subcutaneous adipose tissue is a common consequence of long term WD consumption (26, 62). Evidence has been provided that lack of ATM, attenuates fat accumulation (56). However, little is known regarding the effect of WD on A-T carriers. Previously, we have shown that ATM deficiency in male mice associate with accelerated weight gain during weeks 5, 6, 7, 8, and 10 in response to WD vs WT. At the completion of WD (14-weeks post-WD), male WD mice did not exhibit any differences in adipose accumulation (subcutaneous and visceral), total serum cholesterol and triglyceride levels, and displayed no lipid deposition in the myocardium (64). Here we observed that female ATM deficient mice experience attenuated weight gain, fat accumulation and increased serum levels of cholesterol and triglycerides in response to WD. The lower weight gain in female ATM deficient mice was observed one-week post-WD, which continued to be lower throughout the 14 week observation period. The attenuation of WD-induced fat accumulation in female ATM deficient mice is consistent with previous a report that linked ATM abolishment with decreased adipocyte expansion (56). Taken together with our previous observation (64), it appears that ATM deficiency affects body weight and fat accumulation in a sex-specific manner. It is speculated that ATM deficiency in female mice may inhibit adipocyte development and maturation. This notion is supported by the observation of increased serum cholesterol and triglyceride levels in ATM deficient females as they may have a reduced ability to store excess energy as fat.

High fat diets and ATM deficiency independently associate with the development of insulin resistance (1, 4, 52, 61, 65). Fasting glucose levels in male mice were not significantly different between normal chow groups. However, WD led to significant increase in fasting blood glucose levels in WT group, not in hKO (64). Conversely, we observed higher fasting glucose levels in ATM deficient females on normal chow. WD failed to increase fasting glucose levels in

WT group, and fasting serum glucose levels remained significantly higher in hKO-WD group vs WT-WD. The observed changes in glucose levels point to the possibility insulin resistance is exacerbated in ATM deficient mice without WD and remains in the setting of WD.

The severity and duration of excess fat accumulation and/or WD can directly influence the degree of cardiac mechanical and structural dysfunction (1, 11, 21, 26). The increased circulating blood volume observed in obesity can contribute to chamber dilation, leading to systolic dysfunction (4). Previously, we demonstrated that WD in male mice induce similar systolic dysfunction between genotypes with decreased %FS and %EF, and increased LVESD and LVESV (64). Diastolic dysfunction was also noted with WT-WD male group presenting with increased afterload, while hKO-WD male group displayed increased preload. Here, we demonstrate that WD only induces systolic dysfunction in female WT mice with decreased %FS and %EF, and increased LVESD and LVESV. However, all these systolic parameters remained unchanged in response to WD during ATM deficiency. Further, WD did not induce any diastolic dysfunction in either genotype. Interestingly, increases in visceral adiposity is an independent indicator of the development of cardiovascular dysfunction (2, 50). Therefore, reduced WD-induced fat accumulation in female ATM deficient mice may play a role in the preservation of cardiac function.

WD, adiposity and ATM deficiency independently associate with increased cardiac fibrosis, hypertrophy, and cardiac dysfunction (4, 19, 20, 24, 27). Previously, we demonstrated that ATM deficiency in male mice associated with increased fibrosis and hypertrophy at basal levels on NC. WD induced a significant increase in fibrosis in the male WT group compared to NC. However, WD failed to increase fibrosis in hKO group compared to NC counterpart. Further,

hKO-WD exhibited significantly increased hypertrophy compared to WT-WD (64). Here we observed that female ATM deficient mice also exhibit increased levels of fibrosis and hypertrophy at basal levels. WD significantly increased fibrosis and hypertrophy in WT-WD vs WT-NC. Interestingly, myocyte hypertrophy as measured by increased myocyte cross-sectional area was higher in hKO-WD vs WT-WD. Obesity with concomitant changes in hemodynamics can exacerbate fibrosis and hypertrophy. Further, the degree of adipose tissue accumulation correlates with increased cardiac remodeling (4, 27). As fibrosis generally precedes the development of hypertrophy (4), it is possible that the observed basal increase in cardiac fibrosis and hypertrophy observed in female ATM deficient mice are serving as a protective mechanism against WD-induced cardiac dysfunction.

The continual increases in blood volume associated with obesity can induce excessive cardiac remodeling and lead to cardiac cell apoptosis (4). Further, WD and adiposity may exacerbate cardiac apoptosis due to lipotoxicity, decreased metabolic flexibility and associated increase in toxic byproducts and increased oxidative stress (3, 10, 14, 26, 35, 63, 67). Previously, ATM deficiency in male mice associated with increased myocyte and total cell apoptosis at basal levels compared to WT. WD induced a significant increase in myocyte apoptosis in both genotypes. However, male hKO-WD groups exhibited a ~ 2.5-fold increase in myocyte apoptosis compared to WT-WD. Here we observed that ATM deficiency in female mice also associates with increase in myocyte and total apoptosis compared to WT. WD increased myocyte and total apoptosis in both genotypes. However, apoptosis was significantly lower in ATM deficient mice. In fact, total cell apoptosis was found to be ~2 fold less in hKO-WD compared to WT-WD. The lower level of apoptosis in the myocardium of ATM deficient female mice may serve as an additional cardioprotective mechanism during WD-induced cardiac remodeling.

Previously, male ATM deficient mice associated with basal increases in fibrosis and collagen-1α1 compared to WT (64). Here we show that female ATM mice also display increased collagen-1α1 and fibrosis at basal levels. Interestingly, WD exacerbated the expression of collagen-1α1 in ATM deficient mice compared to WT-WD and hKO-NC. While collagen-1α1 in WT-WD trended higher compared to WT-NC, it was not found to be statistically significant. MMP-2 and MMP-9 play an important role in fibrotic signaling and ECM degradation (36, 57). It was interesting to observe that male (64) and female ATM deficient mice exhibit similar modulation of MMP-2 and MMP-9 expression under basal conditions and in response to WD. MMP-2 and MMP-9 expression increased in hKO-NC compared to WT-NC. MMP-2 and MMP-9 expression increased in WT-WD compared to NC, while hKO-WD remained unchanged compared to hKO-NC. MMP expression is tightly controlled to maintain balance between ECM degradation and synthesis (57). Thus, ATM deficiency appears to modulate MMP expression as increased fibrosis was noted at basal levels. WD may also alter MMP-2 and MMP-9 expression since increases in fibrosis was observed in WT-WD vs WT-NC.

Previously we provided evidence that under basal conditions male ATM deficient mice associate with increased expression of Bax (64), a pro-apoptotic protein, which modulates mitochondrial membrane potential to initiate the apoptotic cascade (19, 49). Interestingly, WD increased Bax expression in male WT-WD group compared to WT-NC, but WD failed to increase expression in hKO group vs hKO-NC (64). Here, we provide evidence that female ATM deficient mice also display increased Bax expression during basal conditions compared to WT-NC. WD increased Bax expression in WT-WD vs WT-NC. Conversely, hKO-WD displayed decreased Bax expression compared to WT-WD and hKO-NC. Therefore, the increase in Bax expression observed in WT-WD groups corresponds with the increase in myocyte and total apoptosis

observed compared to WT-NC. This suggests that the apoptosis observed following WD in WT mice is Bax-dependent. Further, the decrease in Bax expression in hKO-WD compared to WT-WD and hKO-NC correlates to the decrease in myocyte and total apoptosis observed in hKO-WD group. PARP-1 is an important DNA damage sensor for single stranded and double stranded DNA breaks (48). Therefore, increased levels of active (full length, 117kDa) PARP-1 would aid in facilitation of DNA damage repair, thereby protecting against apoptosis (48). Previously, we reported that WD induced a decrease in PARP-1 expression during ATM deficiency in male mice compared to WT-WD (64). This study shows that PARP-1 in female mice associates with decreased PARP-1 expression in WT-WD compared to hKO-WD. Increases in PARP-1 expression associate with decreased apoptosis (48). Therefore, the increase in PARP-1 and decrease in Bax expression supports the decrease in apoptosis observed in hKO-WD compared to WT-WD and hKO-NC.

Diet induced obesity modulates important metabolism and cell survival proteins such as Akt and mTOR (39, 40, 43). Interestingly, short-term Akt activation can be cardioprotective as it promotes physiological hypertrophy. However, long-term Akt activation associates with pathological hypertrophy with concomitant increase in fibrosis (16). Previously, ATM deficiency in male mice associated with increased activation of Akt at basal levels vs WT. Following WD, Akt activation increased in WT-WD vs WT-NC, while hKO group remained unchanged. Here we provide evidence that ATM deficiency in female mice also associates with increased Akt activation at basal levels. However, Akt activation remained unchanged following WD during ATM deficiency. The increased activation of Akt at basal conditions may help to explain why female ATM deficient mice exhibit significantly higher levels of fibrosis and hypertrophy compared to WT. Further, the WD-induced activation of Akt in WT may help explain the increased fibrosis and

hypertrophy observed compared to WT-NC. Transgenic mice overexpressing mTOR are shown to display attenuated high fat diet-induced fibrosis and cardiac dysfunction following ischemia-reperfusion injury (7). Previously, we have shown that ATM deficiency in male mice associates with increased mTOR activation at basal levels. Following WD, activation of mTOR significantly decreased in ATM deficient mice, and remained unchanged in WT group (64). Here we also observed higher activation of mTOR in female ATM deficient mice at basal levels compared to WT-NC. Interestingly, WD led to significant decrease in mTOR activation in WT group and mTOR activation remained higher in hKO-WD vs WT-WD. The maintained mTOR activation observed in hKO-WD may also serve a role in the preservation of cardiac function, and maintained level of fibrosis observed in hKO-WD group. Meanwhile, the WD induced decrease in mTOR activation in WT-WD group may help to explain the increase in fibrosis and decrease in cardiac function observed in WT-WD group.

In summary, ATM deficiency in female mice attenuated WD-induced adipose accumulation following 14 weeks of WD. WD induced cardiac dysfunction with decreased %FS, %EF, and increased LVESD and LVESV female WT group. In contrast, ATM deficiency in female mice associated with preserved cardiac function in response to WD. Further, female attenuation of WD-induced fibrosis and apoptosis was observed in the myocardium of female ATM deficient mice, suggesting that cardioprotective effects of ATM deficiency may involve decreased fibrosis and apoptosis. It should be noted that all the observations are made with 14 weeks of WD feeding. Therefore, additional studies involving longer WD feeding would help to elucidate the time-specific cardioprotective role of ATM deficiency in female mice following WD.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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CHAPTER 5. WESTERN-TYPE DIET DURING DEFICIENCY OF ATAXIA-TELANGIECTASIA MUTATED KINASE MODULATES FUNCTIONAL AND BIOCHEMICAL PARAMETERS OF THE HEART IN RESPONSE TO MYOCARDIAL INFARCTION

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Abstract

Ataxia telangiectasia mutated kinase (ATM) deficiency modulates Western-type diet (WD) induced cardiac dysfunction in a sex-specific manner. Male ATM deficient mice on WD exhibit accelerated weight gain, systolic dysfunction, increased preload, and cardiac remodeling. Conversely, female ATM deficient mice on WD exhibit attenuated weight gain, preserved cardiac function, and decreased apoptosis. The objective of this study was to elucidate the sex-specific interactions of WD and ATM deficiency with respect to cardiac structural and functional parameters following myocardial infarction (MI). Age matched male and female wild-type (WT) and ATM heterozygous knockout (hKO) mice were placed on WD for 14 weeks followed by MI via the ligation of the left anterior descending artery. Functional and biochemical parameters of the heart were assessed 1 day post-MI. M-mode echocardiographic analysis revealed that MI significantly decreases percent fractional shortening (%FS), ejection fraction (%EF), stroke volume, and increases LV end systolic diameter (LVESD) and LV end systolic volume (LVESV) in both male and female genotypes vs their respective baseline WD-fed controls. No significant difference in systolic parameters were observed between WT-M-MI vs hKO-M-MI. However, LVEDD and LVEDV were significantly greater in hKO-F-MI vs WT-F-MI. Further, LVESD, LVEDD, LVESV and LVEDV were significantly higher in WT-M-MI vs WT-F-MI. Pulse wave doppler analysis revealed that MI decreases aortic ejection time in all groups. MI decreased A-wave in hKO-F-MI and hKO-M-MI vs their respective WD-fed groups. However, A-wave was found to be statistically increased in WT-M-MI vs hKO-M-MI. E-wave was lower in hKO-M-MI vs hKO-M-B. Other diastolic parameters (isovolumetric contraction time, isovolumetric relaxation time) remained unchanged among all the groups and both sexes. MI induced a similar increase in cardiac cell apoptosis, infarct size and infarct thickness in all groups. Thus, 1) MI induces cardiac

dysfunction in WD-fed WT and ATM deficient mice; 2) the cardioprotective effects observed with WD during ATM deficiency in female mice are abolished 1 day post-MI and 3) ATM deficiency in WD-fed female mice associated with increased LV dilation 1 day post-MI.

New & Noteworthy

Ataxia telangiectasia mutated kinase (ATM) deficiency in human's associates with enhanced predisposition to ischemic heart disease. Previously we provided evidence that Western-type diet (WD) during ATM deficiency in male mice is injurious with systolic dysfunction, increased preload, apoptosis, hypertrophy and inflammation. Conversely, WD during ATM deficiency in female mice associated with cardioprotective effects and decreased apoptosis. Here, we provide evidence that ATM deficiency in female mice is no longer cardioprotective in WD fed mice 1 day following myocardial infarction (MI). Further investigations of the role of ATM deficiency in WD- and chronic MI-induced alterations in function and biochemical parameters of the heart may elucidate mechanisms and appropriate treatment options for patients with ATM deficiency and increased susceptibility to ischemic heart disease.

Introduction

Western-type diet (WD) associates with increased adipose accumulation, chronic low-grade inflammation, increased oxidative stress, mitochondrial dysfunction and cardiac structural and functional alterations (3, 6, 22–24). The duration of WD and degree of obesity directly contributes to the changes in hemodynamics, cardiac remodeling and preload and afterload dysfunction (2, 6). Therefore, WD, obesity and cardiac remodeling independently increase the risk of cardiovascular events (37, 45). Interestingly, WD alone increases the risk of myocardial infarction (MI) by ~35% (5). While early cardiac remodeling following MI can be protective, excessive cardiac remodeling is injurious (29, 41). Consequently, MI significantly contributes to the development of heart disease, a leading cause of death in the United States (40, 45). Animal studies have shown diets high in fat exacerbate cardiac damage and increase infarct size following MI (12).

Ataxia telangiectasia mutated kinase (ATM) generally becomes activated through double stranded DNA breaks, increased oxidative stress and damage, ionizing radiation and other genotoxic stressors (7, 16, 38, 44). While ATM is an important facilitator of DNA damage repair, other important functions include metabolic regulation, mitochondrial function and regulation of vesicle transport (7, 16, 38, 44). As ATM is involved in many nuclear and cytoplasmic functions, mutations in the ATM gene cause a multisystem disorder called ataxia-telangiectasia (A-T). Total abolishment of the ATM function results in the most severe A-T phenotype which is characterized by neurological, immunological, endocrinological and cardiovascular abnormalities (27, 32, 35, 38). However, ~1.4-2% of the general population are A-T carriers (mutation in one allele) which increases susceptibility to ischemic heart disease (44). We provided evidence that WD induces sex-specific alterations in the heart during ATM deficiency. Male ATM deficient mice exhibit

accelerated weight gain, systolic dysfunction, increased preload, and exacerbated cardiac remodeling following WD (43). Conversely, ATM deficiency in female mice displays a cardioprotective role against the injurious effects of WD with preserved cardiac function and decreased WD-induced apoptosis (manuscript in preparation). We have also provided evidence that ATM deficiency early post-MI associates with lower attenuation of cardiac function, delayed inflammatory response, and increased fibrosis and apoptosis (43). However, ATM deficiency late post-MI (14-28 days post-MI) associates with exacerbated cardiac structural and functional parameters of the heart (10, 11). However, WD influence on cardiac structural and functional parameters during ATM deficiency post-MI remains to be investigated. The objective of this study was to elucidate the sex-specific interactions of WD and ATM deficiency with respect to cardiac structure and function 1 day post-MI. The major finding is that the cardioprotective effects of ATM deficiency in female mice are abolished 1 day post-MI. MI induced similar systolic and diastolic dysfunction in all groups compared to baseline. However, some sex-specific differences were noted as WT-M-MI group exhibited increased ventricular diameters and volumes (systolic and diastolic) versus WT-F-MI. MI also increased left ventricular diastolic diameter and volumes in hKO-F-MI vs WT-F-MI. Thus, LV dilation is higher in hKO-F-MI vs WT-F-MI. However, no difference in infarct size, infarct thickness and apoptosis were observed among the groups 1 day post-MI.

Materials and Methods

Vertebrate animals and diets.

This study conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). East Tennessee State

University Committee on Animal Care approved all protocols utilized in this study. Breeding pairs of ATM deficient mice (129S6/SvEvTac) were purchased from Jackson Laboratory (stock #002753). The homozygous KO mice are infertile and die ~2 months of age. Therefore, ATM heterozygous knockout (hKO; deficient) mice were used for breeding providing us with littermate WT and hKO mice. Primers suggested by the Jackson Laboratory were used to genotype the mice using PCR. Six-week-old male WT and hKO mice were placed on a normal chow (NC; Envigo 8604) or Western-type diet (WD; Envigo TD 88137) for 14 weeks. The energy composition of NC is - 32% kcal protein, 14% kcal fat, 54% kcal carbohydrate and 4% sugar (by weight). The energy composition of WD is - 15.2% kcal protein, 42.0% kcal fat, 42.7% kcal carbohydrate and 34% sugar (by weight). All mice were kept on a 12 hour-dark/light cycle with food and water available *ad libitum*.

Myocardial Infarction.

Myocardial infarction (MI) was performed as previously described(10). Briefly, mice were anesthetized with a mixture of isoflurane (2%) and oxygen (0.5 l/min) inhalation and ventilated using a rodent ventilator (Harvard Apparatus). Body temperature was maintained at ~37°C using a heating pad. The heart was exposed by a left thoracotomy. The left anterior descending coronary artery (LAD) was ligated using a 7-0 polypropylene suture. Sham-operated mice underwent the same procedure without ligation of the LAD. At the end of the study period (1 day following LAD ligation), heart function was analyzed by echocardiography followed by excision of heart through an opening of the diaphragm region. The heart was perfused with Krebs-Henseleit buffer to ensure blood clearance, arrested in diastole using 30 mM KCl and weighed. The heart was then divided into two transverse sections (base/mid and apex) and embedded in paraffin.

Echocardiography.

Structural and functional parameters of the heart were measured following 14 weeks WD and 1 day post-MI using a Vevo 1100 imaging system (VisualSonics, Fujifilm) equipped with a 22- to 55-MHz MS550D transducer (9, 34). Therefore, some mice were used for measurement of heart function pre-and post-MI. For this, mice were anesthetized using a mixture of isoflurane (2%) and oxygen (0.6 L/min). Heating pad was used to maintain body temperature ~37˚C. M-mode recordings, obtained using transthoracic short axis view at mid-papillary level, were used to measure/calculated % ejection fraction (%EF), % fractional shortening (%FS), LV end systolic diameter (LVESD), LV end diastolic diameter (LVEDD), LV end systolic volume (LVESV), LV end diastolic volume (LVEDV), and stroke volume. Doppler tracings, acquired from apical four-chamber view were used to measure peak velocity of early ventricular filling (E wave), late ventricular filling (A wave), aortic ejection time (AET), isovolumetric relaxation time (IVRT; measured from the aortic valve closure to the mitral valve opening) and isovolumetric contraction time (IVCT; measured from the closing of the mitral valve to the opening of the aortic valve) as described (34).

Terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay.

Tunnel assay was performed according to the manufacturer's instructions (In Situ Cell Death Detection Kit, Roche) (10). Tissue sections (5μm thick) were also stained with rhodamine-conjugated wheat germ agglutinin (WGA, Rl-1022, Vector) to visualize myocytes and Hoechst 33258 (10 μM; Sigma) to visualize nuclei. Hoechst-positive stained nuclei served as an index to count the total number of nuclei. Apoptotic myocytes were identified by TUNEL-positive staining clearly seen within WGA-stained cells and Hoechst-positively stained nuclei. The index of

myocyte apoptosis was calculated as the percentage myocyte apoptotic nuclei/total nuclei. Total cardiac cells apoptosis was measured by counting the TUNEL-positive and Hoechst-positive nuclei. The index of cardiac cell apoptosis was calculated as the percentage of total apoptotic nuclei/total nuclei.

Morphometric analysis.

Mid-cardiac transverse sections (5μm thick) were stained with TUNEL staining were also used to measure infarct size and infarct thickness. For infarct size, area of infarct and non-infarct was measured Nikon NIS software as previously described (11). Percent infarction was calculated by dividing total area of infarct by the non-infarcted heart section and multiplying by 100. To measure infarct thickness, six separate transverse measurements were averaged from each infarct area of the heart.

Statistical analysis.

Data are expressed as means ± SE. Data were analyzed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test or 2-tailed Student's t-test. Probability (p) values of <0.05 were considered to be significant.

Results

Structural and functional parameters as measured by echocardiography:

WD vs WD-MI Comparison.

The WD alone group is defined as baseline (B), while male and female mice are abbreviated as M and F, respectively. M-mode echocardiography revealed MI induces a significant decrease in %EF,

%FS, stroke volume and increases LVESD, LVEDD, LVESV, and LVEDV in WT-M-MI vs. WT-M-B. On the other hand, MI significantly decreased %EF, %FS and stroke volume, and increased LVESD and LVESV in hKO-M-MI vs hKO-M-B (Table 5.1). Interestingly, LVEDD and LVEDV remained unchanged between hKO-M-MI vs hKO-M-B.

Table 5.1 M-Mode Echocardiographic Parameters in Male Mice Following MI

Parameters	**WT-M-B**	**WT-M-MI**	**hKO-M-B**	**hKO-M-MI**
%EF	49.86 ± 1.5	27.72 ± 2.6*****	52.12 ± 1.5	29.58 ± 2.4*****
%FS	24.96 ± 0.9	12.91 ± 1.3*****	26.4 ± 0.9	13.78 ± 1.2*****
LVESD (mm)	2.97 ± 0.1	3.84 ± 0.2*****	2.94 ± 0.1	3.55 ± 0.1*****
LVEDD (mm)	3.95 ± 0.1	4.41 ± 0.2*****	3.99 ± 0.1	4.11 ± 0.1
LVESV (µL)	34.33 ± 1.9	65.05 ± 6.4*****	33.65 ± 1.8	53.25 ± 3.1*****
LVEDV (µL)	68.35 ± 2.8	89.46 ± 7.8*****	69.98 ± 2.2	75.4 ± 3.4
Stroke Volume (µL)	34.02 ± 1.6	24.4 ± 2.7*****	36.32 ± 1.1	22.18 ± 1.9*****

Values are means ± SE, n=9-10. *p<0.05 vs Baseline; WT-M-B, wild-type male baseline; hKO-M-B, heterozygous knockout male baseline; WT-F-B, wild-type female baseline; hKO-F-B, heterozygous knockout female baseline; WT-M-MI, wild-type male MI; hKO-M-MI, heterozygous knockout male MI; WT-F-MI, wild-type female MI; hKO-F-MI, heterozygous knockout female MI. BPM, beats per minute.

In female mice, we have observed that WD does not affect structural and functional parameters of the heart during ATM deficiency (manuscript in preparation), suggesting that ATM deficiency in female mice associates with cardioprotective effects in response to WD. Here, MI induced a significant decrease in %EF, %FS and stroke volume and increased LVESD and LVESV in WT group (WT-F-MI vs WT-F-B). Likewise, MI caused a significant decrease in %EF, %FS and stroke volume and increased LVESD and LVESV in ATM deficient group (hKO-F-MI vs hKO-F-B) (Table 5.2). LVEDD and LVEDV remained unchanged in WT (p=.10) and hKO (p=.056) groups post-MI.

Table 5.2 M-Mode Echocardiographic Parameters in Female Mice Following MI

Parameters	WT-F-B	WT-F-MI	hKO-F-B	hKO-F-MI
%EF	52.50 ± 1.6	32.55 ± 2.2*	58.48 ± 1.4	32.03 ± 2.8*
%FS	26.55 ± 1.0	15.18 ± 1.1*	30.32 ± 0.8	15.06 ± 1.5*
LVESD (mm)	2.82 ± 0.1	3.18 ± 0.1*&	2.57 ± 0.1	3.43 ± 0.1*
LVEDD (mm)	3.84 ± 0.1	3.75 ± 0.1&	3.68 ± 0.1	4.03 ± 0.1$
LVESV (µL)	30.51 ± 2.1	40.81 ± 2.2*&	24.73 ± 2.8	49.25 ± 4.3*
LVEDV (µL)	64.07 ± 3.4	60.39 ± 2.3&	58.39 ± 4.8	72.15 ± 4.9$
Stroke Volume (µL)	33.57 ± 1.9	19.57 ± 1.4*	33.67 ± 2.2	22.90 ± 2.2*

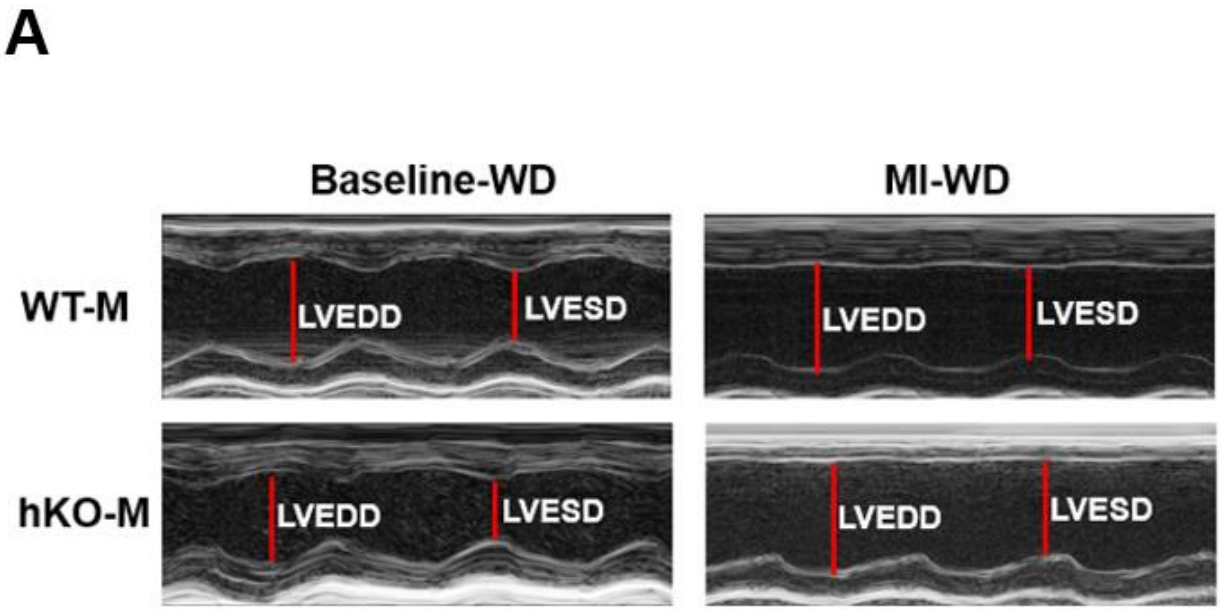
Values are means ± SE, n=9-10. *p<0.05 vs Baseline, $p<0.05 vs WT-F-MI, &p<0.05 vs WT-M-MI; WT-M-B, wild-type male baseline; hKO-M-B, heterozygous knockout male baseline; WT-F-B, wild-type female baseline; hKO-F-B, heterozygous knockout female baseline; WT-M-MI, wild-type male MI; hKO-M-MI, heterozygous knockout male MI; WT-F-MI, wild-type female MI; hKO-F-MI, heterozygous knockout female MI. BPM, beats per minute.

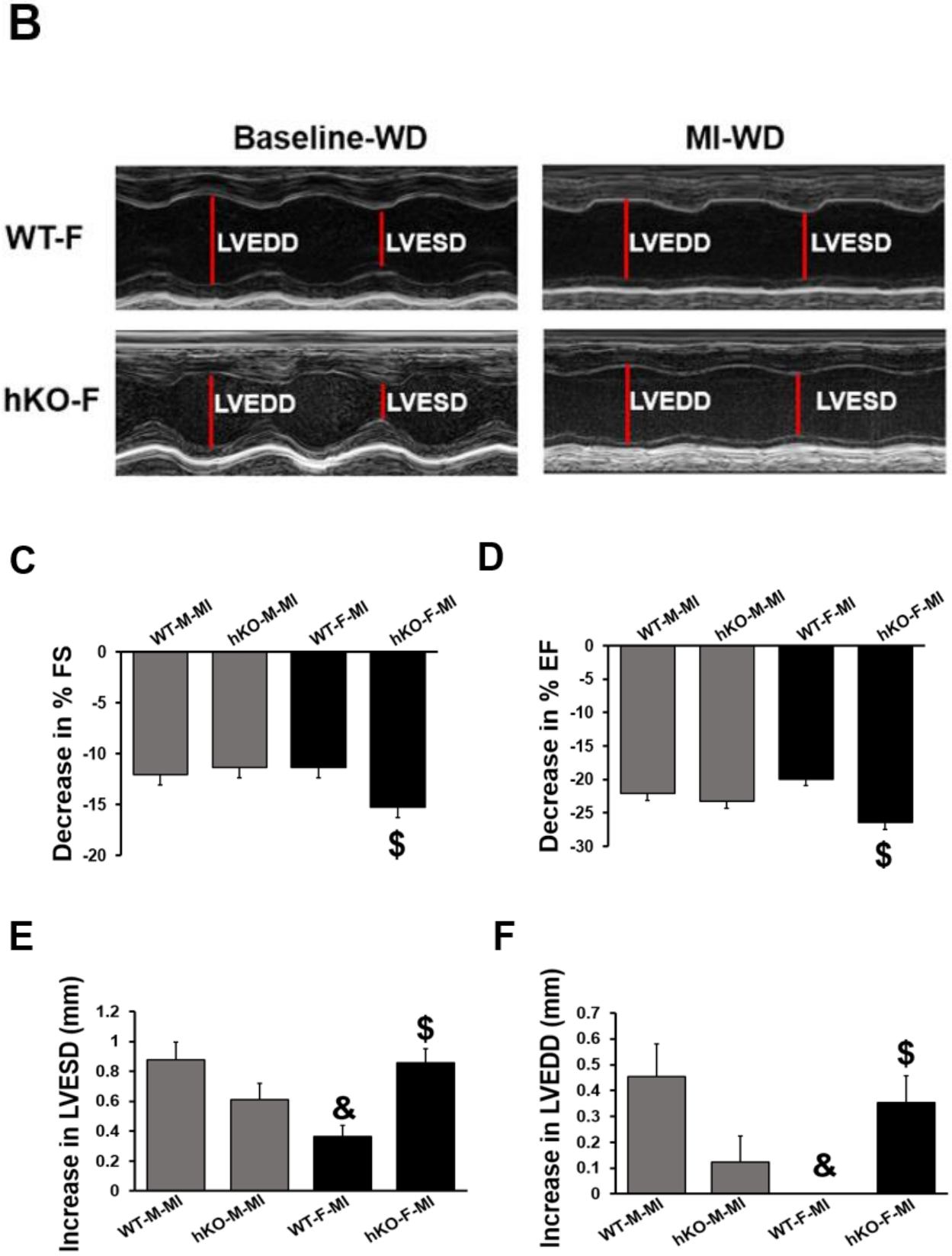
MI-to-MI comparison.

Previously, our lab has provided evidence that ATM deficiency associates with cardioprotective effects 1 day post-MI, as MI-induced decline in %FS and %EF was greater in WT-MI group vs hKO-MI (10). Here, we observed an equal decrease in systolic parameters in WT-M-MI and hKO-M-MI vs their WD counterparts 1 day post-MI. In female mice, although %FS, %EF, LVESD and LVESV were equally effected by MI, LVEDD and LVEDV were significantly higher in ATM deficient females vs WT (hKO-F-MI vs WT-F-MI) (Table 5.1 and Table 5.2). Further, a sex specific difference was observed in WT group as LVEDD, LVESD, LVEDV and LVESV were significantly higher in WT-M-MI vs WT-F-MI 1 day post-MI (Table 5.1 and Table 5.2).

Average systolic change from baseline to MI.

Previously, WD induced a significant decrease in systolic parameters in WT-M-B, hKO-M-B (43), and WT-F-B groups (manuscript in preparation). However, hKO-F-B group displayed preserved systolic parameters in response to WD (manuscript in preparation). Therefore, to assess if sex or genotype played a role in MI-induced structural and functional alterations, the average change in systolic parameters was calculated (change from day 0 to 1 day post-MI). The average MI-induced decrease in %FS and %EF was significantly greater, while MI-induced increased LVESD, LVEDD, LVESV and LVEDD in hKO-F-MI vs. WT-F-MI (Fig 5.1 A-H). In addition, LVESD, LVEDD, LVESV and LVEDV were significantly lower in WT-F vs WT-M 1 day post-MI.




B
Baseline-WD
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WT-F
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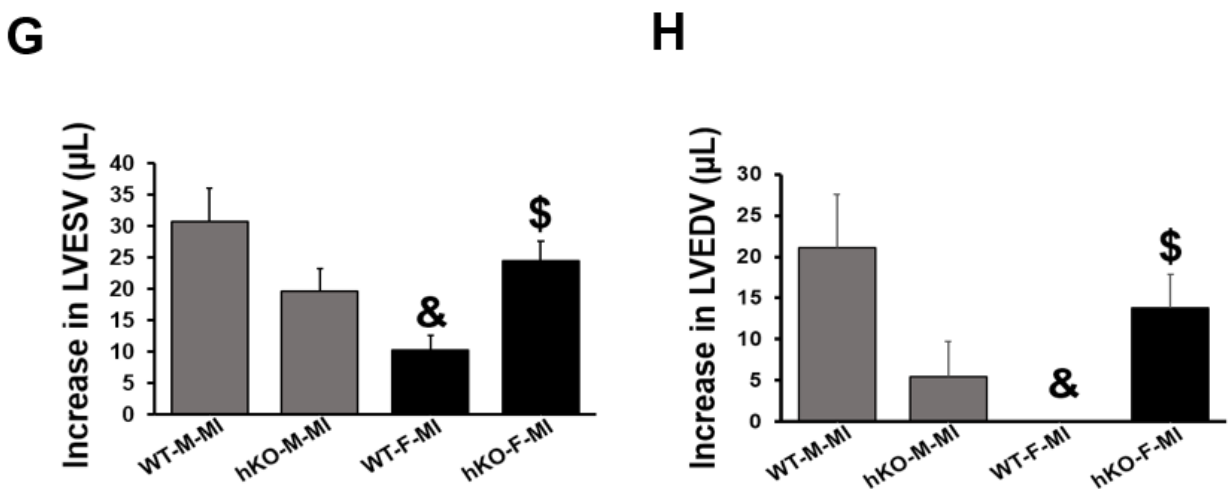


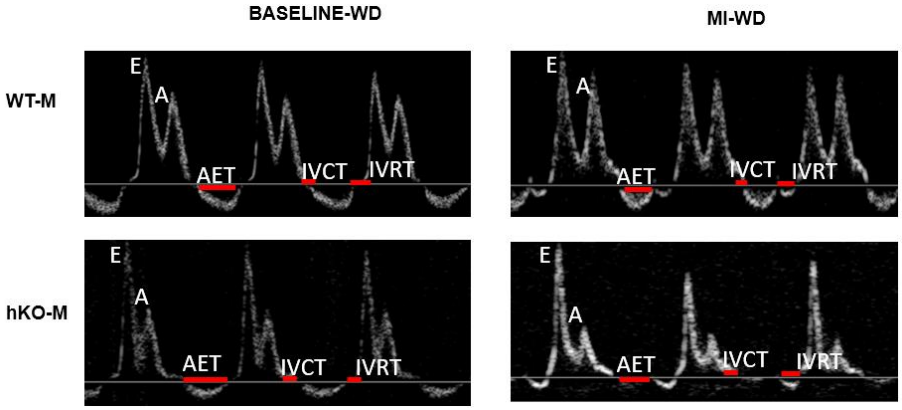
Figure 5.1 WD during ATM deficiency Effect on Average Change in M-mode Parameters of the Heart 1 Day Post-MI.

Indices of m-mode parameters: % fractional shortening (%FS), % ejection fraction (%EF), LV end systolic diameter (LVESD), LV end diastolic diameter (LVEDD), LV end systolic volume (LVESV), and LV end diastolic volume (LVEDV) were measured using M-mode echocardiographic images 1 day post-MI. (A) Representative M-mode tracings for male at baseline and post-MI tracings; (B) Representative M-mode tracings for female at baseline and post-MI tracings; (C) change in %FS; (D) change in %EF; (E) increase in LVESD; (F) change in LVEDD; (G) change in LVESV; and (H) change in LVEDV from baseline to 1 day post-MI. $^{\$}p<0.05$ vs WT-F-MI, $^{\&}p<0.05$ vs WT-M-MI, n=10

Diastolic Function.

Previously, WT-M-B associated with increased afterload with significantly decreased E-wave and AET, and increased IVRT and Tei index in response to WD. However, hKO-M-B mice exhibited increased preload with preserved AET, decreased IVCT, IVRT and Tei index in response to WD (43). Here, MI induced a significant decrease in AET in all groups (Table 5.3 and Table 5.4) with no significant differences among the groups. A-wave was significantly lower in hKO-M-MI vs hKO-M-B and in hKO-F-MI vs hKO-F-B. While, E-wave was significantly lower in hKO-M-MI vs hKO-M-B and in hKO-M-MI vs WT-M-MI (Table 5.3 and Table 5.4).

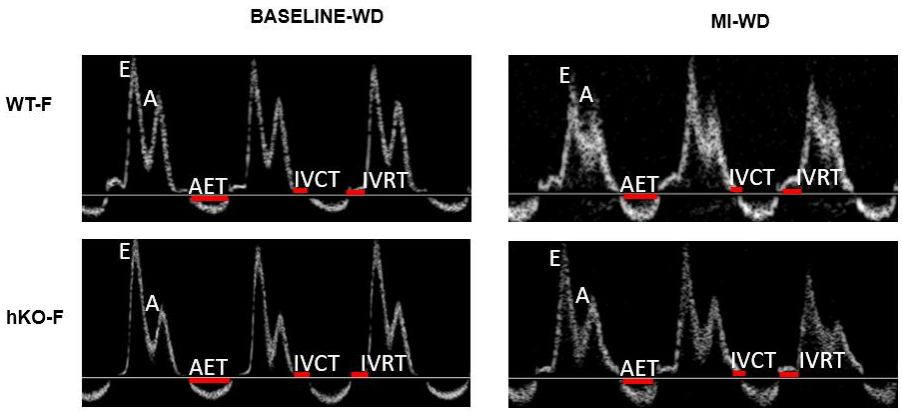
Table 5.3 Male PW Echocardiographic Parameters in Post-MI



Parameters	WT-M-B	WT-M-MI	hKO-M-B	hKO-M-MI
E-wave (mm/s)	499.6 ± 58	390.6 ± 25	538.9 ± 24	317.7 ± 43*
A-wave (mm/s)	361.9 ± 46	283.3 ± 19	325.7 ± 25	167.8 ± 33*&
AET (msec)	50.44 ± 1.5	41.23 ± 2.6*	57.5 ± 0.9	41.27 ± 1.2*
IVCT (msec)	17.51 ± 1.7	14.93 ± 0.7	13.2 ± 1.0	13.67 ± 0.7
IVRT (msec)	27.44 ± 3.1	22.65 ± 2.3	18.7 ± 0.5	20.13 ± 1.7
Tei Index	0.89 ± 0.05	0.92 ± 0.06	0.55 ± 0.1	0.82 ± 0.04

Values are means ± SE, n=7. *p<0.05 vs Baseline, &p<0.05 vs WT-M-MI. WT-M-B, wild-type male baseline; hKO-M-B, heterozygous knockout male baseline; WT-F-B, wild-type female baseline; hKO-F-B, heterozygous knockout female baseline; WT-M-MI, wild-type male MI; hKO-M-MI, heterozygous knockout male MI; WT-F-MI, wild-type female MI; hKO-F-MI, heterozygous knockout female MI. *p<0.05 vs Baseline; AET, aortic ejection time; IVCT, isovolumetric contraction time; and IVRT, isovolumetric relaxation time.

Table 5.4 Female PW Echocardiographic Parameters in Post-MI



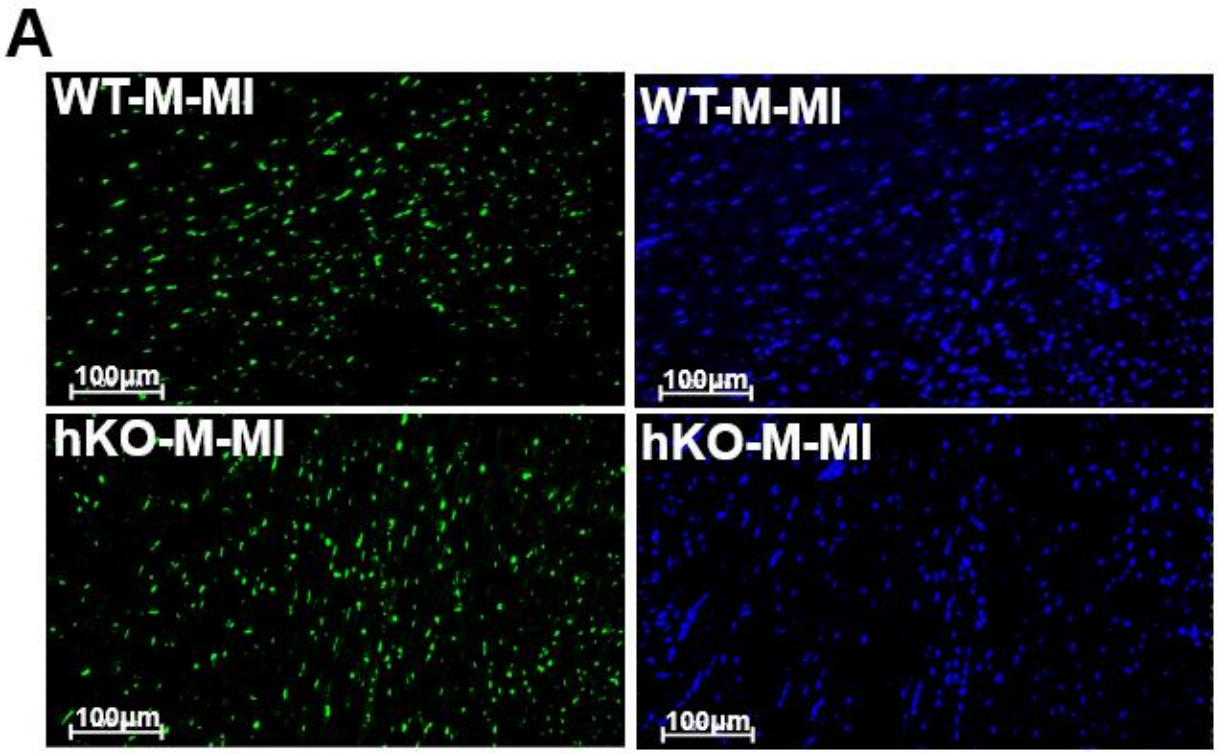
Parameters	WT-F-B	WT-F-MI	hKO-F-B	hKO-F-MI
E-wave (mm/s)	459.3 ± 42	312.03 ± 27	519.39 ± 39	396.47 ± 49
A-wave (mm/s)	267.5 ± 24	166.6 ± 30	338.87 ± 14	231.7 ± 19*****
AET (msec)	57.91 ± 1.3	41.90 ± 1.4*****	54.72 ± 2.5	43.20 ± 1.5*****
IVCT (msec)	19.86 ± 1.4	15.87 ± 1.2	16.52 ± 0.8	14.24 ± 1.6
IVRT (msec)	23.73 ± 1.2	22.85 ± 1.9	22.25 ± 1.3	20.26 ± 1.8
Tei Index	0.75 ± 0.04	0.92 ± 0.05	0.72 ± 0.04	0.80 ± 0.06

Values are means ± SE, n=7. *p<0.05 vs Baseline. WT-M-B, wild-type male baseline; hKO-M-B, heterozygous knockout male baseline; WT-F-B, wild-type female baseline; hKO-F-B, heterozygous knockout female baseline; WT-M-MI, wild-type male MI; hKO-M-MI, heterozygous knockout male MI; WT-F-MI, wild-type female MI; hKO-F-MI, heterozygous knockout female MI. *p<0.05 vs Baseline; AET, aortic ejection time; IVCT, isovolumetric contraction time; and IVRT, isovolumetric relaxation time.

Apoptosis, infarct size and infarct thickness.

Increased myocyte and total apoptosis is often observed with WD and MI (10, 15). Previously, we have shown that WD induces a significant increase in cardiac cell apoptosis in male WT and ATM deficient male hearts vs their normal chow control groups (43). However, WD-fed ATM deficient male group exhibited ~2 fold greater increase in cardiac cell apoptosis vs the WT

counterpart (43). While WD also increased myocyte and total cell apoptosis in female WT and ATM deficient hearts compared to their normal chow groups, WD-induced apoptosis was significantly higher in WT females vs ATM deficient females (manuscript in preparation). Here, we show that MI induces apoptosis in all groups with a similar increase in apoptosis in the infarct region 1 day post-MI (Fig 5.2 A-C). Previously, ATM deficiency on normal chow is shown to have no effect on infarct size 1 day post-MI (10). However, infarct thickness was found to be higher in ATM deficient mice fed with normal chow 7 days post-MI (14). Here, infarct size and infarct thickness showed no differences among the groups (Fig 5.3A-B).



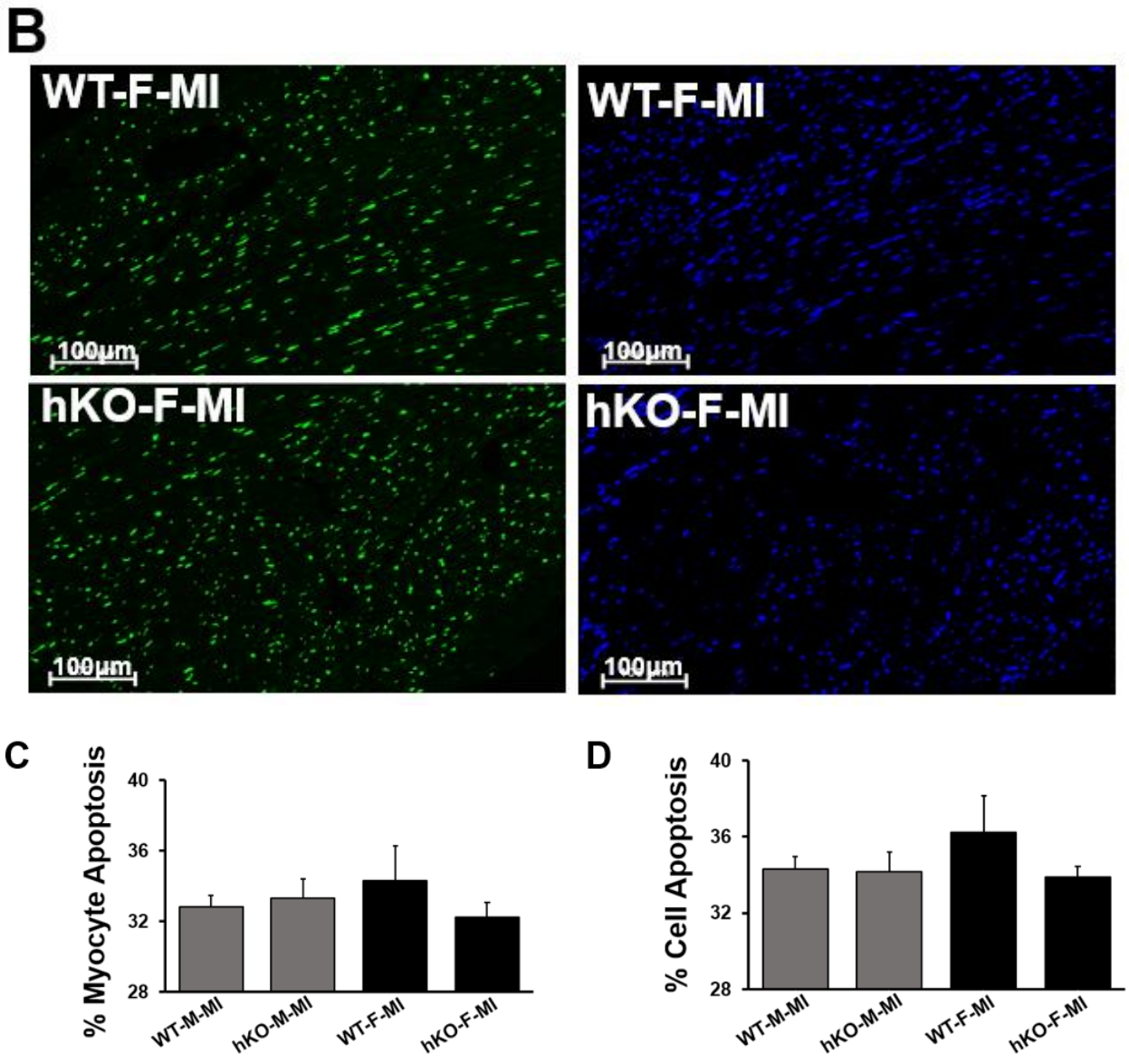
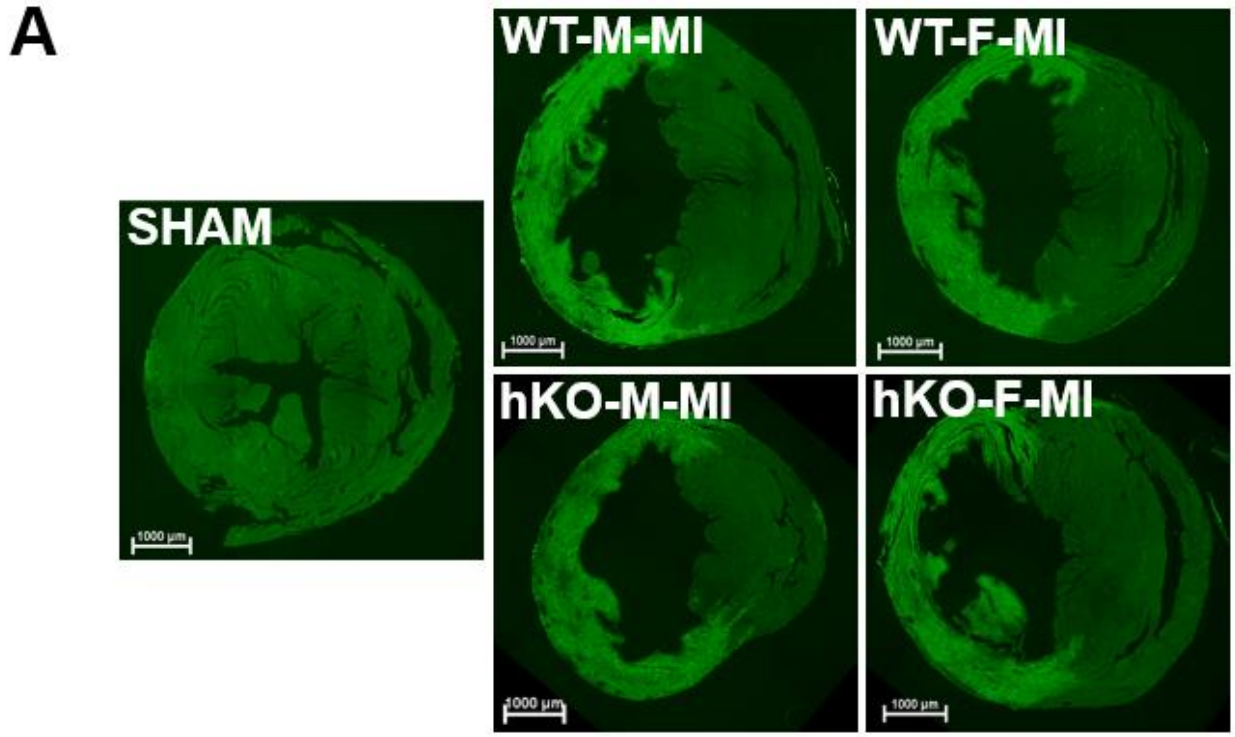
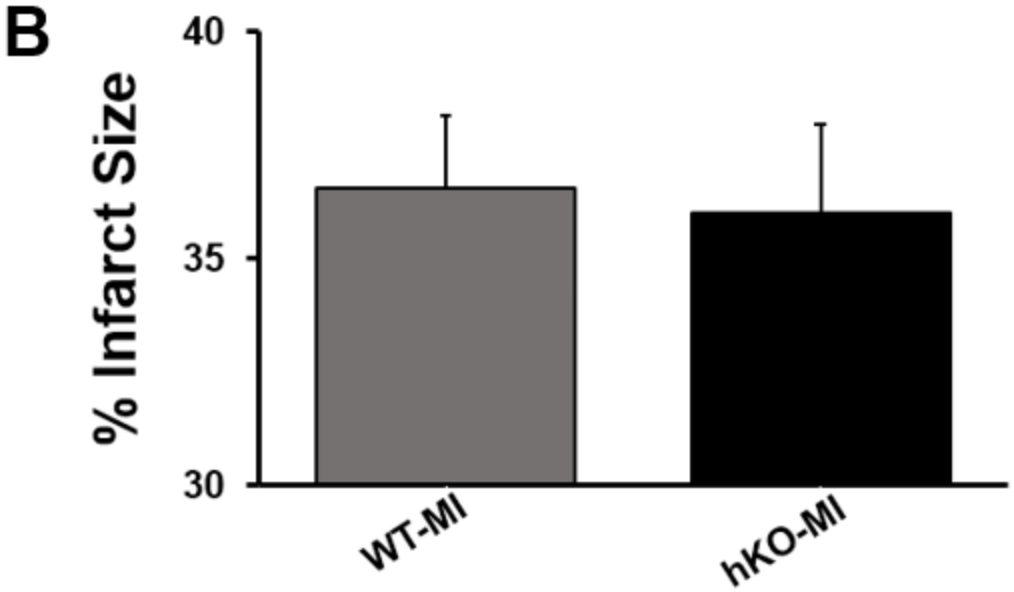


Figure 5.2 WD during ATM Deficiency has No Effect on Apoptosis in the Heart 1 Day Post-MI.

A. Representative images of TUNEL (green), WGA (red) and Hoechst (blue) stained hearts. B. Quantitative analysis of myocyte apoptosis in infarct LV. C. Quantitative analysis of cardiac cell apoptosis. n=5-6.


A
SHAM
WT-M-MI
WT-F-MI
hKO-M-MI
hKO-F-MI
1000 µm


B
% Infarct Size
40
35
30
WT-MI
hKO-MI

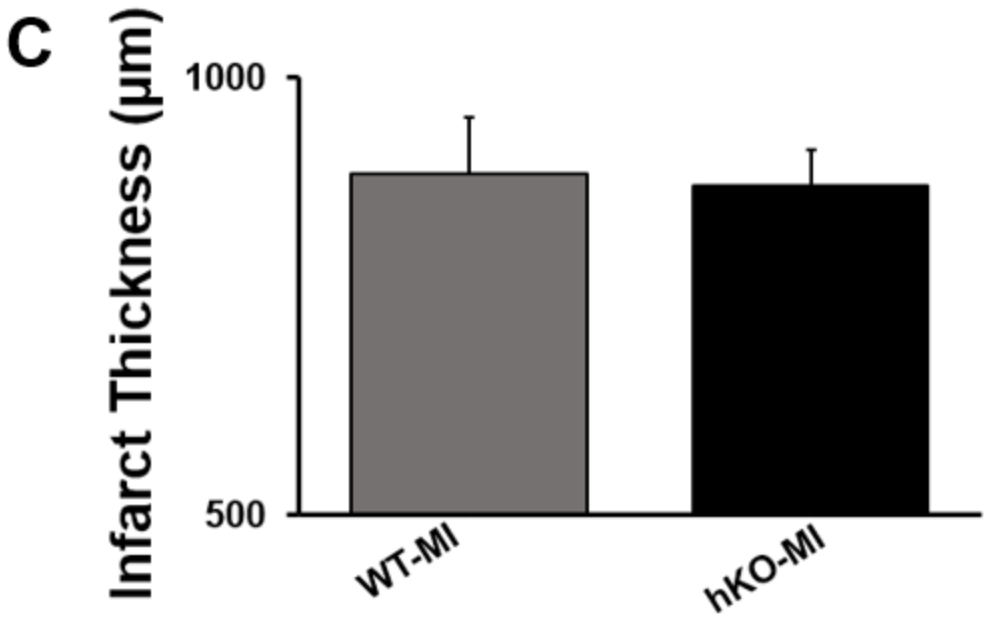


Figure 5.3 WD during ATM Deficiency has No Effect on Infarct Size or Infarct Thickness 1 Day Post-MI.

A. Representative images of TUNEL-stained cross-sections of the heart depicting area of infarct. B. Quantitative analysis of percent infarct size. C. Quantitative analysis of infarct thickness. n=5-6.

Discussion

Many factors increase the risk of ischemic heart disease including diet, obesity, metabolic disorders, sedentary lifestyle and gender (19, 25). Two-thirds of the American population are obese and consume a diet high in fat and sugar, which independently increases the risk of MI by 35% (5, 8, 17, 26, 28). A-T carriers preferentially consume diets high in fat and sugar, have increased susceptibility to ischemic heart disease and account for ~1.4-2.0% of the general population (44). Therefore, WD during ATM deficiency further increases the risk of MI (16, 44). Previously, WD in male ATM deficient mice associated with accelerated weight gain, systolic dysfunction, increased preload and exacerbation of cardiac remodeling (43). Conversely, ATM deficiency in female mice associated with attenuated weight gain, preserved cardiac function, and decreased apoptosis in response to WD (manuscript in preparation). This study examined the sex-specific

interactions of WD and ATM deficiency in respect to cardiac function and structure following early (1 day) MI. The major findings of the study are that WD and early MI during ATM deficiency- 1) decreases systolic parameters compared to WD baseline in all groups; 2) abolishes the cardioprotective effect of ATM deficiency in female mice and increases LV diastolic diameter and volume; 3) causes genotype and sex-specific alterations in systolic parameters post-MI; 4) decreases diastolic function; 5) induces similar cardiac cell apoptosis with no effect on infarct size and thickness.

The degree and duration of adiposity and/or WD directly influences the severity of cardiac mechanical and structural dysfunction (1, 6, 13, 15). Interestingly, obesity, WD, and ATM deficiency independently increase the risk of MI and HF (5, 19, 25, 38, 44). Previously, WD induced systolic dysfunction with decreased %FS and %EF, and increased LVESD and LVESV to a similar extent in male mice of both genotypes (WT and hKO) (43). Conversely, WD had no effect on systolic function in ATM deficient female mice, while it decreased systolic parameters of the heart in WT female mice (manuscript in preparation). Further, WT male mice exhibited increased afterload, while ATM deficient male mice displayed increased preload in response to WD (43). Conversely, WD had no effect on diastolic function in WT or ATM deficient female groups (manuscript in preparation). Here, we demonstrate that MI following 14 weeks of WD significantly decreases %EF, %FS, stroke volume and increases LV dilation in male groups of both genotypes vs their respective control groups (WD-fed mice). Likewise, MI significantly decreased %EF, %FS and stroke volume and increased LVESD and LVESV in WT-F-MI and hKO-F-MI compared to WD-baseline counterparts. These data suggests that MI decreases systolic function in both genotypes and sexes. Interestingly, LV diastolic diameter and volume were

significantly greater in ATM deficient females vs WT females, suggesting that ATM deficiency during WD and MI exaggerates cardiac dysfunction.

Previously, we provided evidence that ATM deficient mice fed normal chow display lower cardiac dysfunction following early MI compared to WT (10). Therefore, ATM deficiency is suggested to be cardioprotective with decreased dilative remodeling. It should be noted here that this study did not analyze the data in a sex-specific manner. Here, all systolic parameters were equally decreased in ATM deficient and WT males 1 day post-MI. Age and sex contribute to the occurrence of MI. The incidence of MI is ~2.5 times more likely to occur in males vs females under the age of 45 (30). However, after 55 years of age, the sex-specific risk is removed. While the incidence of MI is less prevalent in females than in males, females have a higher mortality rate following MI (20). Interestingly obesity mitigates the sex-specific differences in mortality (20). Here, sex-specific differences were noted as WT male mice exhibited increased LV dilation as observed by increased LVESD, LVEDD, LVESV and LVEDV compared to the WT females. Further, ATM deficiency led to significant increase in LV diastolic diameter and volume 1 day post-MI. This suggests that WD exacerbates cardiac dysfunction and remodeling in a sex-specific manner during ATM deficiency early post-MI.

Previously, sex-specific differences were noted during baseline conditions. WD induced systolic dysfunction in WT and ATM deficient males to a similar extent (43). Conversely, ATM deficient females showed maintained systolic function and structure in response to WD (manuscript in preparation). Evaluation of average change from WD to 1 day post-MI showed that MI induces a significant increase in chamber size and volume (LVESV, LVESD, LVEDD and LVEDV) in WT-M-MI compared to WT-F-MI. Interestingly, MI-induced decrease in %FS and

%EF, and increase in LV systolic and diastolic diameters and volumes were significantly greater in ATM deficient females vs the WT group 1 day post-MI. Therefore, the cardioprotective effect of ATM deficiency in female mice following WD is abolished 1 day post-MI.

The increase in blood volume observed with obesity is linked to the development of preload and afterload dysfunction (4, 33, 39). Previously, we demonstrated that WD induced adiposity and diastolic dysfunction in both male groups with WT male group exhibiting increased afterload and ATM deficient mice exhibiting increased preload in response to WD (43). However, WD had no effect on diastolic parameters in female groups (manuscript in preparation). Here, AET significantly decreased in both genotypes and sexes 1 day post-MI. Obesity has been linked to increased, decreased or unchanged E-wave velocities. The differences in loading conditions, degree and stage of diastolic dysfunction is speculated to be the cause of the inconsistency of the effect of obesity on E-wave velocity (39). Here, MI significantly decreased E- and A-wave in ATM deficient males (vs WD-fed ATM deficient males), A-wave was significantly lower in ATM deficient females (vs WD-fed ATM deficient females) 1 day post-MI. In addition, E wave was found to be lower in ATM deficient males vs the WT males 1 day post-MI. These data suggests that ATM deficiency affects ventricular filling in a sex-specific manner in response to WD and MI.

High fat diets and obesity directly contribute to cardiac cell apoptosis through changes in hemodynamics, lipotoxicity, increased oxidative stress and toxic byproducts (15, 21, 31, 42). Increased cardiac cell apoptosis greatly increases the risk of MI and development of HF (18, 36, 45). Previously, we have shown that WD significantly increases myocyte and total cell apoptosis in the myocardium of male mice of both genotypes (43). However, WD-induced apoptosis was ~

2.5-fold higher in ATM deficient male mice when compared to their WT counterpart (43). While WD also significantly increased myocyte and total cell apoptosis in the myocardium of WT and ATM deficient female groups, apoptosis was ~2 fold lower in ATM deficient females vs their WT counterpart (manuscript in preparation). Increased apoptosis was also observed in the myocardium of ATM deficient mice fed normal chow 1 day post-MI, although data was pooled male and female mice of same genotype (10). Here, myocyte and total cell apoptosis similarly increased in all groups 1 day post-MI. This suggests that WD during ATM deficiency does not affect cardiac cell apoptosis during early post-MI.

Previously, we provided evidence that ATM deficiency in (pooled male and female) mice fed normal chow has no effect on infarct size (10). Here, infarct sizes were similar in both genotypes and sexes with no difference among the groups. Infarct thickness was also found to be the same in all MI groups. These data suggests that ATM deficiency 1 day post-MI does not affect infarct size or thickness following 14 weeks of WD. Of note, infarct thickness was found to be higher in ATM deficient mice on normal chow 7 days post-MI (14).

In summary, MI induced systolic dysfunction in both WD genotypes and sexes 1 day post-MI compared to WD-baseline. MI induced similar changes in systolic parameters in male mice of both genotypes. However, MI significantly increased diastolic diameter and volume in ATM deficient females vs their WT counterpart. Further, some sex-specific differences were observed as WT male mice displayed significantly increased LV dilation and volumes compared to WT female mice. The average change in systolic parameters from WD to 1 day post-MI provided evidence that WD during ATM deficiency in female mice exacerbates MI-induced cardiac dysfunction with significant increase in diameters and volumes, and significant decrease in %FS

and %EF vs their WT counterpart. MI induced a similar decrease in AET in both genotypes and sexes. Interestingly, MI induced a similar increase in percent myocyte, total cell apoptosis, infarct size and infarct thickness in all groups and sexes. Therefore, the cardioprotective effects observed with WD during ATM deficiency in female mice are abolished 1 day post-MI, as all groups exhibit decreased cardiac function and similar levels of apoptosis. It should be noted that all the observations are made during early (1 day) post-MI. Therefore, cardiac structural and functional parameters should be assessed at additional time points to assess the interaction of WD and ATM deficiency in male and female mice during late MI.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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CHAPTER 6. CONCLUSION

The majority of the American population consumes a WD and is overweight or obese [23,50,79,81]. A-T carriers preferentially consume diets which are high in fat and sugar and account for ~2% of the population[89]. As aforementioned, ATM deficiency, WD and adiposity are independently associated with metabolic dysfunction, exacerbated cardiac remodeling, MI, heart disease, HF and cardiac-related mortality[60,69,70,80,83,90,94]. This means that millions of A-T carriers are consuming a WD with an exponentially increased risk of morbidity and mortality. However, the elucidation of the cardiovascular structural and functional effects of WD during ATM deficiency is yet to be studied. The primary findings of this investigation is that WD exacerbates cardiac dysfunction in male ATM deficient mice. Conversely, ATM deficiency in female mice is was found to be protective from the injurious effects of WD. However, after induction of an MI, the protective effect observed in female ATM deficient mice was eradicated. Male ATM deficient mice on WD exhibited time-specific acceleration of weight gain, systolic and preload dysfunction, exacerbated cardiac remodeling with associated increases in myocyte and total apoptosis, hypertrophy, sustained levels of fibrosis, metabolic and inflammatory signaling alterations, and increased cytokines and chemokines[112]. Female ATM deficient mice on WD displayed a decrease in weight gain and total body fat accumulation, and increased cholesterol, triglycerides, and fasting glucose. Preservation of systolic and diastolic function, maintained percent fibrosis, increased cardiac hypertrophy and a significantly decreased percent apoptosis was also observed in ATM deficient female mice on WD vs WT-WD. In male and female ATM deficient mice after 14 weeks after WD led to similar increases in cardiac cell apoptosis, infarct size and infarct thickness in both genotypes and sexes 1 day post-MI. Thereby, abolishing the protective effect in female ATM deficient mice exhibited 14 weeks post-WD. Overall, this data

suggests that ATM deficiency in male mice on WD exacerbates cardiac dysfunction. However, ATM deficiency in female mice on WD is cardioprotective under baseline conditions with preserved cardiac function. After ischemia, the cardioprotective effect of ATM deficiency in female mice is abolished as both male and female ATM deficient mice on WD exhibit similar decreases in cardiac function and percent apoptosis compared to WT counterpart.

Male ATM Deficient Mice on WD

WD induces metabolic disorders such as obesity and insulin resistance. A-T patients generally display low body weights and limited adipocyte maturation[89,98] However, WD-induced adipose accumulation in A-T carriers has not yet been elucidated. Our laboratory has provided evidence that after 14 weeks of WD, male ATM deficient mice exhibit time-specific accelerated body weight gain compared to WT[112]. Increases in triglycerides and cholesterol has been noted in A-T patients as well as in an ATM deficient/ApoE null mice[12,114], and reduction of apoB-48 carrying lipoprotein clearance[114]. However, WD induced similar increases in serum cholesterol and triglycerides in both male genotypes after 14 weeks of WD. Many studies have shown that increases in body weight and fat storage alter cardiac function and structure[1,15,45,113]. While short durations of WD (8 weeks) have been shown to induce structural and functional impairment in the heart[22], extended durations of WD and adiposity correlate with the severity of cardiac dysfunction[1,37]. Interestingly, this study showed that the severity of WD/obesity-induced systolic dysfunction was similar in WT and ATM deficient mice. However, WD-induced diastolic dysfunction was significantly different between genotypes. WT mice exhibited increased afterload associated cardiac dysfunction, while ATM deficient mice displayed preload associated cardiac dysfunction in response to WD[112]. Further examination of atrial and LV blood pressure

is needed to better elucidate the difference in WD-induced diastolic dysfunction between genotypes.

Multiple studies demonstrate that high fat diets and obesity exacerbate cardiac dysfunction and remodeling with increased in fibrosis, hypertrophy and apoptosis. As previously mentioned, WD and adiposity individually associate with increases in cardiac fibrosis and hypertrophy, which occur as compensatory mechanisms to stabilize the myocardium following WD/obesity related cardiac injury[4,29,45,53,66,84]. Fibrosis was found to be increased in ATM hKO mice on NC compared to WT, but was maintained with WD. Further, we noted that WD exacerbated cardiac hypertrophy in the ATM deficient group[112]. While fibrosis and hypertrophy are protective during early cardiac remodeling, they can lead to cardiac cell apoptosis[46,112]. Apoptosis and expression of Bax, a pro-apoptotic protein, was significantly increased in ATM deficient mice on WD. This suggests that ATM deficiency exacerbates cardiac remodeling in response to WD. Insulin resistance is also a hallmark of metabolic syndrome as well as ATM deficiency[45,113]. While WD did increase fasting glucose levels in WT mice, ATM deficient mice were unaffected. However, Akt and Glut-4 were increased in ATM deficient mice on NC, which may indicate a potential shift toward glucose metabolism[112]. This may also suggest that an extended duration WD may be required to induce insulin resistance in male ATM deficient mice. ATM deficiency is associated with increased inflammatory regulators (NFκB, IKKα/β), suggesting ATM plays a role in inflammatory signaling on NC.[112] While WD did not further exacerbate activation/expression of metabolic signals (Akt, Glut-4) or inflammatory regulators (NFκB, IKKα/β), WD did induce an increase in circulating levels of inflammatory cytokines/chemokines (IL-12(p70), eotaxin, IFN-γ, MIP-1α and MIP-1β)[112]. However, the

observation of metabolic and immunological abnormalities observed during ATM deficiency in response to WD is consistent with previous studies[38,63,80,104,108].

In summary, the data provides evidence that WD during ATM deficiency in male mice augments weight gain, induces systolic dysfunction with increased preload, exacerbates cardiac remodeling with increased hypertrophy and apoptosis, and increases circulating inflammatory mediators.

Female ATM Deficient Mice on WD

WD commonly associates with altered metabolic signaling and excess adipose tissue accumulation[60,69,70]. While A-T patients experience minimal weight gain and dysfunctional adipocyte maturation, there is limited information regarding sex-specific differences in A-T carriers in response to WD [89,98]. Previously, we demonstrated that ATM deficiency in male mice associates with accelerated weight gain, systolic dysfunction with increased preload and increased cardiac cell apoptosis in response to WD[112]. Interestingly, following 14-weeks of WD, female ATM deficient mice exhibit attenuated body weight and fat gain in response to WD compared to WT. Metabolic dysfunction with defective lipoprotein clearance and concomitant increases in serum cholesterol and triglyceride levels have been observed during lack or deficiency of ATM[12,114]. Similarly, we demonstrated that female ATM deficient mice display increased cholesterol, triglyceride and fasting glucose levels in response to WD. Increases in adipose tissue and WD associate with altered hemodynamics, cardiac remodeling and functional abnormalities[1,15,45,113]. The degree of cardiac dysfunction associates with the duration and severity of adiposity[1,37]. The data presented here demonstrates that following 14-weeks of WD, there is reduced adipose tissue accumulation in female ATM deficient mice which associated

with preserved cardiac systolic and diastolic function. These observations are in contrast with WT female mice where, WT-WD mice displayed increased body and fat weight gain with decreased systolic function. Adiposity and WD associate with exacerbated fibrosis, hypertrophy and apoptosis[1,44,72]. WD did induce an increase in fibrosis and hypertrophy in WT mice. Interestingly, WD failed to increase fibrosis and hypertrophy in ATM deficient mice when compared to the normal chow group. However, hypertrophy was significantly higher in ATM deficient group vs WT in response to WD. As previously mentioned, the compensatory development of fibrosis and hypertrophy following WD and/or adiposity is initially protective. However, continual compensation can lead to cardiac apoptosis[46,112]. This study provides evidence that ATM deficiency associates with decreased apoptosis, and Bax expression and increased levels of intact PARP-1 protein in response to WD. Conversely, WT mice on WD exhibited increased cardiac cell apoptosis, and Bax expression and decreased levels of intact PARP-1 protein. These data suggests that the decrease in fat accumulation and cardiac remodeling observed during ATM deficiency in female mice provided cardioprotective effects in response to WD. Meanwhile, increased fat accumulation, myocardial fibrosis and myocyte hypertrophy, contributed to the increase in apoptosis observed in WT mice in response to WD. Changes in metabolic pathways and cell survival proteins are commonly observed with WD and obesity[55,61,64,113]. Extended activation of Akt, which is an important modulator of metabolic and cell survival, can exacerbate fibrosis and cardiac hypertropy[25]. Consistent with this observation, WD failed to increase Akt activation in female ATM deficient mice, while WT exhibited increased activation of Akt compared to WT-NC. Overexpression of mTOR has been linked to decreased diet induced cardiac remodeling and dysfunction following myocardial ischemia reperfusion injury[10]. Our data provided evidence that WD decreases activation of mTOR in the

hearts of WT group, while ATM deficient hearts exhibited increased mTOR activation when compared to their WT counterpart 14 weeks post-WD.

In summary, ATM deficiency in female mice mitigates bodyweight and fat gain, preserves systolic and diastolic function, attenuates cardiac apoptosis, and alters expression and activation of apoptotic, fibrotic, and metabolic signaling molecules. Further, ATM deficiency associated with increased serum cholesterol, triglycerides, and fasting glucose levels in response to WD.

WD-Induced Cardiac Effects in ATM Deficient Mice Following MI

Many factors such as diet, excess adipose tissue, metabolic disorders, sedentary lifestyle and gender influence the risk of myocardial infarction (MI)[58,77]. The majority of Americans are obese and consume a WD, which independently increases the risk of MI by 35 % alone[8,24,50,79,81]. Interestingly, males under the age of 45 have a 2.5 times greater incidence of MI than females[87]. While MI is less prevalent in females under the age of 45, the mortality rate is higher in females post-MI[59]. Males and females are equally affected by *ATM* gene mutations[34]. A-T carriers, with a heterozygous knockout mutation in the *ATM* gene, account for approximately 1.4-2% of the general population. A-T patients prefer to consume diets high in fat in sugar similar to WD[34,113]. WD, adiposity and ATM deficiency independently increase the risk of ischemic heart disease[1,44,47,113]. Previously, we demonstrated that ATM deficiency in male mice associates with accelerated weight gain, systolic dysfunction with increased preload, and increased apoptosis in response to WD[112]. Conversely, ATM deficiency in female mice display attenuated body weight gain, preserved cardiac function and decreased apoptosis in response to WD (manuscript in preparation). In male mice, WD induced systolic dysfunction as observed by decreased %FS,

and %EF, and increased LVESV and LVESD to similar extent in both WT and ATM deficient mice when compared to their normal chow counterparts[112]. In contrast, WD induced systolic dysfunction, as observed by decreased %FS and %EF, and increased LVESV and LVESD, only in WT female mice when compared to normal chow counterpart. However, WD had no effect on cardiac function in ATM deficient in female mice (manuscript in preparation). Those data highlight the sex-specific role of ATM deficiency in response to WD. To further investigate the sex-specific differences of ATM deficiency during ischemic heart disease and WD, MI was performed in male and female mice fed with WD for 14 weeks. Analysis of heart function 1 day post-MI revealed that, all WD-MI groups display exacerbated systolic dysfunction compared to their WD alone counterparts. Previously, ATM deficiency on normal chow was shown to associate with attenuated dilative remodeling during early post-MI[31]. This study provides evidence that MI induces similar systolic dysfunction in male counterparts on WD 1 day post-MI. However, LV dilation as observed by increased LVEDD and LVEDV was significantly higher in female ATM deficient mice on WD vs WT group (hKO-F-MI vs WT-F-MI) 1 day post-MI.

As previously mentioned, age and sex contribute to the incidence of MI[87]. Here, some sex specific differences were observed as MI significantly increased systolic and diastolic diameters and volumes (LVESV, LVESD, LVEDD and LVEDV) in WT males vs WT females (WT-M-MI vs WT-F-MI). Our work provides evidence that WD during ATM deficiency in female mice does not affect systolic or diastolic parameters (manuscript in preparation). Therefore, we next evaluated cardiac structural and functional parameters pre and 1 day post-MI. This analysis revealed sex and genotype-specific differences as male WT mice exhibit increased systolic and diastolic chamber size and volume (LVESV, LVESD, LVEDD and LVEDV) compared to WT

female group (WT-M-MI vs WT-F-MI). The decrease in %FS and %EF and increase in systolic and diastolic diameters and volumes 1 day post-MI were significantly higher in female ATM deficient mice when compared to WT female mice (hKO-F-MI vs WT-F-MI). It was interesting to note that ATM deficiency in female mice was cardioprotective in WD-fed mice, however, MI exacerbated cardiac dysfunction. Thus, the cardioprotective effect of ATM deficiency in female mice during WD conditions were abolished 1 day post-MI. Further, MI induced a decrease in AET in both genotypes and sexes. E- and A-waves were also significantly decreased in WD-fed ATM deficient mice 1 day post-MI (hKO-M-MI) vs its control ATM deficient male group (hKO-M-B). On the other hand, A-wave was only significantly lower in WD-fed ATM deficient female mice 1 day post-MI and E-wave was significant lower in WD-fed ATM deficient group vs its WT counterpart. Previously, WD induced a significant increase in cardiac cell apoptosis in all groups and sexes. However, ATM deficient males exhibited significantly more apoptosis in their myocardium vs WT males[112]. Conversely, ATM deficiency in female mice associated with decreased cardiac cell apoptosis vs the WT female group (manuscript in preparation). Normal chow fed ATM deficient mice are shown to have increased cardiac cell apoptosis in the infarct region of the heart 1 day post-MI with similar infarct size[31]. It should be noted that this study did not analyze male and female separately[31]. Here, we demonstrate that both WD-fed genotypes and sexes displayed similar increases in myocyte and total cell apoptosis 1 day post-MI. Similarly, WD feeding did not affect infarct size 1 day post-MI. While infarct thickness was previously found to be increased during ATM deficiency 7 days post-MI[41], here infarct thickness remained unchanged between genotypes and sexes.

In summary, MI induced systolic dysfunction in both genotypes and sexes compared to WD-baseline. The average change in systolic parameters post-MI showed that WD during ATM

deficiency in female mice exacerbates cardiac dilative remodeling 1 day post-MI. Thus, the cardioprotective effects of ATM deficiency in female mice observed in response to WD are abolished 1 day post-MI. Further, MI induced similar decreases in apoptosis with no effect on infarct size and thickness in both genotypes and sexes.

Future Directions

The current investigation explored the sex-specific WD-induced cardiac structural, functional and biochemical alterations during ATM deficiency at baseline and under ischemic conditions (1 day post-MI). However, additional studies are needed to further elucidate the sex-specific role of ATM deficiency in WD/MI-induced cardiac dysfunction.

We have provided evidence that male WT and ATM deficient mice exhibit significant decrease in systolic and diastolic function in response to 14 weeks of WD. The introduction of a normal chow diet with and without access to an exercise regimen 8 weeks post-WD will help elucidate if cardiac function can be improved or restored in the male group.

We have also shown that female WT mice exhibit systolic dysfunction and increased cardiac cell apoptosis in response to WD. However, female ATM deficient mice maintained cardiac function and displayed decreased cardiac cell apoptosis in response to WD. It would be interesting to investigate if estrogen played a role in the attenuation of WD-induced cardiac dysfunction and cardiac cell apoptosis in female ATM deficient mice. An ovariectomy could be performed on WT and ATM deficient mice at six weeks of age. After which, the mice can be placed on WD for 14 weeks to assess the role of estrogen in the cardioprotective effects of ATM deficiency in response to WD.

We provided evidence that 1 day post-MI, both genotypes and sexes exhibited cardiac dysfunction with similar increases in apoptosis, infarct size and infarct thickness. Thus, the protective effect observed with WD during ATM deficiency in female mice was abolished 1 day post-MI. However, these observations were made during early (1 day post-MI). Therefore, cardiac functional and biochemical parameters should be assessed during late MI to better understand the role of ATM deficiency in the cardiac structural and functional alterations occurring with WD and late MI. Lastly, it would be interesting to perform echocardiography on a weekly basis during the 14 weeks of WD feeding and post-MI to identify specific time points when development of WD/MI-induced systolic and diastolic dysfunction starts to occur in both genotypes and sexes.

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