ABSTRACT

Title of Dissertation:	THE EFFECTS OF AGE, SARCOPENIA, AND RESISTANCE EXERCISE TRAINING ON MITOCHONDRIAL STRUCTURAL DYNAMICS IN SKELETAL MUSCLE
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Sarcopenia, the progressive, age-related loss of skeletal muscle mass, contributes to older adults' risk of falls, hospitalization, and loss of independence. Mitochondrial dysfunction is a hallmark trait of aging and sarcopenia that may be mediated by changes to mitochondrial structure and location through the involvement of mitochondrial fusion, fission, and mitophagy (collectively referred to as mitochondrial quality control). Therefore, the purpose of this dissertation was to investigate whether mitochondrial quality control is altered by age or sarcopenia. The first study performed in a rat model of aging demonstrated that expression of proteins regulating fusion and mitophagy was higher in skeletal and cardiac muscle from old vs. young rats, and this was accompanied by reduced expression of fission proteins in skeletal muscle in the old rats. The second study included older humans and revealed no differences in mitochondrial quality control protein expression in skeletal muscle from sarcopenic vs. non-sarcopenic older adults. Furthermore, twelve weeks of resistance exercise training did not alter the expression of mitochondrial quality control proteins in the sarcopenic individuals. The third study investigated

morphological differences in mitochondrial subpopulations and lipid droplets from the sarcopenic individuals from study two, both before and after resistance exercise training. Peripherally located and intermyofibrillar mitochondrial content and morphology did not change significantly after resistance exercise training. Lipid droplets from the intermyofibrillar region were similarly unchanged, but lipid droplets from the peripheral region had minor morphological changes after resistance exercise training. Together, this dissertation indicates that mitochondrial quality control proteins in skeletal and cardiac muscle are altered in response to aging and may contribute to mitochondrial dysfunction, but mitochondrial structural dynamics in skeletal muscle do not appear to be altered in older adults with a moderate degree of sarcopenia. This suggests that other, non-mitochondrial factors may play larger roles in the pathophysiology of sarcopenia. While the sarcopenic participants did improve muscular strength after resistance training, this was not accompanied by changes in mitochondrial content, morphology, or quality control. Therefore, resistance exercise training may not be an effective strategy to enhance mitochondrial structural dynamics in sarcopenia.

THE EFFECTS OF AGE, SARCOPENIA, AND RESISTANCE EXERCISE TRAINING ON MITOCHONDRIAL STRUCTURAL DYNAMICS IN SKELETAL MUSCLE

by

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List of Abbreviations

ALM – Appendicular lean mass

ATP – Adenosine triphosphate

AWGS – Asian Working Group for Sarcopenia

BNIP3 – Bcl-2 19kD interacting protein

BMI – Body mass index

BSA – Bovine serum albumin

CAS – Catalog number

CIV – Complex IV

CS – Citrate synthase

DRP1 – Dynamin-related protein 1

ER – Endoplasmic Reticulum

FIS1 – Fission-1

HRP – Horseradish peroxidase

IgG – Immunoglobulin G

IMF – Intermyofibrillar

IMM – Inner mitochondrial membrane

mAb – Monoclonal antibody

MFF – Mitochondrial fission factor

MFN1 – Mitofusin 1

MFN2 – Mitofusin 2

Mid49 – Mitochondrial dynamics protein of 49 kDa

Mid 51 – Mitochondrial dynamics protein of 51 kDa

mtDNA – Mitochondrial DNA

OMM – Outer mitochondrial membrane

OPA1 – Optic atrophy type 1

OPA1-Long – Optic atrophy type 1 long isoforms

OPA1-Short – Optic atrophy type 1 short isoforms

p62 – Sequestosome 1

pAb – Polyclonal antibody

PGC-1α – Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha

Pink1 – PTEN-induced kinase 1

PL – Peripherally Located

PLIN – Perilipin

PTEN – Phosphatase and tensin homolog

PVDF – Polyvinylidene fluoride

ROS – Reactive oxygen species

SMI – Skeletal muscle mass index

SOD – Superoxide dismutase

TA – Tibialis anterior

TBS – Tris buffered saline

TBS-T – Tris buffered saline with 0.1% Tween 20

VO_{2max} – Maximal oxygen consumption

Chapter 1: Introduction

The older adult population is the fastest growing age group in the United States and has more than doubled in size over the past 50 years[1]. In fact, the population of adults aged 65 years and over is expected to surpass the population of children and adolescents age 18 years and under by 2035[2]. This shift in population size is expected to result in increased government spending on healthcare due to age-related injuries and hospitalizations. Older adults are at an increased risk of frailty and falls largely due to the progressive age-related decline in skeletal muscle mass, referred to as sarcopenia. Beginning at age 40, adults experience a loss in skeletal muscle mass ranging between 3%-5% per decade[3]. The rate of muscle loss accelerates with advanced age as longitudinal studies reporting a loss of 6.5-10% per decade after age 75[3]. An estimated 11% and 9% of independently living men and women, respectively, over the age of 60 are sarcopenic worldwide[4,5]. The prevalence of sarcopenia immensely increases when evaluating older adults in nursing homes, where 51% of men and 31% of women are sarcopenic[4].

Sarcopenia is a multifactorial disease that is characterized by a decrease in myofiber number and cross-sectional area. Sarcopenia is largely caused by both modifiable and non-modifiable factors associated with aging including reduced caloric intake, physical inactivity, hormonal changes, decreased myofibril synthesis, and sporadic cycling of denervation and reinnervation of muscle fibers[6,7]. Regardless of the cause, sarcopenia is accompanied by decreased oxidative capacity and muscle capillarization, resulting in greater fatigability[8,9]. These decreases in muscle quantity and function negatively impact physical function and ability to complete activities of daily living. Diminished function increases risk of falls and hospitalization, leading to

loss of independence and need for caretaking. Despite the numerous factors that contribute to the onset of sarcopenia, currently there are no pharmaceutical treatments for the debilitating disease.

Mitochondrial Quality Control

Mitochondrial dysfunction has been implicated in the process of biological aging. Mitochondria are responsible for calcium storage, initiating apoptosis, and, importantly, producing energy in the form of adenosine triphosphate (ATP). Proper mitochondrial function is vital for healthy muscle and when dysfunction occurs, neurodegenerative, metabolic, and cardiovascular diseases can arise[10,11]. It has been well established that sarcopenic individuals exhibit mitochondrial dysfunction[12,13]. Aged skeletal muscles exhibit altered mitochondria regulatory protein expression, accumulation of genetic damage and genomic instability, and increased reactive oxygen species (ROS) accumulation[6,11,14].

Mitochondrial function is highly dependent on mitochondrial structure. Skeletal muscle mitochondria exist in a dynamic reticulum where structure is regulated by fusion, fission, and mitophagy[15]. Collectively, these processes are referred to as mitochondrial quality control. Fission aids in maintaining mitochondrial function by cleaving off damaged or dysfunctional portions of mitochondria. After being isolated, the damaged portion can subsequently be degraded through mitochondrial-initiated mitophagy. On the other hand, fusion is responsible for the growth of the mitochondrial reticulum and can improve mitochondrial efficiency, stabilize mitochondrial DNA, and promote cristae remodeling[16,17]. A balance of both fission and fusion is essential to maintain properly functioning mitochondria, an excess of either process can be detrimental to skeletal muscle health. Uncontrolled fission leads to fragmented mitochondria, limiting their functional

capacity; whereas uncontrolled fusion results in hyperfused mitochondria, reducing mitophagy and the ability to remove dysfunctional mitochondria[17].

As a double membraned organelle, mitochondria are dependent on both outer (OMM) and inner (IMM) mitochondrial membrane fusion proteins (Figure 1). Fusion is initiated by OMM-bound GTPases mitofusin 1 (MFN1) and mitofusin 2 (MFN2)[18]. After two adjacent mitochondria are tethered by MFN, they undergo a conformational change that physically brings the two mitochondria closer together[18,19]. GTP hydrolysis is necessary for fusion, although the exact mechanism on how fusion occurs is not fully understood. One theory is amphipathic helixes located next to MFN facilitates the merger of the OMMs since amphipathic helixes naturally perturb the OMM without causing lysis[20]. While MFN1 and MFN2 work in tandem to induce fusion of the OMM, they have slight structural differences that are believed to affect their specific roles. However, both have been shown to be required for fusion[21,22].

After OMM fusion, IMM fusion is mediated by dynamin-like GTPase optic atrophy type 1 (OPA1). OPA1 function is dependent on proteolysis that creates 8 different isoforms which are classified as either long (OPA1-Long) or short (OPA1-Short) isoforms[19,23]. Mitochondrial processing peptide cleaves OPA1 to create OPA1-Long isoforms, which can be cleaved again by proteases OMA1 or YME1L to create OPA1-Short isoforms[24]. While OPA1-Long isoforms are bound to the IMM, OPA1-Short isoforms are soluble and located in the intermembrane space. As such, the precise function of the OPA1-Short isoforms is debated since the OPA1-Long isoforms alone are sufficient to induce fusion[21,25]. Recently, OPA1-Short has been implicated in cristae regulation and fusion inhibition[25,26]. Under normal conditions, YME1L is constitutively active, whereas OMA1 is induced when ATP production decreases or membrane potential dissipates[23,25]. By inducing OMA1, the presence of OPA1-Short isoforms increases and the OPA1-Long isoforms

decrease, which is thought to prevent damaged mitochondria from fusing together. Interestingly, the OMMs can still fuse despite an increase in OPA1-Short isoforms. Rather than having one IMM, the mitochondrion will have multiple IMMs, thus inhibiting the sharing of matrix components and membrane potential[27,28].

When mitochondrial membrane potential dissipates, fission is triggered in order for dysfunctional mitochondria to be removed via mitophagy (Figure 1)[29]. Fission is largely dependent on the GTPase dynamin-related protein 1 (DRP1) and the endoplasmic reticulum (ER). Fission often occurs at mitochondrial-ER contact sites where the ER wraps around the mitochondria[18]. DRP1 is recruited from the cytosol to the mitochondrial-ER sites via OMM-bound receptors fission-1 (Fis1), mitochondrial fission factor (MFF), and mitochondrial dynamics proteins of 49 and 51 kDa (Mid49 and Mid51)[27,29]. DRP1 undergoes oligomerization to form larger dimers and tetramers that will encircle the OMM[30]. Hydrolysis of GTP results in a conformational change that will physically constrict the OMM, resulting in two separate mitochondria[30].

Following fission, mitophagy can occur. Mitophagy is mitochondrial specific autophagy that can selectively degrade dysfunctional mitochondria (Figure 1). The best understood mitophagy system is the ubiquitin-dependent Phosphatase and tensin homolog (PTEN)-induced kinase 1 (Pink1) and Parkin pathway. Under normal conditions, Pink1 is imported to the IMM where it is cleaved by mitochondrial processing peptidase and presenilin associated rhomboid-like protease before being degraded[31]. When mitochondria depolarize, Pink1 is unable to translocate to the IMM causing it to accumulate on the surface of the mitochondria which will recruit ubiquitin ligase Parkin to the OMM[32]. The activation of Parkin through phosphorylation by Pink1 at Ser65 allows for ubiquitination of OMM proteins[18,33]. Autophagic adaptor proteins, including p62 and NDP52, serve as the connection between the ubiquitin and autophagy degradation pathways.

Autophagic adaptor proteins will bind to ubiquitin and tether mitochondria to autophagosomes[32]. Mitophagy is completed when the autophagosome fully engulfs the mitochondria and fuses with a lysosome.

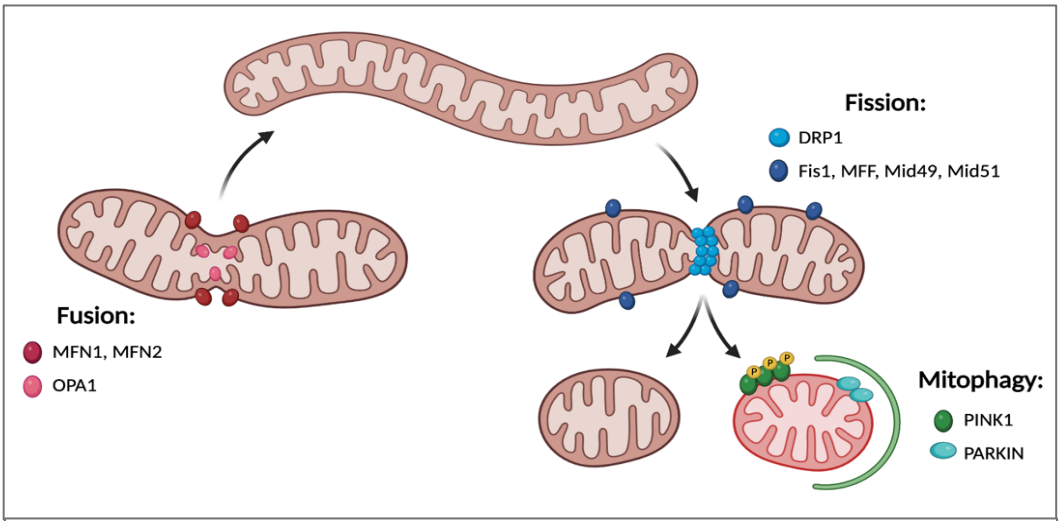

Figure 1. Schematic of mitochondrial quality control. Fusion joins adjacent mitochondria resulting in elongated mitochondria. Fusion is regulated by outer mitochondrial membrane proteins MFN1 & MFN2 and inner membrane protein OPA1. Conversely, fission separates mitochondria and is regulated by DRP1. Outer membrane proteins FIS1, MFF, Mid49, and Mid51 recruit DRP1, which will constrict leading to fragmented mitochondria. When mitochondria are damaged and membrane potential dissipates, PINK1 accumulates on the outer membrane. The phosphorylation of PINK1 recruits PARKIN to the outer membrane which will also undergo PINK1-dependent phosphorylation. Parkin triggers ubiquitination of outer membrane proteins which will serve as binding sites for phagophores before ultimately being consumed and degraded.

Mitochondrial turnover is kept in balance by mitochondrial biogenesis. The production of new mitochondria is regulated by peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 α)[34]. PGC-1 α is enriched in mitochondrial dense tissues such as skeletal and cardiac muscle and is inducted by increased energy demands, such as aerobic exercise and starvation[34,35]. Translocation of PGC-1 α to the nucleus induces other downstream transcription co-activators including nuclear respiratory factor 1 and 2, which in turn will activate mitochondrial

transcription factor[34]. Mitochondrial transcription factor will translocate to the mitochondria where it will promote mitochondrial DNA replication and transcription[36].

Sarcopenia and Mitochondrial Quality Control

Sarcopenia is a multifaceted disease that ultimately results in the reduction of skeletal muscle mass and cross-sectional area. One of the main pathways controlling muscle mass is the IGF1-Akt axis. Protein synthesis is stimulated when Akt activates mTOR, whereas protein breakdown is stimulated when Akt activates the family of transcription factors known as FoxO[37]. FoxOs induce the ubiquitin-proteosome and autophagy-lysosome systems by recruiting several atrogenes including Atrogin-1, MuRF-1, MUSA1, BNIP3, LC3, and ATF3[37–40]. Mitochondrial quality control impacts skeletal muscle mass maintenance, at least partially by regulating these pathways. Inducible knockout models have demonstrated skeletal muscle specific knockout of fusion proteins MFN2 and OPA1 result in increased ROS production, mitochondrial fragmentation, impaired autophagy, and decreased skeletal muscle cross sectional area[41,42]. These impairments are also accompanied by higher expression of FoxO3, Atrogin-1, MuRF-1, and transcription factor ATF4, which upregulates atrophy related gene FGF21[42]. Inducible double knockout of DRP1 and OPA1 in skeletal mouse increased protein expression of FoxO3 as well as expression of several of its downstream targets including Atrogin-1, MuRF-1, and MUSA1[43]. DRP1 inhibition also leads to reduced mitochondrial function and skeletal muscle cross sectional area[44–46]. Similarly, double overexpression of DRP1 and FIS1 produces skeletal muscle atrophy through the activation of FoxO3 by AMPK[44]. Together these studies demonstrate skeletal muscle mass maintenance is interconnected with mitochondrial quality control pathways.

Previous studies have indicated mitochondrial quality control is altered in skeletal muscle with age, although there is some conflicting literature. Some rodent studies have reported that fusion, fission, and mitophagy increase with age, with older rats having higher Parkin, MFN2, OPA1, and FIS1 protein expression compared with young rats[47,48]. However, other rodent and human studies have found no differences in MFN1/2, OPA1, and FIS1 protein expression between young and older counterparts[49–51]. Conversely, others have reported decreases in gene and protein expression of fusion and fission proteins in humans and rodents[42,52,53]. Thus, the effects of age on mitochondrial quality control proteins are unclear.

The effects of sarcopenia on mitochondrial quality control are even less understood, as only two studies have compared mitochondrial quality control expression in human sarcopenic and non-sarcopenic skeletal muscle[54,55]. The first found mixed results with MFN2 protein expression being downregulated in sarcopenic individuals, while other fusion proteins (OPA1, MFN1), as well as FIS1 did not differ from to non-sarcopenic age-matched individuals[54]. However, that study had several limitations as all participants were frail and hospitalized due to accidental falls resulting in hip fractures. Additionally, the participants were 80 years of age and older, which only accounts for ~16% of the older adult population[56]. A separate study compared mitochondrial quality control gene expression between sarcopenic and non-sarcopenic men using genome-wide transcriptomics data acquired from the Singapore sarcopenia study[55]. The investigators found lower OPA1 and FIS1 gene expression, but no differences in MFN2 expression between sarcopenic and non-sarcopenic older men[55]. This study had limitations as the authors did not quantify mitophagy-related gene expression, nor did they quantify protein expression for any mitochondrial quality control proteins. It is also important to note that only male participants were used so results could have been sex-specific and sex difference could be missed.

Sarcopenia and Intracellular Organization

Mitochondrial localization also impacts mitochondrial function. Unlike other tissues, skeletal muscle mitochondria function in a dynamic network to optimize ATP production and minimize ROS production. Recent work has discovered morphological differences between peripherally located mitochondria positioned below the sarcolemma and those located deeper within the myofiber (intermyofibrillar). Although both subpopulations work in tandem, morphological differences have led researchers to believe the two subpopulations contribute differently to energy distribution. Peripherally located (PL) mitochondria are believed to have a higher propensity to generate membrane potential, which is then utilized by intermyofibrillar (IMF) mitochondria to generate ATP[57]. This discovery was made when immunohistochemistry revealed a higher concentration of complex IV near the sarcolemma and a higher concentration of complex V deep within the myofiber[15,57].

Limited research has been conducted evaluating morphological changes of mitochondrial subpopulations with age. Decreased mitochondrial content within skeletal muscle is characteristic of aging, but this may be an oversimplification. One study reported increased fragmentation of IMF mitochondria in aged mice compared to young but observed no changes in the total area of all IMF or PL mitochondria normalized to the area of the micrograph taken (hereby referred to as fractional area)[58]. A separate study found no differences between IMF and PL mitochondrial number or fractional area in young and older adults[59]. That study did, however, report that IMF mitochondria were smaller on average in the older adults compared with young adults[59]. Similarly, another study found IMF mitochondria were smaller in older adults, while PL mitochondrial size did not differ from young adults[60]. While it appears age may affect IMF mitochondria, it is not known whether sarcopenia influences mitochondrial subpopulations. PL mitochondria are less

numerous in skeletal muscle of people with type 2 diabetes compared with lean non-diabetic individuals, and this is accompanied by decreased ETC activity[61], suggesting specific mitochondrial subpopulation deficits may contribute to various pathologies. Whether this is true in sarcopenia is unknown as no study has compared mitochondrial subpopulation in sarcopenic and non-sarcopenic skeletal older adults.

Previous studies have also demonstrated that intracellular fat, stored as lipid droplets, is often localized near mitochondria. Lipid droplets are found more abundantly in the IMF region and are frequently tethered to IMF mitochondria[57]. Lipid-mitochondrial contact can aid in energy production in healthy muscle through fatty acid oxidation following quick fatty acid import to the mitochondria[17]. However, with age, intracellular lipid droplet content increases and mitochondrial-lipid droplet contacts decrease indicating a shift away from lipid oxidation and towards storage[60]. Rather than being a source of quick energy, these droplets take up space that displace myofibrils[15] and may reduce muscular health.

Exercise Training Attenuates Sarcopenia and Regulates Mitochondria

The role of physical activity in attenuating sarcopenia is well supported[62]. Resistance exercise training is particularly important for older and sarcopenic adults as it not only improves muscle mass and strength, but also improves balance and mobility. Sarcopenic older adults who underwent 12 weeks of full-body resistance band and seated chair exercises saw improvements in regular walking speed, leg muscle mass, body fat, and upper body strength[63–65]. Piastra et al.[66] demonstrated 9 months of low to moderate intensity full-body resistance exercise training increased skeletal muscle mass, handgrip strength, and improved static balance in moderately sarcopenic women. Similarly, low volume resistance exercise training can also elicit positive

adaptations as 12 weeks of resistance exercise only consisting of leg extension and press training increased knee extensor force production along with gait length and width in sarcopenic men[67]. In addition to whole muscle improvements, resistance exercise training also can also decrease inflammation and oxidative stress in sarcopenic individuals. Twelve weeks of resistance band training decreased antioxidant scavenger superoxide dismutase (SOD) in sarcopenic skeletal muscle from older adults[65]. Further, 12 weeks of full-body kettle ball training decreased circulating C-reactive protein in sarcopenic women[68].

Resistance exercise training can also induce mitochondrial improvements in older adults. Previously sedentary older adults who underwent moderate resistance exercise training increased mitochondrial volume and electron transport chain complex content[69–71]. Improvements in mitochondrial function have also been reported; 12 weeks of resistance exercise training increased maximal mitochondrial respiration as well as increased activity of fatty acid metabolism enzyme β-hydroxyacyl Coenzyme A dehydrogenase in older men[72,73]. While no study has evaluated mitochondrial structure after resistance exercise training, few have investigated and reported changes to mitochondrial structure regulating proteins. Mesquita et al. found that after 12 weeks of whole-body resistance exercise training MFN1, MFN2, OPA1, and Drp1 protein expression all increased in older adults[74]. Conversely, Zampieri et al.[75] reported no changes to MFN2 protein expression but lower OPA1 protein expression in older adults following nine weeks of leg press training. No study has evaluated mitochondrial quality control proteins nor mitochondrial morphology in response to resistance exercise training in sarcopenic individuals. Given the negative effects dysregulation of fusion, fission, and mitophagy can elicit on muscle mass, the beneficial regulation of mitochondrial quality control via resistance training may help to combat sarcopenia.

The effect of exercise training on mitochondrial IMF and PL subpopulations is not well understood. Indeed, few studies have evaluated the changes that occur in sarcopenic or older skeletal muscle following exercise training. In aged mice, IMF mitochondrial fragmentation decreased following aerobic exercise training to levels seen in young mice[58]. Similarly, older adults with advanced-stage knee osteoarthritis increased IMF mitochondria number and fractional area following 12-weeks of lower body resistance training[76]. In both studies, PL mitochondria were unaffected, indicating IMF and PL mitochondria may respond differently to aging and resistance exercise training.

Therefore, the overall goal of this dissertation was to determine how mitochondrial quality control and morphology are impacted by age and age-related sarcopenia. The first study investigated age-related changes in mitochondrial quality control using a rat model of aging. To ascertain whether mitochondrial quality control alterations were unique to skeletal muscle with age, fusion, fission, mitophagy, and biogenesis proteins were assessed in cardiac muscles. The second study compared expression of mitochondrial quality control proteins sarcopenic and non-sarcopenic older adults. Fusion, fission, mitophagy, and biogenesis proteins were assessed in sarcopenic older adults after 12 weeks of resistance exercise training. The final study quantified mitochondrial subpopulation and lipid droplet morphology in sarcopenic skeletal muscle before and after resistance exercise training.

Chapter 2: Age and Sex-Specific Changes in Mitochondrial Quality Control in Skeletal and Cardiac Muscle

Introduction

Mitochondria serve an essential role in not only energy production in the form of adenosine triphosphate (ATP), but also in reactive oxygen species (ROS) signaling, calcium handling, and apoptosis. Unfortunately, mitochondrial dysfunction is one of the hallmark characteristics of aging, and previous studies have reported decreases in mitochondrial respiration[77,78], reduced time to open the mitochondrial permeability transition pore[77,79], and increased mitochondrial DNA damage[80] in older skeletal and cardiac muscle. These changes negatively impact muscle health and function with age. Studies have also reported alterations in the structure of mitochondria in skeletal and cardiac muscle of aged animals compared to young counterparts[81,82]. Since mitochondrial structure, in part, determines its function, recent research has been investigating how mitochondrial structure regulating proteins are impacted by age.

In healthy skeletal and cardiac muscle, mitochondria are organized in a reticulum that is modulated to efficiently produce ATP and minimize ROS production. The mitochondrial reticulum is also impacted by the energetic demand of the muscle it resides in. Mitochondria make up ~35% of cardiac muscle volume, but only account for 3-10% of skeletal muscle volume on average[83,84]. Individual mitochondrial volume is also larger in cardiac muscle as opposed to skeletal[83]. These stark differences in mitochondrial volume are due to cardiac muscle relying almost exclusively on oxidative metabolism, thus resulting in large masses of mitochondria to meet the continual energy demand[85]. Mitochondrial volume varies in skeletal muscle based on fiber type. Slow twitch oxidative fibers have higher mitochondrial volume than fast twitch glycolytic

fibers[79,83]. However, mitochondrial volume in oxidative muscles is still lower than cardiac muscle[79,83].

The mitochondrial reticulum in skeletal and cardiac muscles is maintained by multiple processes that are collectively referred to as mitochondrial quality control. Together, the processes of fusion, fission, mitophagy, and biogenesis ensure the mitochondrial reticulum is able to meet the energetic demands of the cell, while mitigating dysfunction. Constant cycles of fusion and fission ensure the mitochondrial reticulum functions optimally by redistributing matrix components and preserving membrane potential[86]. Fusion joins adjacent mitochondria together, creating a more expansive reticulum. Fusion relies on separate proteins to ensure proper fusion of both the outer (OMM) and inner mitochondrial membrane (IMM). OMM-bond proteins mitofusin 1 and 2 (MFN1 and MFN2) allow for OMM fusion, while IMM protein optic atrophy 1 (OPA1) fuses the IMM. Opposing fusion, is the process of fission which separates dysfunctional portions from the reticulum[87]. OMM proteins such as fission-1 (FIS1) and mitochondrial fission factor (MFF) recruit dynamin-related protein 1 (DRP1) to the surface of the mitochondria[88]. DRP1 physically cinches the mitochondrion causing it to cleave into two distinct sections of mitochondria[87].

Cleaved mitochondria can subsequently undergo selected degradation via mitophagy. The primary mitophagy pathway is the phosphatase and tensin homologue-induced putative kinase 1 (Pink1) and Parkin pathway which occurs on the outer mitochondrial membrane (OMM). The accumulation of Pink1 on the OMM recruits and phosphorylates E3 ubiquitin ligase Parkin and causes ubiquitin chains to form[89]. The ubiquitin chain serves as a binding site for a phagophore, which will engulf the dysfunctional mitochondria before merging with a lysosome and degrading the encapsulated mitochondria. Conversely, mitochondrial biogenesis is regulated by the

transcriptional coactivator peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α)[90]. PGC-1α translocates to the nucleus and activates several downstream transcription factors to promote the synthesis of new mitochondria[58]. Carefully balanced mitophagy and biogenesis are therefore crucial for quality control and maintaining the mitochondrial reticulum.

Disruption to mitochondrial quality control not only results in abnormal mitochondrial shape and function but can also impact muscle ultrastructure. Skeletal muscle specific knockout of fusion disrupts myofibril organization, increases mitochondrial fragmentation, and increases ROS production[42,91]. In cardiac muscle, fusion knockout results in pre-mature death, fragmented mitochondria with disrupted cristae, displaced myofibrils, and decreased maximal respiration[92–95]. Knockdown and knockout of fission proteins decreases cross sectional area and maximal respiration in skeletal muscle[45], while in cardiac muscle it causes cardiac enlargement and increased fibrosis[96]. Mitophagy upregulation has differential effects in skeletal and cardiac muscles. Skeletal muscle upregulation of Parkin expression increases muscle mass and cross-sectional area but fragments the mitochondrial network[97]. However, Parkin overexpression in mouse heart does not blatantly affect impact cardiac or mitochondrial structure[96].

Mitochondrial structure regulating proteins may be differently affected by age and may underlie age-related mitochondrial dysfunction. The majority of studies previously conducted have found MFN1 and MFN2 protein content decreases in skeletal muscle from old animals[48,98–102] while OPA1 is largely unchanged[51,98,100]. FIS1 protein expression is increased in skeletal muscle of old animals[48,99–101,103], but there are mixed reports on how DRP1 is affected by age in skeletal muscle. Previous studies have reported DRP1 protein expression stays the same[41,98,100] or increases[101] in skeletal muscle from old animals. Mitophagy related proteins have not been studied as extensively in the context of skeletal muscle aging, but the few studies conducted agree Parkin

protein expression increases with age[47,48,104]. Although there is limited research, it appears mitochondrial quality control proteins are affected differently by age in cardiac tissue. MFN1 and MFN2 protein expression are reduced[41,105], while OPA1 protein expression may be increased in aged hearts[106]. DRP1 protein expression does not appear to change[105], but FIS1, Parkin, and Pink1 have not been extensively researched in cardiac tissue with age. Several studies discussed here also reported declines in maximal respiration and/or increases in ROS production[41,47,100,106], suggesting dysregulation to mitochondrial quality control proteins may be linked to mitochondrial dysfunction with age.

Despite several studies investigating the effects of age on mitochondrial quality control, most studies have lacked a comprehensive approach. Studies often focus on fusion or fission and most do not quantify fusion, fission, mitophagy, and biogenesis in the same study. Further, limited studies have been conducted on mitochondrial quality control in aged cardiac tissue likely stemming from the previously held notion that fusion and fission are unnecessary for normal heart function[107]. Additionally, much of the available literature has been conducted on male subjects with only three of the aforementioned studies including females, and only one comparing male and female animals. Triolo et al.[47] found higher Parkin protein expression in old versus young mice, but also found Parkin expression to be higher in the female mice, compared to male counterparts. Researchers in that study also found higher expression of autophagy-related protein Beclin-1 in the old animals when both sexes were combined[47]. However, when sexes were separated, only the male mice had significantly higher Beclin-1 protein expression in the old group. While, no fusion or fission proteins were evaluated in that study, Triolo et al.[47] demonstrates the exclusion of females, or grouping with male subjects may potentially mask sex-specific changes with age. Considering the current gaps in knowledge, we sought to determine the effects of age

and sex on mitochondrial quality control proteins regulating biogenesis, mitophagy, fusion and fission in both cardiac and skeletal muscle (tibialis anterior) of rats.

Methods

Animals:

All animal research procedures were approved and in accordance with the University of Maryland Institutional Animal Care and Use Committee. 16 young (2-5 months) and 16 old (21-34 months) adult Hilltop SD rats (Rattus norvegicus, Scottdale, PA, USA) were fed ad-libitum with standard chow and unrestricted access to wheel running. Groups were further stratified by sex resulting in four groups with eight rats in each group. Power calculations estimated 5-7 animals per group was needed to detect differences between young and old groups at $1-\beta = 0.80$. No animals were excluded from analysis, nor were there any inclusionary or exclusionary criteria for the rats aside from age.

When rats reached the appropriate ages, they were euthanized. On the day of sacrifice, rats were massed and anaesthetized with 2-4% isoflurane with 100% oxygen. Rats were placed supine on the surgical area and upon cessation of toe and tail-pinch reflexes, a thoracotomy was performed. Hearts were quickly removed and cannulated via the aorta and rinsed with phosphate buffer saline. Death was confirmed via exsanguination. After euthanization, the skin on the hindlimbs of the mice was removed, and the TA from both legs were quickly and carefully dissected and removed. Heart and TA weights were collected before storing at -20°C until analysis.

Western blots:

Mitochondrial quality control protein content was quantified using western blotting. MFN2 and OPA1 were used as markers of mitochondrial fusion, while DRP1 and FIS1 were used as markers of mitochondrial fission. Parkin and Pink1 were used as markers of mitochondrial mitophagy and mitochondrial biogenesis was measured by PGC-1α protein expression. Mitochondrial content was quantified by CIV protein expression.

Proteins were extracted from cells in 1% Triton buffer with HALT protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific 78430 and 78428, Waltman, MA, USA). Whole muscle homogenates were made for both the TA and heart by homogenizing and centrifuging samples at 500 x G for 5 minutes at 4º C. Supernatants were collected and total protein content was quantified by Pierce bicinchoninic acid assay (Thermo Fisher Scientific 23225, Waltham, MA, USA). Equal amounts of protein (30ug for TA; 60ug for heart) were separated by SDS-PAGE on 4-15% Bio-Rad TGX stain free gels (4568085, Hercules, CA, USA) before photoactivation of gel. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad 1704156, Hercules, CA, USA). Protein transfer was confirmed by imaging total protein fluorescence. Membranes were blocked for 2 hours at room temperature in tris buffered saline (TBS; Bio-Rad 1706435, Hercules, CA, USA) with 5% bovine serum albumin (BSA; Fisher Scientific BP1600-100, Hampton, NH, USA) and 0.1% Tween-20 (Bio-Rad 1610781, Hercules, CA, USA). Membranes were incubated overnight at 4º C with primary antibodies and dilutions as listed in Table 1. Due to manufacturer supply issues, we were unable to analyze Pink1 protein expression in cardiac muscle. A secondary antibody conjugated to horseradish peroxidase (Table 1) was used to detect bands in conjunction with Clarity Western Enhanced Chemiluminescent substrate from Bio-Rad (Hercules, CA, USA). Band intensities were

captured using a Bio-Rad ChemiDoc XRS and analyzed using Image Lab 8.0 (Bio-Rad, Hercules, CA, USA). Molecular weights of proteins were confirmed by comparing to a protein standard (Bio-Rad 1610373, Hercules, CA, USA). Band intensities were normalized to total protein in their corresponding lane. To account for differences in mitochondrial content, MFN2, OPA1, Fis1, Drp1, Parkin, and Pink1 content were expressed relative to complex IV (CIV) content. Data are presented normalized to total protein only, and with the secondary analysis relative to CIV protein content. Protein expression for long and short isoforms of OPA1 were quantified separately.

Statistical Analysis:

Statistical analyses were performed using GraphPad Prism 9. Mitochondrial quality control protein expression was analyzed separately for TA and cardiac muscle using two-way ANOVAs with age and sex as factors. Following significant interaction effects, post-hoc comparisons using Fisher's LSD tests were made. All tests were two-tailed with statistical significance set at $P < 0.05$. Data are presented as means ± standard error of the mean.

Table 1. Chapter 2 Antibodies

Antibody	Manufacturer	CAS number	Research Resource Identifiers	Dilution
Anti-rabbit IgG HRP linked antibody	Cell Signaling Technology, Danvers, MA, USA	7074S	AB_2099233	1:2000 in TBS-T with 5% BSA or milk
CIV (3E11) Rabbit mAb	Cell Signaling Technology, Danvers, MA, USA	4850S	AB_2085424	1:1000 in TBS-T with 5% BSA
DRP1 (D6C7) mAb	Cell Signaling Technology, Danvers, MA, USA	8570S	AB_10950498	1:1000 in TBS-T with 5% BSA
Fis1 pAb	Thermo Fisher Scientific, Waltham, MA, USA	PA5-22142	AB_11152577	1:1000 in TBS-T with 5% BSA
GAPDH pAb	Thermo Fisher Scientific, Waltham, MA, USA	PA1-987	AB_2107311	1:5000 in TBS-T with 5% BSA
Mitofusin-2 Rabbit mAb	Cell Signaling Technology, Danvers, MA, USA	83667S	AB_2800025	1:1000 in TBS-T with 5% milk
OPA1 (D6U6N) Rabbit mAb	Cell Signaling Technology, Danvers, MA, USA	80471S	AB_2734117	1:1000 in TBS-T with 5% BSA
Parkin Recombinant Rabbit mAb (21H24L9)	Thermo Fisher Scientific, Waltham, MA, USA	702785	AB_2842252	1:500 in TBS-T with 5% BSA
PGC-1α pAb	Thermo Fisher Scientific, Waltham, MA, USA	PA5-72948	AB_2718802	1:500 in TBS-T with 5% milk
Pink1 Rabbit mAb	Thermo Fisher Scientific, Waltham, MA, USA	PA1-16604	AB_2164267	1:500 in TBS-T with 5% BSA

Legend = CAS, Catalog Number; IgG, Immunoglobulin G; HRP, Horseradish Peroxidase; mAb, monoclonal; pAb, polyclonal; TBS-T, tris buffer saline with 0.1% Tween 20; BSA, bovine serum albumin

Results

Rat Characteristics

Rat characteristics are summarized in Table 2. On average, the aged rats were ~91 weeks older ($P < 0.001$) and had significantly larger body masses ($P < 0.001$) than young counterparts. In the old rats, there was 83% heavier heart mass ($P < 0.001$) and 39% heavier TA mass ($P < 0.001$) compared to young rats. Old animals also had lower skeletal muscle mass relative to body mass than young rats as evident by 31% lower TA to body mass ratio ($P < 0.001$). Heart masses relative to body mass were similar in young and old rats ($P = 0.21$).

On average, young female rats were ~5 weeks younger than young male rats ($P = 0.039$), but old female rats did not differ in age from old male rats ($P = 0.143$). Compared to young males, young females had 66% lower body masses ($P < 0.001$), 54% lower heart masses ($P < 0.001$), and 66% lower TA masses ($P < 0.001$). Similarly, old females had 71% lower body masses ($P < 0.001$), 50% lower heart masses ($P < 0.001$), 70% lower TA masses ($P < 0.001$) than old males. Neither young nor old females TA to body mass ratio differed from males, signifying relative skeletal muscle mass loss was similar between sexes ($P = 0.60$ and $P = 0.99$, respectively).

Table 2. Rat Characteristics

	Young Female (n= 8)	**Young Male (n = 8)**	**Old Female (n = 8)**	**Old Male (n = 8)**
Age (weeks)	8.5 ± 0.2*#	13.3 ± 2.0*	109.9 ± 10.1	93.5 ± 2.7
Body mass (g)	200 ± 4.9*#	331 ± 24.5*	393 ± 18.4#	697 ± 46.1
Heart mass (g)	0.746 ± 0.016*#	1.2 ± 0.046*	1.4 ± 0.078#	2.1 ± 0.123
TA mass (g)	0.395 ± 0.006*#	0.655 ± 0.047*	0.547 ± 0.041#	0.931 ± 0.040
TA/body mass ratio	0.00195 ± 0.00004*	0.00198 ± 0.00004*	0.00136 ± 0.00008	0.00136 ± 0.00006
Heart/body mass ratio	0.00369 ± 0.00008	0.00355 ± 0.00019	0.00358 ± 0.00036	0.00311 ± 0.00019

Legend = TA, tibialis anterior. Data are means ± SEM. *$P < 0.05$ within sex, #$P < 0.05$ within age.

Tibialis anterior mitochondrial content

Mitochondrial content, as measured by CIV protein expression, was 40% lower in the older TAs compared to the young counterparts (main effect of age $P < 0.001$; Figure 2A). While GAPDH was initially going to be used to normalize protein expression, we found GAPDH protein content to be 1.3-fold higher in the old TAs compared to the young (main effect of age $P < 0.005$; Figure 2B). Thus, protein content was normalized to total protein in each sample as acquired from stain-free imaging. To account for potential differences in mitochondrial content, mitochondrial quality control protein expression was also expressed relative to CIV expression. No sex-specific changes were observed in CIV nor GAPDH protein expression.

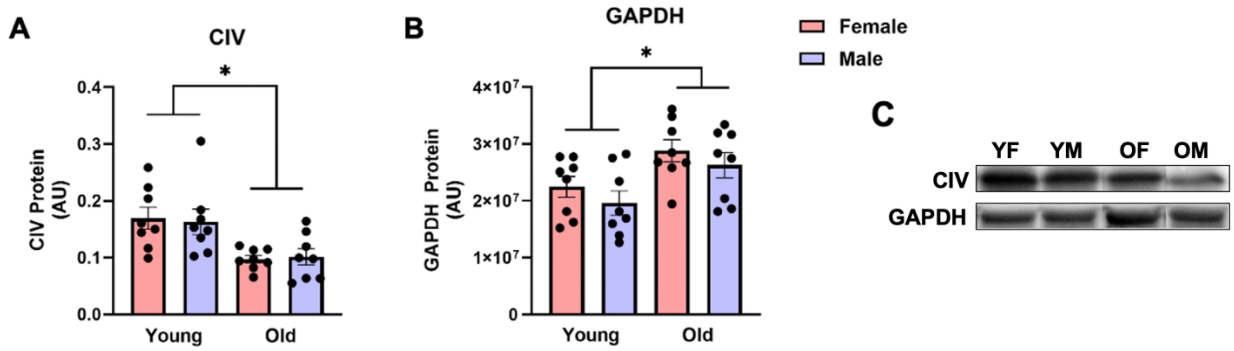

Figure 2. CIV protein is lower, while GAPDH is higher in old tibialis anterior muscles. Legend = AU, arbitrary units; YF, young female; YM, young male; OF, old female; OM, old male. *Significant main effect of age ($P < 0.05$). Data are presented as means $\pm$ SEM, n = 8 per group.

Tibialis anterior mitochondrial fusion

In order to determine whether mitochondrial fusion was affected by age or sex we quantified OMM fusion protein MFN2 and IMM fusion protein OPA1 expression. The long and short isoform of OPA1 were analyzed separately. MFN2 protein levels were 2-fold higher in the TAs from the old animals compared with young animals (main effect of age $P < 0.005$; Figure 3A). Conversely, OPA1-Short and OPA1-Long protein content were unaffected by age or sex (Figure 3B and 3C, respectively). When MFN2, OPA1-Short, and OPA1-Long protein content

were subsequently expressed relative to CIV content, the same results persisted. MFN2 protein levels were 3.5-fold higher in old TAs compared to young when normalized to CIV expression (main effect of age $P < 0.001$; Figure 3D). OPA1-Short or OPA1-Long protein content remained unchanged when expressed relative to CIV expression (Figure 3E and 3F, respectively). No fusion proteins were significantly impacted by sex regardless of CIV normalization.

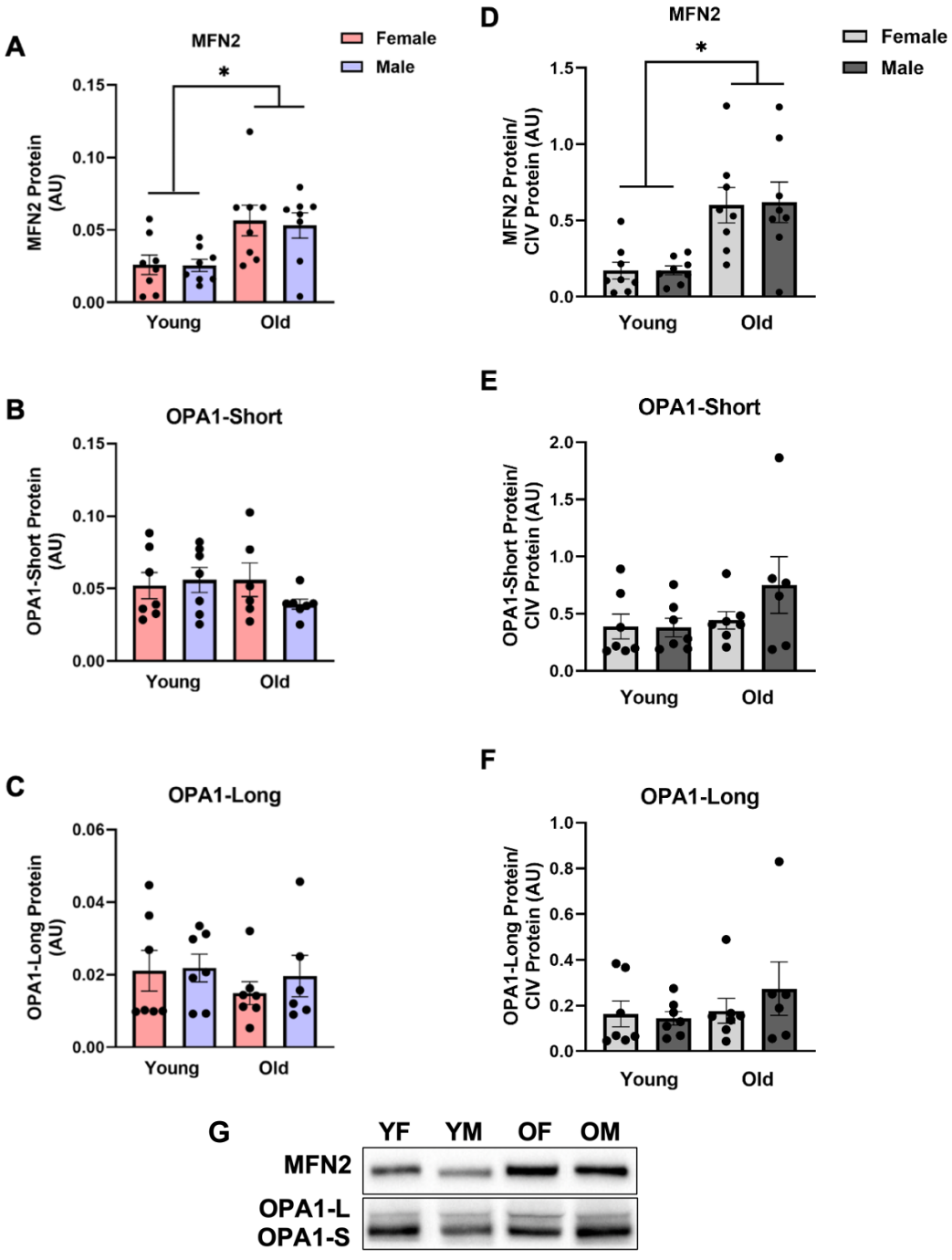

Figure 3. MFN2 protein is higher from tibialis anterior from old rats, while OPA1 is unaffected. Figures A, B, and C are normalized to total protein. Figures D, E, and F are normalized to total protein and subsequently expressed relative to CIV expression. Legend = AU, arbitrary units; YF, young female; YM, young male; OF, old female; OM, old male. *Significant main effect of age ($P < 0.05$). Data are presented as means + SEM, n = 7-8 per group.

Tibialis anterior mitochondrial fission

DRP1 was 40% lower in the old rat TAs compared with young (Main effect of age P = 0.014; Figure 4A). However, there was a significant sex * age interaction effect for Fis1 protein expression (Interaction P = 0.047; Figure 4B), but host-hoc analysis revealed groups were not different from each other. The age and sex-related differences in DRP1 and Fis1 protein expression were likely due to differences in mitochondrial content as these differences were abolished when DRP1 and Fis1 were expressed relative to CIV content (Figure 4C and 4D, respectively).

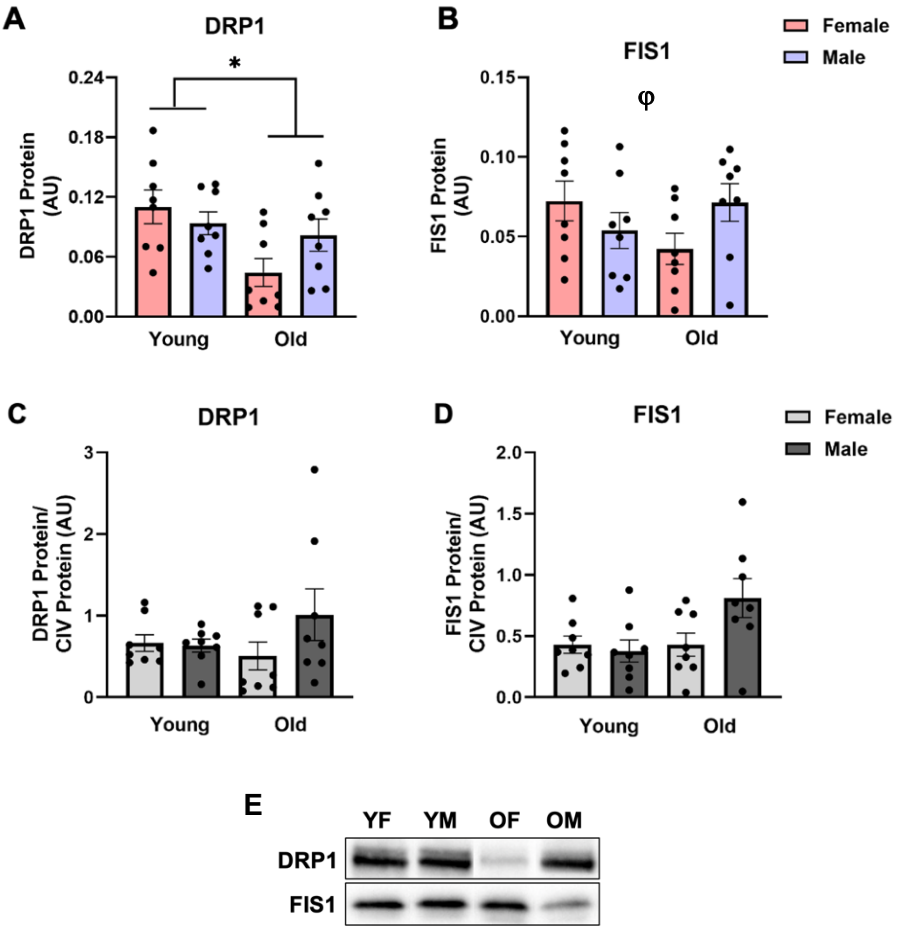

Figure 4. Fis1 protein is impacted by age and sex, while DRP1 is lower in tibialis anteriors from old rats.
Figures A, and B are normalized to total protein. Figures C and D are normalized to total protein and subsequently expressed relative to CIV expression. Legend = AU, arbitrary units; YF, young female; YM, young male; OF, old female; OM, old male. *Significant main effect of age, φSignificant interaction effect ($P < 0.05$). Data are presented as means ± SEM, n = 8 per group.

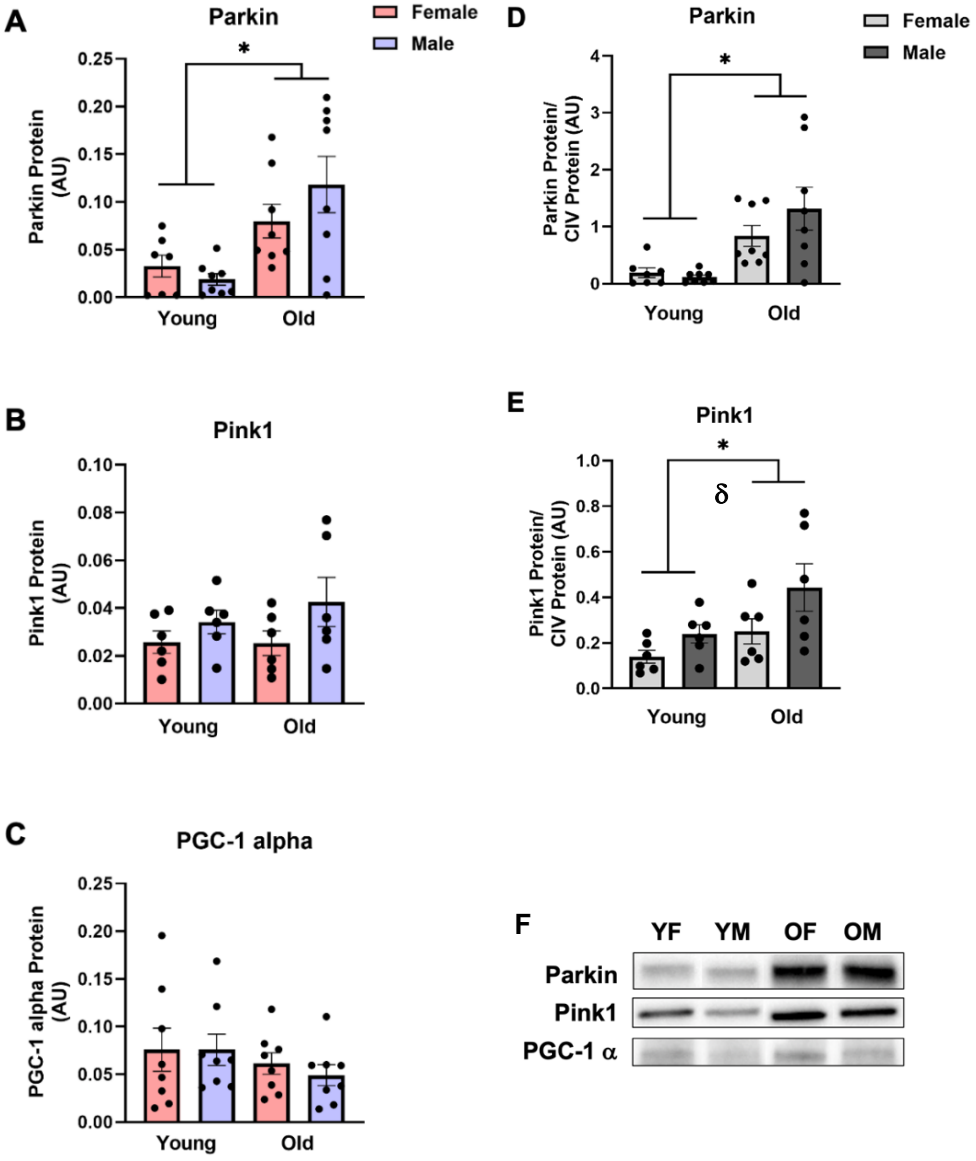

Figure 5. Mitophagy proteins are higher in old tibialis anterior muscles, but biogenesis is unaffected by age and sex. Figures A, B, and C are normalized to total protein. Figures D and E are normalized to total protein and subsequently expressed relative to CIV expression. Legend = AU, arbitrary units; YF, young female; YM, young male; OF, old female; OM, old male. δSignificant main effect of sex, *Significant main effect of age ($P < 0.05$). Data are presented as means + SEM, n = 6-8 per group.

Tibialis anterior mitochondrial mitophagy and biogenesis

Parkin protein expression was almost 4-fold higher in old TAs compared to young counterparts (main effect of age $P < 0.001$; Figure 5A). Pink1 expression did not differ in young and old rat TAs (Figure 5B). When Parkin was expressed relative to CIV, the age differences were further exacerbated with old rat TAs having 7-fold higher Parkin compared to young animals (Main effect of age $P < 0.001$; Figure 5D). Pink1 expression was 83% higher in the old animals compared to young, as well as 75% higher in males compared to females when Pink1 was expressed relative to CIV (Main effect of age $P = 0.023$ and main effect of sex $P = 0.033$; Figure 5E). We also measured PGC-1α as a marker of mitochondrial biogenesis but found PGC-1α protein expression did not differ between young and old rats (main effect of age $P = 0.211$; Figure 5C). There were no sex-specific changes in PGC-1α protein expression in TAs from young and old rats.

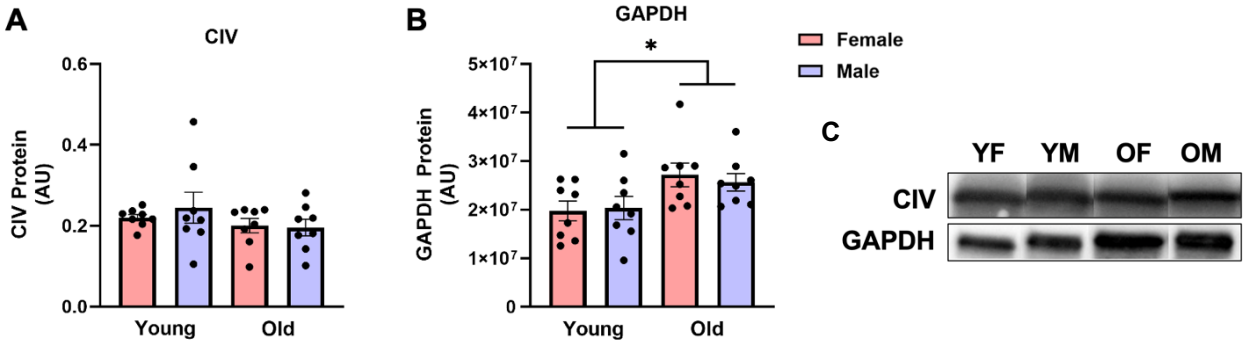

Figure 6. CIV protein is unaffected by age, but GAPDH is higher in old cardiac muscle. Legend = AU, arbitrary units; YF, young female; YM, young male; OF, old female; OM, old male. *Significant main effect of age ($P < 0.05$). Data are presented as means + SEM, n = 8 per group.

Cardiac mitochondrial content

We analyzed the same mitochondrial content and quality control proteins as done in the TAs to determine whether sex or age impacted these processes in cardiac tissue. Unlike the TAs, CIV protein expression did not differ between young and old rat cardiac muscle (Figure 6A).

Conversely, GAPDH expression in cardiac muscle was 40% higher in the old rats compared to young (main effect of age P =0.007; Figure 6B). Because mitochondrial quality control proteins are dependent, in part, on mitochondrial content, markers of fusion, fission, and mitophagy were also expressed relative to CIV expression.

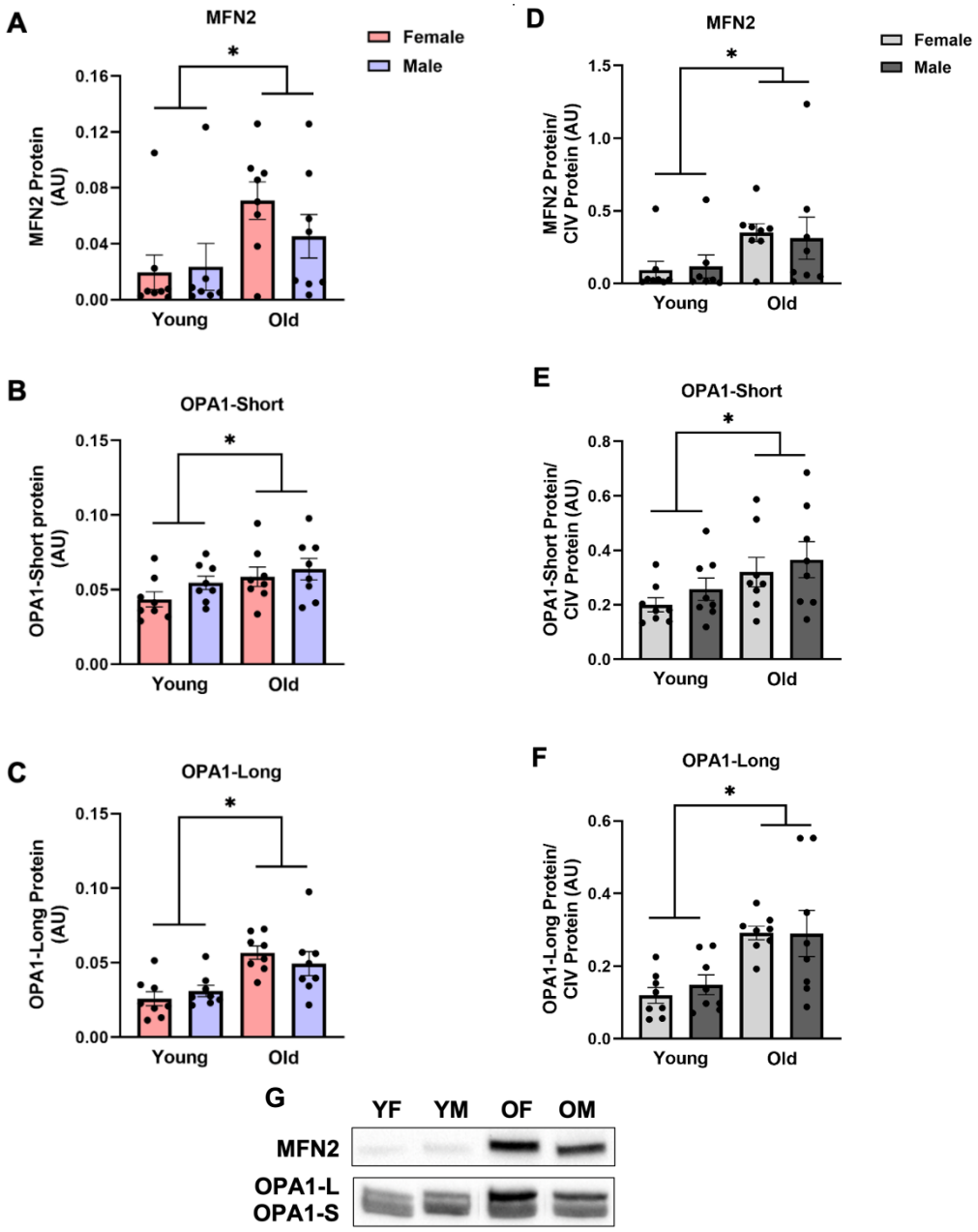

Figure 7. Fusion proteins are higher in old cardiac muscle. Figures A, B, and C are normalized to total protein. Figures D, E, and F are normalized to total protein and subsequently expressed relative to CIV expression. Legend = AU, arbitrary units; YF, young female; YM, young male;

OF, old female; OM, old male. *Significant main effect of age ($P < 0.05$). Data are presented as means ± SEM, n = 7-8 per group.

Cardiac mitochondrial fusion

Age significantly impacted fusion protein expression in cardiac muscles, while sex did not. MFN2 was over 2-fold higher in old cardiac muscles compared to young (main effect of age $P = 0.018$; Figure 7A). Similarly, OPA1-Short and OPA1-Long protein expression were 1.2- and 1.8-fold higher in the cardiac muscle from old rats compared to young, respectively (main effects of age $P = 0.049$ and $P < 0.001$; Figure 7B and figure 7C). This pattern persisted when MFN2, OPA1-S and OPA1-L were expressed relative to CIV expression. In old cardiac muscle, MFN2 protein expression was 3-fold higher, OPA1-Short protein expression was 1.5-fold higher, and OPA1-Long protein expression was 2-fold higher than young rats when expressed relative to CIV expression (man effect of age all $P < 0.03$; Figure 7).

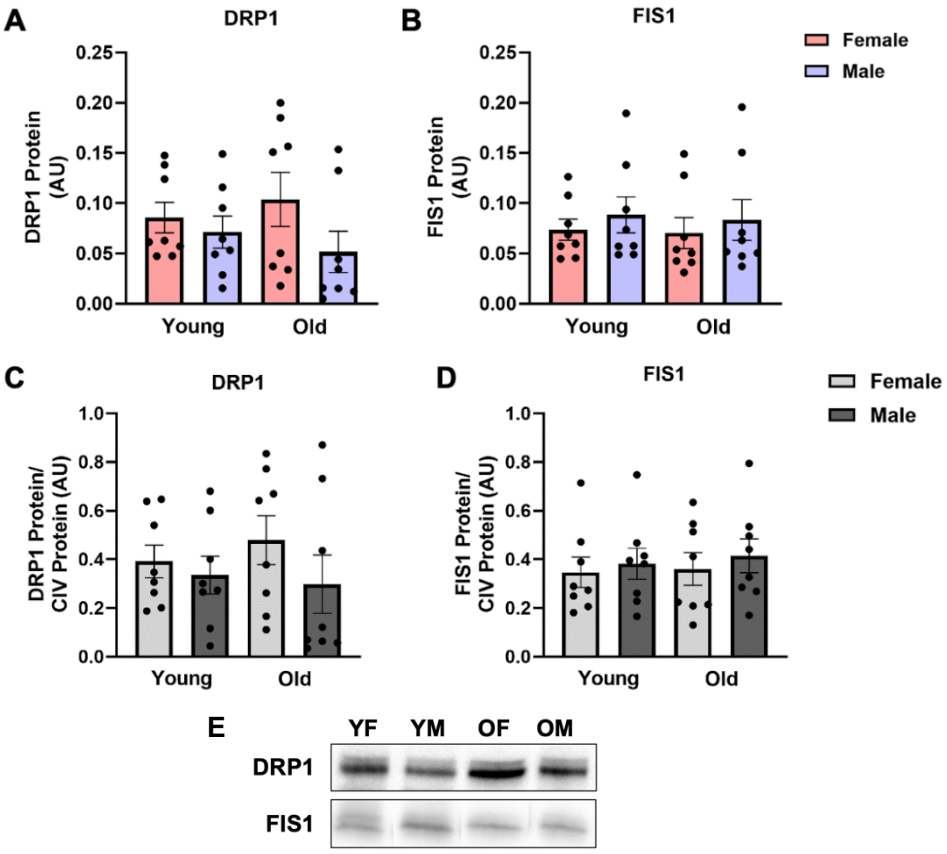

Figure 8. Fission proteins are similar in cardiac muscle regardless of age or sex. Figures A and B are normalized to total protein. Figures C and D are normalized to total protein and subsequently expressed relative to CIV expression. Legend = AU, arbitrary units; YF, young female; YM, young male; OF, old female; OM, old male. Data are presented as means ± SEM, n = 8 per group.

Cardiac mitochondrial fission

We also measured fission protein expression to determine if specific age or sex differences exists in cardiac muscles, however, there were no significant differences in male and female DRP1 and FIS1 protein expression between young or old animals (Figure 8A and figure 8B, respectively). Expressing DRP1 and FIS1 protein expression relative to CIV expression did not alter these results (Figure 8C and figure 8D, respectively).

Cardiac mitochondrial mitophagy and biogenesis

Parkin and PGC-1α were quantified in cardiac muscles from young and old, female and male rats as markers of mitochondrial mitophagy and biogenesis, respectively. Parkin protein expression was over 5-fold higher in the cardiac muscle from old rats compared to young counterparts (Main effect of age $P = 0.019$; Figure 9A). Parkin protein expression remained over 5-fold higher in old cardiac muscle when Parkin was expressed relative to CIV expression (main effect of age $P = 0.020$; Figure 9C). Unlike the TA, PGC-1α protein expression in old cardiac muscles was almost 1.5-fold higher than young cardiac muscles (Main effect of age $P < 0.005$; Figure 9B). No sex-specific differences in markers of mitophagy or biogenesis were detected between young and old rat cardiac muscles.

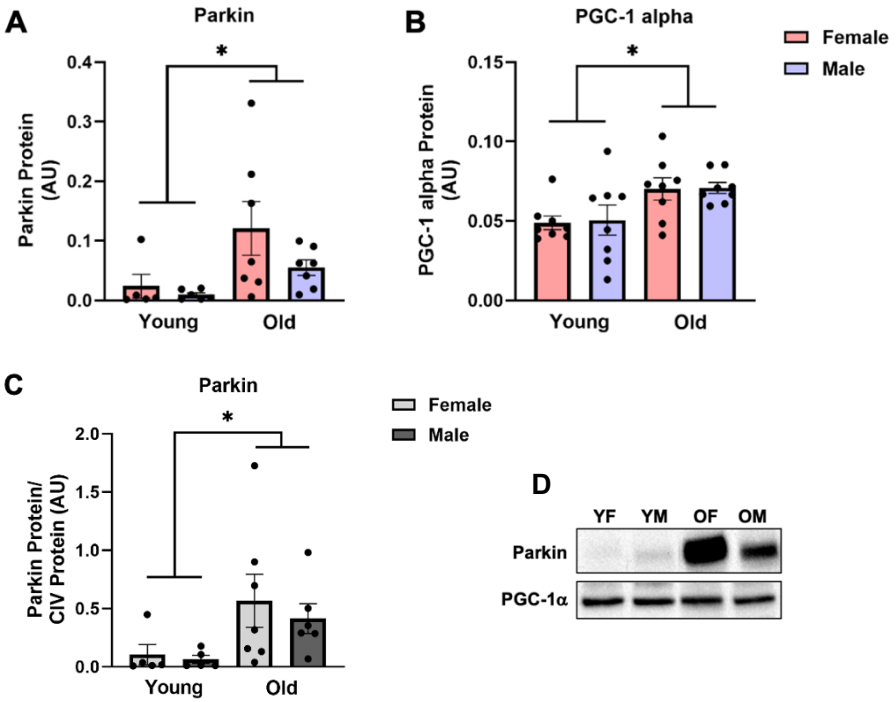

Figure 9. Mitophagy and biogenesis proteins are higher in cardiac muscle from old rats. Figures A and B are normalized to total protein. Figure C is normalized to total protein and subsequently expressed relative to CIV expression. Legend = AU, arbitrary units; YF, young female; YM, young male; OF, old female; OM, old male. *Significant main effect of age ($P < 0.05$). Data are presented as means ± SEM, n = 5-8 per group.

Discussion

The main findings of this study were that proteins regulating mitochondrial fusion, fission, mitophagy, and biogenesis are impacted by age in skeletal and cardiac muscle, without major differences between male and female sexes. In TA muscles, old rats had markedly higher expression of proteins regulating mitophagy and outer mitochondrial membrane fusion. Older animals also had lower mitochondrial content as well as reduced fission protein DRP1 in the TA. A sex and age interaction effect was observed for fission protein FIS1, while the only sex-specific difference observed in the TA was higher Pink1 expression in male versus female rats regardless of age. Cardiac muscle from old rats had higher protein expression of markers of mitochondrial biogenesis, mitophagy, and inner and outer mitochondrial membrane fusion. Conversely, markers

of fission and mitochondrial content did not differ between young and old rats. No sex differences were observed in any mitochondrial quality control marker in cardiac muscle. Together our results demonstrate alterations to expression of mitochondrial quality control proteins with age that differ by muscle type but are largely not sex dependent.

Tibialis Anterior Mitochondrial Quality Control

We found lower mitochondrial content, as measured by CIV, in the TA muscles of old male and female rats, as compared to their younger counterparts. This was not surprising as previous studies have demonstrated mitochondrial content can decrease in skeletal muscle of aged animals. Murine studies have reported lower CIV protein expression as well as citrate synthase activity in old versus young counterparts[99,100]. Further, using electron microscopy, researchers have found mitochondrial density (mitochondria per um^2) is lower in old skeletal muscle than young[41]. To account for differences in mitochondrial content that would impact mitochondrial-dependent proteins, such as fusion, fission, and mitophagy proteins, we presented our protein content data relative to total protein in each sample, as well as relative to CIV expression.

Interestingly, we did not observe any declines in PGC-1α protein expression between young and old groups despite previous studies indicating mitochondrial biogenesis may decrease with age in skeletal muscle[99,108]. However, both of those studies utilized male rats that were an average of 4-14 months older than our old male rats. This may suggest with advanced age, there is a more pronounced decrease in mitochondrial biogenesis as measured by PGC-1α protein expression. Despite PGC-1α levels remaining unaffected by age in our study, we observed a decline in mitochondrial content (CIV expression). Thus, the reduced mitochondrial content may be due to increased mitophagy with age. Indeed, Parkin protein expression, as well as Pink1 expression normalized to mitochondrial content (CIV) were higher in old rat TAs. Pink1 protein

normalized to total protein did not differ between groups, signifying higher Pink1 protein expression in old animals is largely driven by differences in mitochondrial content. However, considering the accumulation of Pink1 on the OMM leads to the recruitment of Parkin and ultimately mitochondrial degradation, higher Pink1 protein expression relative to mitochondrial content may indicate more Pink1 accumulation on the OMM due to mitochondrial dysfunction in TAs from old animals compared to young. In agreement, we also found higher Parkin expression in TAs from old than young animals which would also indicate greater mitochondrial dysfunction in old skeletal muscle. Studies conducting functional analysis on aged skeletal muscle have found consistent results as alterations in mitochondrial respiration, mtDNA, and ROS production have been reported in skeletal muscle from older adults and animals[7,14,109].

Our results are also consistent with others who have found higher expression of mitophagy-related proteins in aged skeletal muscle. As previously mentioned, Triolo et al.[47] found higher Parkin expression in old male and female mice quadriceps. Authors also found Parkin-independent mitophagy protein Bcl-2 19kD interacting protein (BNIP3) was higher in old mice compared to young[47]. Others have reported similar findings and have demonstrated higher expression of Parkin and Parkin-independent mitophagy proteins, including BNIP3 and sequestosome 1 (p62), in male rat extensor digitorum longus and TA muscles[48,110]. In our study, we demonstrated male rats had higher Pink1 protein expression than females in skeletal muscle when controlling for mitochondrial content. Sex differences in mitochondrial quality control proteins has largely been unexplored with few studies for comparison. Our results are somewhat in line with Triolo et al.[47] who found higher expression of autophagy protein Beclin-1 in old male rats, but not old females. Given mitophagy is mitochondrial specific autophagy, it is not surprising that autophagy related proteins may also be upregulated with age, similar to mitophagy proteins. It is unclear as to why

sex specific changes exist in mitophagy and autophagy with age. One hypothesis is, more mitophagy and autophagy in old male rat skeletal muscle, might be a consequence of increased ROS production. A 2022 meta-analysis found ROS measures were higher in skeletal muscle from male subjects across a variety of experimental methods (i.e. oligomycin-stimulated, succinate-stimulated)[111]. They also reported ROS production was correlated with age, in that older men had higher ROS production compared to older women[111]. Elevated levels of ROS can activate both autophagy and mitophagy pathways[112,113], therefore it is possible that increased markers of mitophagy in old male rats may be due, in part, to increased ROS.

We also found significant changes in skeletal muscle expression of fission proteins DRP1 and FIS1 when considering sex and age as variables. While DRP1 protein expression was lower in old rat TAs, there was a significant sex * age interaction effect for FIS1 protein expression. These changes, however, were driven by declines in mitochondrial content. When DRP1 and FIS1 protein expression were expressed relative to CIV protein expression there were no differences between groups, indicating the content of fission proteins relative to mitochondrial content did not differ between young and old rats regardless of sex. Ultimately, the declines in mitochondrial content reduced DRP1 and resulted in a significant sex * interaction effect for FIS1 expression. Our results suggest fission may be impaired in skeletal muscle with age and may be partially dependent on sex. To our knowledge, we are the first to report sex-dependent changes in fission proteins in aged skeletal muscle.

Previous studies are mixed on mitochondrial fission with age. Most studies have reported DRP1 protein expression stays the same[41,51,52,98,100,114] in aged human and murine skeletal muscle. It is important to note that the majority of these studies[41,52,98,100,114] used traditional loading controls, which as we illustrated here, can be altered by age (Figure 2). Instead, we used stain free

technology as it has been proven to have lower variability between samples[115]. Leduc-Gaudet et al.[51] normalized their western data to total protein via ponceau staining and found DRP1 protein expression was unaltered by age in male mice. The authors did report higher MFN2 to DRP1 expression and concluded in old skeletal muscle there is a relative decrease in fission and increase in fusion[51]. Our results agree with the notion that the fission, and more specifically DRP1 expression, may be reduced in aged skeletal muscle.

Intriguingly, both age and sex interacted to influence FIS1 protein expression in our study. The literature is divided on the effects of age on FIS 1 expression, with studies reporting decreases[41,98,102], increases[100,101,103], or no differences[49,50,52,114] in FIS1 protein expression in old compared to young skeletal muscle. It is unclear why there are such varied results but methodological differences (age and muscle group analyzed) along with varied species used (mouse, rat, or human), may account for some of the discrepancies. Capitanio et al.[102] conducted a similar study to us using only male rats and found both FIS1 and DRP1 protein expression decreased in gastrocnemius muscles from 22-month versus 3-month-old rats. Conversely, Yeo et al.[100] studied female mice and found FIS1 to be higher, but DRP1 expression to be similar, in TAs from 24-month and 2-month-old mice. These conflicting results highlight the need for future studies into sex-specific changes to mitochondrial related proteins with age.

We found divergent results when we assessed markers of fusion in skeletal muscle of old and young rats. MFN2 protein expression was higher in old male and female TAs, but neither OPA1 isoform (short or long) was impacted by age or sex. This may indicate greater OMM fusion without a concurrent increase in IMM fusion[27]. Indeed, OPA1 knockdown in young male mice produces enlarged mitochondria with disrupted cristae, demonstrating OMM are able to fuse normally without fully-functioning OPA1[116]. Since fusion is normally regarded as a positive

adaptation, higher MFN2 protein expression in aged skeletal muscle may be counterintuitive; but it has also been suggested that increased fusion with age may be a compensatory mechanism to combat mitochondrial dysfunction in skeletal muscle. When mitochondrial membrane potential is disrupted, mitochondria are selected for degradation via mitophagy. In some cases, these dysfunctional mitochondria can fuse with neighboring functional mitochondria as an attempt to rescue membrane potential[27]. The rescuing of mitochondria via fusion can also reduce the proportion of dysfunctional to functional mitochondria[27]. Similarly, fusion allows for the mixing of matrix components, such as mitochondrial DNA (mtDNA), which is thought to reduce the likelihood of mutated mtDNA from reaching pathogenic threshold[42,91]. Diluting mutated mtDNA with normal mtDNA is important for maintaining mitochondrial function as mtDNA encodes for several subunits of the electron transport chain and damage to mtDNA can impact the production and in turn function of these proteins[42,91]. Thus, fusion can be used as a compensatory mechanism in order to maintain membrane potential and dilute mutant mtDNA in skeletal muscle.

Together with our fission results, we have demonstrated old skeletal muscle has a pro-fusion shift, which is consistent with previous studies who have reported increased fusion-to-fission ratio. As previously mentioned, Leduc-Gaudet et al.[51] found MFN2, OPA1, and DRP1 protein expression did not differ between young and old male rats, but found higher MFN2 expression relative to DRP1, indicating a pro-fusion shift in old male skeletal muscle. Other studies have found increases in MFN2 and OPA1 with no changes (or decreases) in FIS1 and DRP1 protein expression, further solidifying old skeletal muscle is in a pro-fusion state[98,102] . Greater fusion with age may indicated a larger need for compensatory fusion due to mitochondrial dysfunction. This further supports the hypothesis that despite an increase in mitophagy proteins, mitochondrial dysfunction persists in old skeletal muscle.

Cardiac Mitochondrial Quality Control

Mitochondrial content did not differ between young and old rat hearts. This was not surprising considering cardiac tissue relies on constant ATP production and losses in mitochondrial content could have severe consequences[117]. Similar to us, others have reported no changes in CIV protein expression or activity with age[105,106]. Consistent with these findings, electron microscopy analysis has demonstrated structural changes in mitochondria of old animals cardiac tissue, but total volume of mitochondria does not change[118]. To account for any numerical, but non-statistical, differences in mitochondrial content, we also expressed mitochondrial quality control proteins in the heart relative to CIV protein. This secondary analysis did not impact any of the results, signifying the changes in mitochondrial quality control proteins were not due to mitochondrial content.

Despite mitochondrial content remaining unchanged in old cardiac muscles, we did observe higher PGC-1α protein expression in old male and female rats hearts compared with young counterparts which would suggest more mitochondrial biogenesis. It has previously been reported that PGC-1α gene expression is upregulated in aged cardiac muscle, likely as a compensatory mechanism to combat mitochondrial dysfunction[119]. The production of new mitochondria likely preserves mitochondrial content in the presence of greater mitochondrial dysfunction and subsequently, degradation. Mitochondrial dysfunction in aged cardiac tissue stemming from mutated mtDNA, increased ROS production, and surplus calcium uptake can lead to poor cardiac function with age[117,119]. Mitophagy serves a crucial role in minimizing the negative effects of impaired mitochondria. As such, we also observed higher Parkin expression in our old rat hearts compared to young rats. Mitophagy is not well described in aged cardiac muscle, but previous studies have demonstrated higher Parkin and p62 protein content as well as higher

autophagy proteins Beclin-2 and LC3-II in aged mice hearts[120–122]. Together, this indicates mitochondria removal pathways are increased in aged cardiac muscles, thus resulting in unchanged mitochondrial content. Little is known regarding mitochondrial quality control in old cardiac muscle compared to young controls. However, abnormal mitochondrial quality control has been implicated in multiple cardiovascular adverse events including myocardial ischemia and reperfusion injury, atherosclerosis, and cardiac hypertrophy[123,124]. This is likely due to the regulatory role mitochondrial structure imparts on function. Indeed, knockout models have also demonstrated fusion, fission, and mitophagy proteins (MFN1, MFN2, OPA1, DRP1, Parkin) are necessary for appropriate mitochondrial structure as well as function[95,96,125–127]. Loss of these proteins also negatively impacts heart structure and can result in cardiac enlargement and hypertrophy.

We also found higher protein expression of all markers of fusion in old cardiac muscles compared to young counterparts, though fission did not differ between groups. This pro-fusion shift supports previous findings of enlarged mitochondria in old mouse hearts[128], although the reason behind enlarged mitochondria in cardiac muscle with age is not completely understood. As previously mentioned, some studies have actually reported higher expression of mitophagy and autophagy proteins in aged hearts[120–122]. However, other studies have indicated impaired autophagy may be partially to blame for the enlarged mitochondria[120,129,130]. Liang et al.[120] isolated mitochondria from aged mouse hearts and separated them based on size by differential centrifugation. Large mitochondria had significantly lower Parkin and LC3-II protein expression compared to small mitochondrial from aged hearts[120]. When both mitochondria were pooled together, Parkin and p62 were both elevated in the old hearts[120]. Together, this suggests smaller mitochondria may be targeted for degradation leading to the accumulation of enlarged

mitochondria, despite an apparent increase in mitophagy. Such a pro-fusion shift in aged hearts may be an adaptive response to inadequate autophagy as an attempt to complement for mitochondrial damage and increased ROS production[131,132].

Conclusions

Collectively, our results indicate fusion and mitophagy protein expression is higher in aged skeletal and cardiac muscle, while markers of fission are reduced in skeletal muscle of old rats. Similarly, we found sex affects select fission and mitophagy proteins in skeletal, but not cardiac, muscle. Greater fusion and mitophagy may serve a compensatory mechanism to mitigate mitochondrial dysfunction with age by restoring membrane potential via fusion and degrading unsalvageable mitochondria via mitophagy. Consistent with the literature, our findings suggest mitochondrial dysfunction is greater in the skeletal and cardiac muscle from old animals compared to young counterparts. Whether these processes can be manipulated (via upregulation or suppression) to improve mitochondrial and muscle health is poorly understood and warrants investigation. Additionally, future studies are needed to understand the underlying mechanisms leading to sex-specific differences and whether sex-specific interventions are needed to address mitochondrial dysfunction with age.

Chapter 3: Impacts of Sarcopenia and Resistance Exercise Training on Mitochondrial Quality Control

Introduction

Aging is accompanied by a progressive decline in skeletal muscle mass, referred to as sarcopenia[3,133]. The stark decrease in skeletal muscle mass leads to declines in muscle strength, which increases risk for falls and fractures. Treatments for sarcopenia are greatly needed as there are currently no proven pharmaceutical options. Mitochondria are a potential target to mitigate the negative effects of sarcopenia due to the mitochondria's role in maintaining energy homeostasis and generating energy. Indeed, mitochondria are involved in adenosine triphosphate (ATP) production, calcium handling, reactive oxygen species (ROS) production, and programmed cell death. A large body of evidence has indicated mitochondrial dysfunction increases with age and may contribute to skeletal muscle wasting[13,52,60,80,134–138]. Despite this growing body of literature, the mechanisms underlying these adverse effects remains unclear.

The mitochondrial reticulum is maintained through multiple cellular processes collectively referred to as mitochondrial quality control. In skeletal muscle the reticulum is dynamic, and its structure is regulated by fusion, fission, mitophagy, and biogenesis[15]. These processes regulate mitochondrial structural dynamics and rely on several proteins to ensure mitochondria function effectively[10,11]. In healthy skeletal muscle, fusion and fission are balanced and undergo constant cycles. Fusion joins adjacent mitochondria together through outer mitochondrial membrane proteins mitofusin-1 and -2 (MFN1 and MFN2) and inner mitochondrial membrane protein optic atrophy type 1 (OPA1)[21,23]. Conversely, segments of the mitochondrial reticulum can be separated into multiple smaller mitochondria via fission. During the process of fission, dynamin-related

protein 1 (DRP1) is recruited to the mitochondrial surface and will encircle and cleave the mitochondria into two[30]. Several outer mitochondrial membrane proteins have been identified as mediators of DRP1 recruitment including fission protein 1 (FIS1), mitochondrial fission factor (MFF), and mitochondrial dynamics proteins of 49 and 51 kDa (MiD49 and MiD51, respectively)[88,139,140].

Mitochondrial biogenesis and breakdown are also in balance in healthy muscle. Mitochondrial degradation is largely regulated by mitophagy proteins PTEN-induced kinase1 (Pink1) and Parkin[32,33] . Fission proceeds and aids mitophagy by separating dysfunctional portions of the mitochondria[87]. The accumulation of Pink1 on the outer surface of the mitochondria then recruits Parkin, which subsequently undergoes ubiquitination, signaling the mitochondrion for degradation. There are also Parkin-independent pathways that are less understood and require further investigation in the context of aging[141]. Balancing mitophagy is mitochondrial biogenesis, which is largely regulated by transcription coactivator peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α)[142,143]. Together these processes control mitochondrial quality and require strict regulation.

Evidence suggests the processes regulating mitochondrial quality control may be disrupted in skeletal muscle with advanced age. Previous studies have reported altered levels of mitochondrial biogenesis, fusion, fission, and mitophagy proteins in skeletal muscle of older adults compared with young adults[52,77], although not all studies agree[49,50,114]. While some have reported MFN2, OPA1, FIS1, DRP1, and Parkin protein expression do not differ between young and older adults when physical activity level is controlled for[49,50,114], others have reported lower PGC-1α, OPA1, and Parkin (when normalized to mitochondrial content) in older adults[52,77,144]. Lower OPA1, MFN1, MFN2, and DRP1 gene expression has also been reported in old sedentary adults

compared to their young counterparts[42,60]. Further suggesting aberrant mitochondrial quality control in aged skeletal muscle, electron microscopy analysis of older adults and mice demonstrated enlarged mitochondria compared to young counterparts[51,81].

Few studies have evaluated mitochondrial quality control in sarcopenic individuals; therefore, our understanding on mitochondrial quality control in sarcopenia versus aging is very limited. Marzetti et al.[54] conducted a study on physically frail and hospitalized sarcopenic adults and found lower MFN2 protein expression in sarcopenic adults compared to non-sarcopenic, age-matched controls. However, no differences were observed in FIS1, MFN1, or OPA1 protein content between the sarcopenic and non-sarcopenic groups[54]. Another study that evaluated transcriptomics data exclusively from males found lower OPA1 and FIS1 gene expression in sarcopenic compared to non-sarcopenic, older men[55]. MFN2 gene expression, however, was similar between the sarcopenic men and healthy, age-matched controls[55]. Given the link between abnormal mitochondrial quality control and muscle atrophy[41,42,44–46,145], the lack of studies on mitochondrial quality control in sarcopenic individuals highlights an under-researched area that may help identify potential targets to combat sarcopenia.

Resistance exercise training is recommended to increase muscle size and strength in older adults with or without sarcopenia[146]. Previous literature has suggested resistance exercise training may also elicit beneficial effects on skeletal muscle mitochondria in older adults. Increased mitochondrial volume and respiration (function) have been observed in older adults after resistance exercise training[69,71]. Further, Mesquita et al.[74] recently reported that older adults who underwent 12 weeks of whole-body resistance exercise training exhibited increased skeletal muscle protein expression of MFN1, MFN2, OPA1, and DRP1 suggesting increases in both fusion and fission; however, these studies did not focus specifically on sarcopenic individuals. Given the lack of

understanding regarding mitochondrial quality control in sarcopenic individuals, we sought to determine whether the expression of proteins regulating mitochondrial quality control differ between sarcopenic and non-sarcopenic older adults. Since resistance exercise training may be an effective lifestyle intervention to reverse decrements in mitochondrial quality control, we further sought to determine whether 12 weeks of lower-body resistance exercise training impacts mitochondrial quality control in lower-limb skeletal muscle of sarcopenic older adults. This work will expand upon our current understanding of the pathophysiology of sarcopenia and may identify mitochondrial targets to mitigate sarcopenia.

Methods

Study Design

The purpose of this study was to compare mitochondrial quality control in sarcopenic and non-sarcopenic older adults and to determine the effects of resistance exercise training on mitochondrial quality control in sarcopenic individuals. To achieve this, mitochondrial fusion (MFN2, OPA1), fission (DRP1, FIS1), mitophagy (Pink1), and biogenesis (PGC-1α)-regulating protein abundance was quantified in skeletal muscle biopsies from sarcopenic and non-sarcopenic participants. At baseline, sarcopenic participants were compared with non-sarcopenic participants of similar age, sex, and body mass index (BMI) to determine mitochondrial quality control differences related to sarcopenia. Sarcopenic participants then completed 12 weeks of supervised lower-body resistance training. Following the intervention, a second muscle biopsy was taken from the sarcopenic participants to quantify the effects of resistance training on mitochondrial fusion, fission, mitophagy, and biogenesis protein expression. Additional measurements of body

composition, aerobic capacity, and muscular strength testing were obtained to characterize participants and responses to resistance training.

Participants

Twenty community-dwelling older adults between the ages of 65-88 were recruited from Baltimore and College Park, Maryland areas for participation in exercise training studies. Ten sarcopenic participants were recruited for the prospective resistance exercise training intervention in this study, and data and muscle specimens from ten non-sarcopenic participants in a previous study were used as a baseline comparison group. Inclusionary criteria for all participants were as follows: BMI = 18-35 kg/m^2, no regular vigorous physical activity (<20 min exercise, 2x/week), and for women, > 1-year past menopause and not on hormone replacement therapy. All participants were free of metabolic, pulmonary, and renal diseases, and did not have a history of cancer, stroke or smoking within the last two years. For inclusion in the sarcopenic group, body composition was measured during screening via bioelectrical impedance analysis in order to calculate skeletal muscle mass index (skeletal muscle mass/height in meters^2). Sarcopenic participants were considered to be at least moderately sarcopenic (men <10.75 kg/m^2; women <6.75 kg/m^2)[147]. All procedures were approved by the University of Maryland Institutional Review Board and conformed with the Declaration of Helsinki. All participants provided written informed consent.

Body Composition

Participant height (nearest 0.1cm) and weight (nearest 0.1 kg) were measuring using a portable stadiometer and physician's scale, respectively, to calculate BMI. Body fat, total lean mass, and appendicular lean mass (ALM) was assessed using dual-energy X-ray absorptiometry

(GE Lunar Prodigy, GE Healthcare, Madison, WI, USA). ALM was subsequently divided by BMI to serve as an index of sarcopenia (ALM/BMI), where higher values are more desirable and would indicate greater lean mass relative to body mass.

Maximal Graded Exercise Test

Maximal aerobic capacity (VO_{2max}) was measure by indirect calorimetry during a graded exercise test on a treadmill as previously described in our lab[148]. Briefly, treadmill speed was kept constant while the incline was increase 2% every 2 minutes until the subject was unable to continue. A true VO_{2max} was confirmed by the achievement of at least two of the following criteria: respiratory exchange ratio ≥ 1.10, maximum heart rate > 90% of age-predicted maximum (220-age), or a plateau in VO_2 (<200mL/min change).

Strength Testing

To verify our resistance training-induced physiological changes in the sarcopenic group, maximal quadriceps force production was measured before and after exercise training on a System 4 Pro dynamometer from Biodex (Shirley, New York). Participants performed three maximal single-leg isometric contractions 60° of knee flexion. The averages of three values were used as the maximal isometric force production for the right leg.

Muscle Sampling

Percutaneous needle biopsies were taken from the vastus lateralis muscle using a Bergstrom needle (Stille, Solna, Sweden), after local anesthesia was administered, as previously described[148,149]. All participants' biopsies were taken on their right leg, except for one sarcopenic participant who requested their biopsy be taken on their left. Approximately 200-300mg of skeletal

muscle was flash frozen and stored at -80ºC until analysis. Biopsies were taken 24-48 hours following the exercise intervention to avoid potential transient acute effect of the last exercise bout.

Western Blotting

Muscle samples were homogenized with equal amount (weight/volume) of 1% triton buffer with protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). Following centrifugation at 16,000 x g for 5 minutes at 4ºC, supernatant protein content was quantified by Pierce BCA Assay (Thermo Fisher Scientific, Waltham, MA, USA). Protein samples containing Laemmli buffer (Bio-Rad) and beta-mercaptoethanol were warmed for 5 min at 70º C. Equal amounts (30ug/well) of protein were loaded into 4-15% Bio-Rad TGX stain free gels (Hercules, CA, USA) and were separated by SDS-PAGE. Gels were activated before transferring onto polyvinylidene difluoride membranes and total protein fluorescence was imaged following transfer to confirm equal loading and transfer of proteins. Membranes incubated with a blocking buffer composed of TBS (Bio-Rad), 0.1% Tween 20 (Bio-Rad), and 5% BSA or milk (Fisher Scientific, Hampton, NH, USA) for 2 hours at room temperature before incubating with primary antibodies overnight at 4º C. Primary antibodies and dilutions are shown in Table 3. The following day, membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (Cell Signaling Technology, 7074S, RRID AB_2099233) and bands were detected with a chemiluminescent substrate from Bio-Rad. Images were acquired using a Bio-Rad ChemiDoc XRS and analyzed on Image Lab 8.0. Band intensities were normalized to the total protein image acquired on day 1 of the protocol. Data are presented normalized to total protein, as well as relative to CIV protein content to account for differences in mitochondrial content. OPA1-short and -long isoforms were quantified separately.

Table 3. Chapter 3 Antibodies

Antibody	Manufacturer	CAS number	Research Resource Identifiers	Dilution
Anti-rabbit IgG HRP linked antibody	Cell Signaling Technology, Danvers, MA, USA	7074S	AB_2099233	1:2000 in TBS-T with 5% BSA or milk
CIV (3E11) Rabbit mAb	Cell Signaling Technology, Danvers, MA, USA	4850S	AB_2085424	1:1000 in TBS-T with 5% BSA
DRP1 (D6C7) mAb	Cell Signaling Technology, Danvers, MA, USA	8570S	AB_10950498	1:1000 in TBS-T with 5% BSA
Fis1 pAb	Thermo Fisher Scientific, Waltham, MA, USA	PA5-22142	AB_11152577	1:1000 in TBS-T with 5% BSA
GAPDH pAb	Thermo Fisher Scientific, Waltham, MA, USA	PA1-987	AB_2107311	1:5000 in TBS-T with 5% BSA
Mitofusin-2 Rabbit mAb	Cell Signaling Technology, Danvers, MA, USA	83667S	AB_2800025	1:1000 in TBS-T with 5% milk
OPA1 (D6U6N) Rabbit mAb	Cell Signaling Technology, Danvers, MA, USA	80471S	AB_2734117	1:1000 in TBS-T with 5% BSA
Parkin Recombinant Rabbit mAb (21H24L9)	Thermo Fisher Scientific, Waltham, MA, USA	702785	AB_2842252	1:500 in TBS-T with 5% BSA
PGC-1 alpha pAb	Thermo Fisher Scientific, Waltham, MA, USA	PA5-72948	AB_2718802	1:500 in TBS-T with 5% milk
Pink1 Rabbit pAb	Abcam, Waltham, MA, USA	ab23707	AB_447627	1:500 in TBS-T with 5% BSA

Legend = CAS, Catalog Number; IgG, Immunoglobulin G; HRP, Horseradish Peroxidase; mAb, monoclonal; pAb, polyclonal; TBS-T, tris buffer saline with 0.1% Tween 20; BSA, bovine serum albumin

Exercise Training Intervention

Following baseline testing, sarcopenic participants underwent 12-weeks of supervised resistance training, 3 days per week. All exercises focused on the lower-extremity muscles and were performed on Keiser K-300 pneumatic resistance machines (Keiser Corporation, Fresno,

CA). To target muscles associated with mobility, the leg press, knee extension, and leg curl were used. Resistance started at 60% of 1-repetition maximum (1-RM; determined before the first day of training) and progressed to 70-75% of 1-RM within the first 2 weeks of training. Resistance was progressively increased throughout the study as strength increased. Criteria for progression was as follow: successful completion of two sets of 12-repeitions at a rate of perceived exertion of the new Borg scale of less than 8. Each time this criterion was met, resistance increased 5%. For standard training, 2 sets of 12 repetitions of each exercise were performed. Proper technique and strength development were prioritized during training.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 9. Independent samples t-tests were used to compare subject characteristics and mitochondrial quality control protein expression between sarcopenic and non-sarcopenic controls at baseline. Dependent samples t-tests were used to determine whether there were changes in characteristics or mitochondrial quality control protein expression within the sarcopenic group before and after resistance exercise training. All tests were two-tailed with statistical significance set at $P < 0.05$. Data are presented as means ± standard error of the mean (SEM).

Results

Participant Characteristics

Participant characteristics can be found in Table 4. There were no significant differences in age, weight, BMI, body fat percentage, or appendicular lean mass (ALM) between the non-sarcopenic and sarcopenic participants at baseline. Sarcopenic participants at baseline had

significantly lower ALM/BMI (P = 0.038), absolute VO_{2max} (P = 0.037), and relative VO_{2max} (P = 0.020) compared to non-sarcopenic participants.

Following 12 weeks of resistance training, sarcopenic participants significantly increased their ALM/BMI compared to pre-training values (0.719 $\pm$ 0.159 vs. 0.736 $\pm$ 0.155; P = 0.030). Weight, BMI, body fat percentage, appendicular lean mass, and absolute and relative VO_{2max} were not significantly affected by the resistance exercise intervention. Knee extension maximal isometric force production increased 13% following resistance training compared to pre-training values (P = 0.017).

Mitochondrial Content

Mitochondrial content, as measured by complex IV (CIV) protein content, did not differ between the non-sarcopenic and sarcopenic adults prior to training (P = 0.075; Figure 10A). CIV protein content also, did not change after training in the sarcopenic individuals (P = 0.280). Similarly, GAPDH protein content did not differ between groups or after training (Figure 10B). As there were no significant differences, but some numeric differences, in mitochondrial content between groups, mitochondrial quality control protein expression is presented independently and relative to CIV protein expression below for full interpretation.

Table 4. Sarcopenic and Non-Sarcopenic Participant Characteristics

Variable	Non-Sarcopenic Control	Sarcopenic Baseline	Sarcopenic 12-Week Resistance Training
Age (years)	71.1 ± 2.5	75.9 ± 2.0	
Sex	F = 7; M = 3	F = 7; M = 3	
Height (cm)	167.7 ± 2.5	164.3 ± 3.0	
Skeletal Muscle Mass Index (kg/m^2)		7.1 ± 0.47	
Weight (kg)	73.8 ± 3.7	74.9 ± 4.2	73.3 ± 4.2$^{\#}$
BMI (kg/m^2)	26.6 ± 1.3	27.6 ± 0.7	26.8 ± 0.6$^{\#}$
Body fat (%)	34.8 ± 2.4	36.8 ± 1.4	37.0 ± 1.4
Total Lean Mass (kg)	47.1 ± 2.3	44.0 ± 3.3	43.9 ± 3.3
ALM (kg)	21.8 ± 1.3	19.9 ± 1.8	19.9 ± 1.7
ALM/BMI	0.865 ± 0.055	0.712 ± 0.055*	0.736 ± 0.049$^{\#}$
Absolute VO_2 (L/min)	1.85 ± 0.10	1.48 ± 0.15	1.52 ± 0.13
Relative VO_2 (mL/kg/min)	24.45 ± 1.4	19.84 ± 1.2*	20.53 ± 1.1
Right Knee Extensor Force† (N*m)		111.3 ± 10.5	125.9 ± 17.7$^{\#}$

Data are means ± SEM. Legend = BMI, body mass index; ALM, appendicular lean mass. *P < 0.05 compared with non-sarcopenic control, $^{\#}$P < 0.05 compared with Sarcopenic Pre-Training. †Includes force production from left leg of one sarcopenic participant.

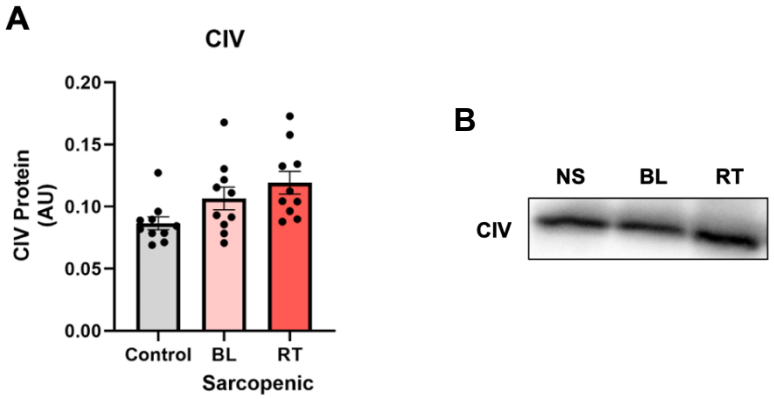

Figure 10. CIV content does not differ between sarcopenic and non-sarcopenic skeletal muscle regardless of exercise training. Legend = AU, arbitrary units; BL, baseline; RT, post resistance exercise training; NS, non-sarcopenic control. Data are presented as means ± SEM, n = 10 per group.

Mitochondrial Fusion

MFN2, OPA1-S, and OPA1-L protein expression did not differ between non-sarcopenic and sarcopenic older adults at baseline (Figure 11A-C). There was a tendency for MFN2 protein to increase after resistance training in skeletal muscle from the sarcopenic group. (P = 0.054; Figure 11A), though that trend was abolished when MFN2 was expressed relative to CIV expression (Figure 11D) There were also no significant differences in OPA1-S or OPA1-L when expressed relative to CIV expression (Figure 11D-F).

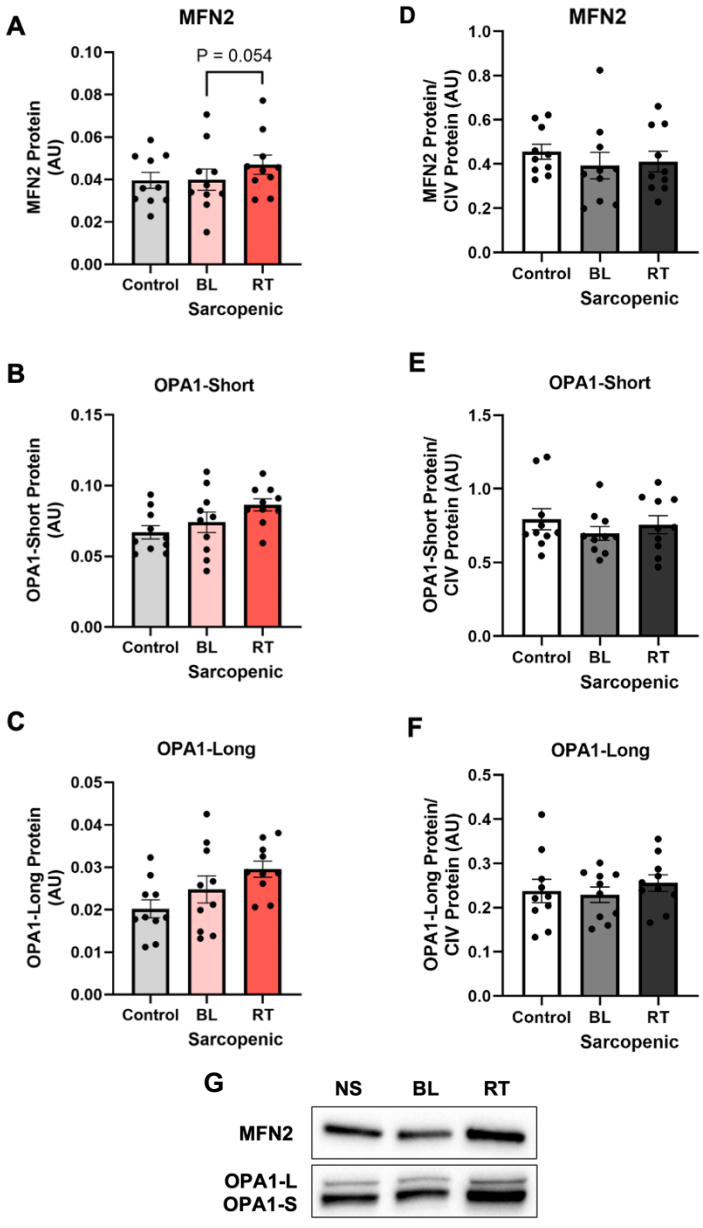

Figure 11. Mitochondrial fusion does not differ between sarcopenic and non-sarcopenic skeletal muscle. Figures A, B, and C are normalized to total protein. Figures D, E, and F are normalized to total protein and subsequently expressed relative to CIV expression. Legend = AU, arbitrary units; BL, baseline; RT, post resistance exercise training; NS, non-sarcopenic control. Data are presented as means ± SEM, n = 10 per group

Mitochondrial Fission

DRP1 protein content did not differ between groups (Figure 12A). Similarly, Fis1 protein abundance did not differ based on sarcopenia status and was unaffected by training (Figure 12B). When expressed relative to CIV expression, there remained no differences in DRP1 or Fis1 between groups (Figure 12C and 12D, respectively).

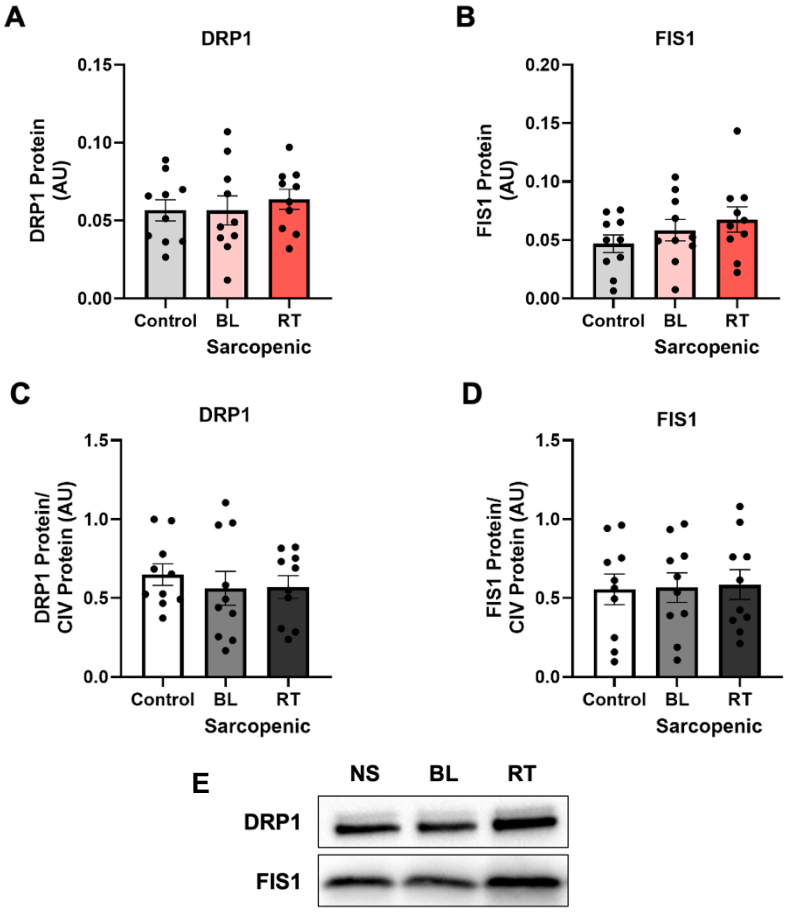

Figure 12. Fission proteins are not impacted by sarcopenia or resistance exercise training. Figures A and B are normalized to total protein. Figures C and D are normalized to total protein and subsequently expressed relative to CIV expression. Legend = AU, arbitrary units; BL, baseline; RT, post resistance exercise training; NS, non-sarcopenic control. Data are presented as means ± SEM, n = 10 per group.

Mitochondrial Mitophagy and Biogenesis

Mitochondrial degradation, as measured by Pink1, and mitochondrial biogenesis, as measured by PGC-1α, did not differ between groups, nor after resistance exercise training in the sarcopenic group (Figure 13A and 13B, respectively). When Pink1 was expressed relative to CIV expression, the same pattern existed (Figure 13C).

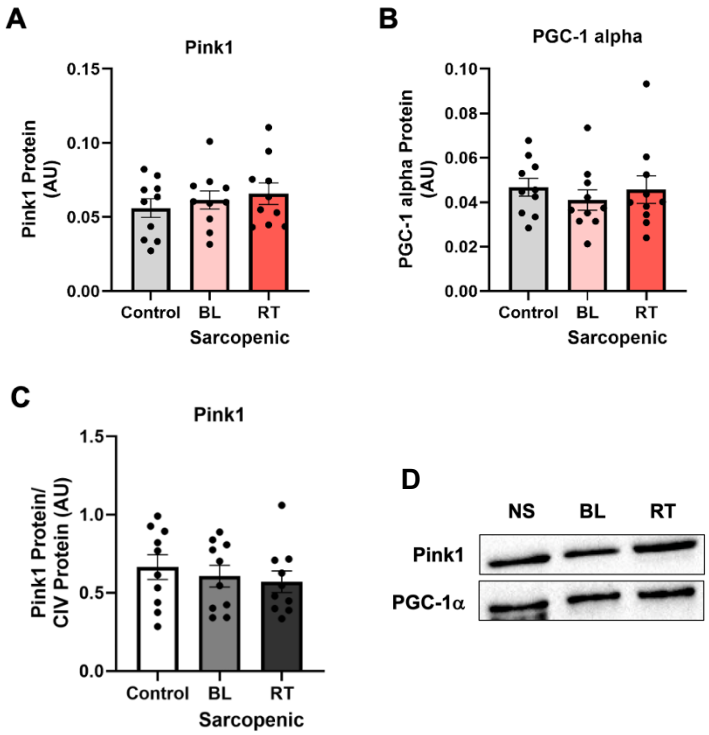

Figure 13. Mitophagy and mitochondrial biogenesis in sarcopenic skeletal muscle is similar to non-sarcopenic skeletal muscle regardless of exercise training. Figures A and B are normalized to total protein. Figure C is normalized to total protein and subsequently expressed relative to CIV expression. Legend = AU, arbitrary units; BL, baseline; RT, post resistance exercise training; NS, non-sarcopenic control. Data are presented as means ± SEM, n = 10 per group.

Mitochondrial Quality Control Proteins and Whole-Body Measures

CIV protein was inversely related to ALM/BMI (Pearson correlation P = 0.012; Figure 14A) but was not correlated with any other body composition measurement (total lean mass, appendicular mass, or total leg mass). Expression of fusion proteins was inversely related to lean

body mass, appendicular lean mass, and ALM/BM (Figure 14B-F). MFN2 and OPA1-S were inversely correlated to total lean body mass (Pearson correlation P = 0.012 and P = 0.022, respectively). Both MFN2 and OPA1-S protein expression were also inversely correlated with ALM (Pearson correlation P = 0.018 and P = 0.018, respectively). Greater ALM/BMI was associated with lower MFN2 content (Pearson correlation P = 0.003) and was borderline associated with lower OPA1-S content (Pearson correlation P = 0.055; r = -0.425). No other proteins were correlated with any other whole-body measures.

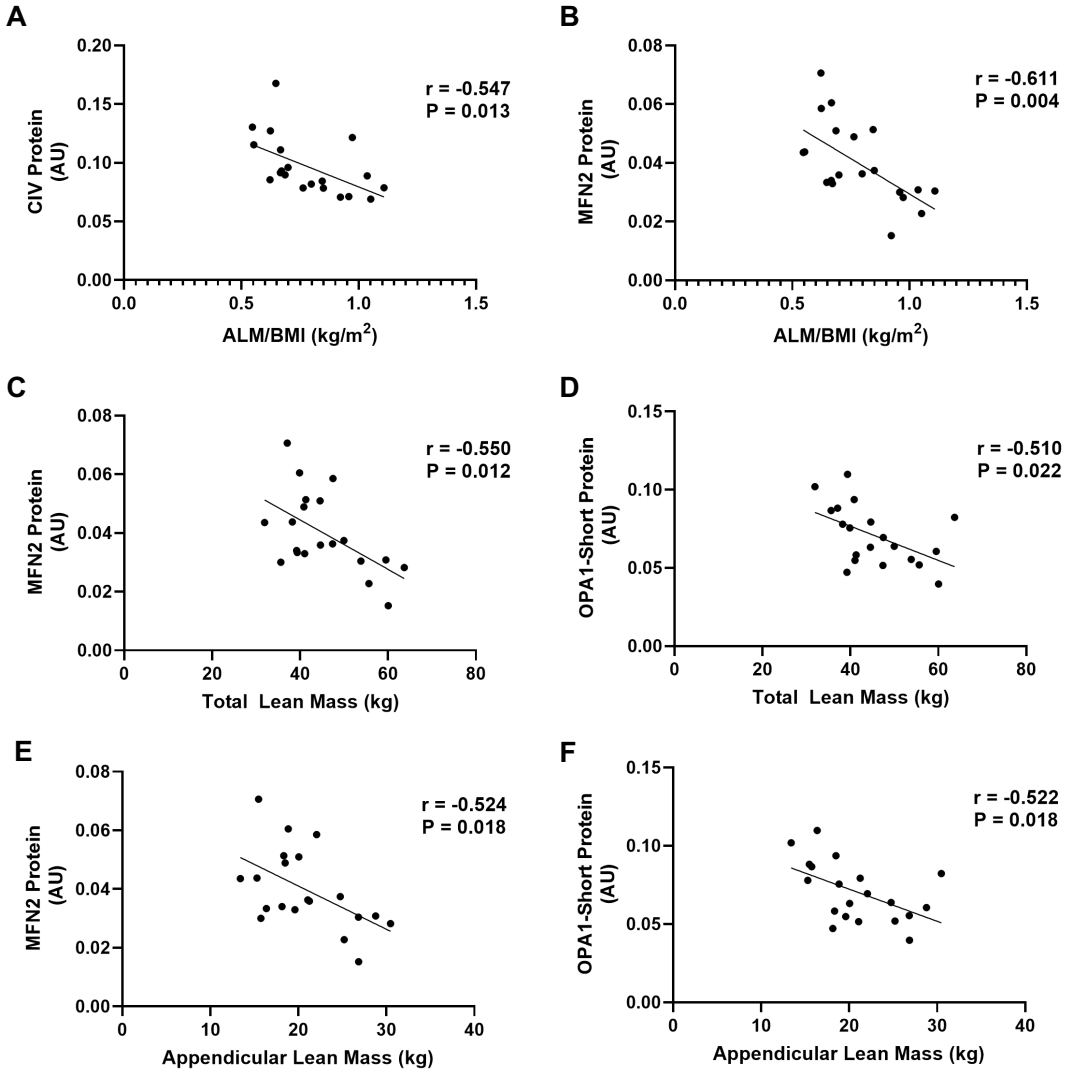

Figure 14. Greater lean mass is associated with lower expression of mitochondrial fusion proteins. Legend = AU, arbitrary units; ALM, appendicular lean mass; BMI, body mass index. Samples include non-sarcopenic controls and sarcopenic participants pre-training (n = 20).

Discussion

We found that within skeletal muscle, markers of mitochondrial biogenesis, fusion, fission, and mitophagy were not different between sarcopenic and non-sarcopenic age-matched control. Twelve weeks of lower-body resistance training was an effective intervention to increase skeletal muscle strength, although it did not alter mitochondrial content or quality control proteins, or ALM in sarcopenic individuals.

Sarcopenic versus non-sarcopenic mitochondrial content and quality control proteins

We observed no differences in mitochondrial content between sarcopenic and non-sarcopenic older adults matched for age, sex, and BMI. These findings were contrary our hypothesis that sarcopenic individuals would have lower mitochondrial content, concomitant with greater quantities of proteins regulating fission and lower quantities of proteins regulating fusion as compared with healthy, aged-matched controls. For our study, CIV was used as a marker for mitochondrial content, but several other mitochondrial content markers exist, including citrate synthase (CS) activity, cardiolipin content, and CIV activity[84]. Miglivacca et al.[55] found that CIV and CS activity were both lower in sarcopenic men compared to non-sarcopenic men. That study, however, used a more stringent criteria for sarcopenia set by the Asian Working Group for Sarcopenia (AWGS) to assess the presence of sarcopenia in their Chinese population[55]. Miglivacca et al.[55] used ALM relative to height, along with low handgrip or gait speed to classify their participants as sarcopenic. Male participants in that study had an ALM relative to height less than 7.0 kg/m^2, while our male participants' ALM relative to height was 8.9 kg/m^2. Similarly, the women in our study (ALM/h^2 = 6.5 kg/m^2) were also above the sarcopenic cut off value set forth

by the AWGS ($ALM/h^2 < 5.7$ kg/m^2)[150]; therefore, we may not have observed declines in mitochondrial content in our study due to our participants not being severely sarcopenic.

Also, contrary to our hypothesis, markers of mitochondrial biogenesis, fusion, fission, and mitophagy did not differ between non-sarcopenic and sarcopenic older adults. Our results are partially in line with the limited other studies on mitochondrial quality control in sarcopenic older adults. Similar to the present study, Marzetti et al.[54] found no differences in PGC-1 α, OPA1, or Fis1 protein content between physically frail sarcopenic older adults and non-sarcopenic age-matched controls. This group did, however, report decreased expression of MFN2 and LC3B, an autophagy-related protein[54], while we observed no differences in MFN2 nor our mitophagy markers. One potential explanation for the difference in findings can be attributed to participant characteristics between studies. Our participants were, on average, less sarcopenic than Marzetti et al.[54] who used skeletal muscle mass relative to height (SMI) to determine the presence of sarcopenia. Our participants had a mean SMI of 7.1 kg/m^2, compared to the participants used by Marzetti et al.[54] who had a mean SMI of 5.9 kg/m^2. Additionally, while we excluded potential participants with pulmonary, cardiovascular, and metabolic diseases, Marzetti et al.[54] included older adults with cardiovascular and metabolic diseases. Indeed, 10% of the sarcopenic individuals studied by Marzetti et al.[54] had congestive heart failure, 20% had coronary heart disease, and 30% had diabetes mellitus. Although the non-sarcopenic and sarcopenic groups had similar prevalence of diseases in that study, cardiovascular and metabolic diseases are known to impact mitochondrial quality control[123,151], which may partially explain the differences between the former and present studies.

Another difference between studies to consider was the participants' mobility status, as all participants in Marzetti et al.[54] were recruited from individuals that were hospitalized as a result

of fall-induced hip fractures. Comparatively, all our participants were independently living and free of mobility issues, which may suggest otherwise healthy sarcopenic older adults do not exhibit altered mitochondrial quality control. Miglivacca et al.[55] found conflicting results to us and the previously mentioned study, and reported FIS1 and OPA1 gene expression were lower in sarcopenic men compared to non-sarcopenic men. However, it is important to note that differences in mRNA expression are not always mirrored by protein abundance due to post-transcriptional modifications. Our findings are in line with a mouse model of sarcopenia that observed no differences in MFN2, OPA1, DRP1, or LC3II protein content between wild type and mitochondrial DNA (mtDNA) proofreading deficient mice[98]. The proofreading deficient mice have previously been shown to exhibit pre-mature aging and muscle wasting, thus making them an animal proxy for sarcopenia[152].

While we did not observe differences in mitochondrial quality control between our sarcopenic and non-sarcopenic older adults, we did find MFN2 and OPA1-short protein content were both inversely correlated with total lean and appendicular mass. MFN2, a marker of fusion, has a protective role in skeletal muscle mass as overexpression of MFN2 in skeletal muscle from old mice can increase muscle mass and cross-sectional area[153]. Conversely, dysfunctional mitochondria can fuse with healthy mitochondria in response to stress as an attempt to rescue membrane potential and preserve function[27,154,155]. The fusion of outer and inner mitochondrial membranes allows for the complementation of dysfunctional mitochondria by sharing membrane potential and matrix components, such as mtDNA[27]. mtDNA encodes for multiple subunits of the electron transport chain, meaning mutations or deletions in mtDNA can have direct consequences on mitochondrial function[156]. Mitochondria carry multiple copies of mtDNA and often has a mixture of normal and mutated DNA, which serves as a protective mechanism against the

detrimental effects of mutated DNA[157,158]. The mixing of mtDNA via fusion is thought to reduce the likelihood of mutated mtDNA reaching pathogenic threshold by diluting the relative proportion of mutated to normal mtDNA[42,91]. However, the disruption of membrane potential can also cleave inner mitochondrial membrane fusion protein OPA1 into its short isoforms, which some have suggested inhibits inner mitochondrial membrane fusion and in turn, the sharing of membrane potential and matrix components[23,159,160]. Together, the higher MFN2 and OPA1-S protein content with lower muscle mass observed in our study may indicate a failing compensatory attempt to preserve mitochondrial function in a deteriorating muscle state. This hypothesis is speculatory, as mitochondria function was not assessed in our study.

Mitochondrial dysfunction has repeatedly been implicated as a contributing factor of sarcopenia[7]. However, our findings suggest these mitochondrial quality control proteins may not be impacted in individuals with moderate sarcopenia. Aberrant mitochondrial quality control may play an additive role in mitochondrial dysfunction, rather than initiate dysfunction except in extreme circumstances. For example, knockout of fusion and fission proteins induces skeletal muscle atrophy in young and old male mice[41,45,46], while upregulation of fusion and mitophagy induces muscle growth in old male mice[97,153]. However, that may represent a non-physiological scenario where extreme alterations in fusion, fission, and mitophagy proteins impact skeletal muscle mass, but smaller changes are tolerated.

Effects of resistance exercise training on mitochondrial quality control proteins

The resistance training intervention utilized in our study was effective in increasing muscle strength. We observed a ~13% increase in knee extension force production, which is consistent with other resistance training studies in older adults that have reported 10-15% greater knee extension force production post training[74,161,162]. Our training improved weight, BMI, and our

sarcopenia index (ALM/BMI). ALM was unaltered after the intervention which would suggest the improvements in the sarcopenia index (ALM/BMI) were due to decreases in BMI. The lack of changes in ALM may be partially explained by our intervention, which only exercised lower body, while ALM take upper and lower body lean mass into account.

We did not observe any significant changes in skeletal muscle mitochondrial content following resistance training. This was somewhat surprising considering other studies have reported altered mitochondrial content following resistance training in older adults. Greater electron transport chain complex protein content has been reported in middle to older adults after 6-10 weeks of full body resistance exercise[74,163]. Similarly, Lamb et al.[74] reported increased CS activity in middle aged adults after 10 weeks of resistance exercise training and Parise et al.[71] found greater CIV activity in older adults following 14 weeks of resistance exercise. However, some studies have found conflicting results. Ruple et al.[163] and Parise et al.[71] reported increases in electron transport chain content and activity but found no increases in CS activity following resistance exercise training. Other studies have also reported no changes in mitochondrial content following resistance exercise training in older adults. Irving et al.[164] found that 8 weeks of resistance training did not increase total electron transport chain protein content in older men and women. Further, 12 weeks of resistance training did not impact CS activity in older men[72,165]. Our results agree with the latter studies that mitochondrial content is not impacted by resistance exercise in sarcopenic individuals.

We found markers of mitochondrial biogenesis, fusion, fission, and mitophagy were unchanged following resistance exercise training in sarcopenic individuals. Our results may suggest these processes are not as responsive to exercise in sarcopenic skeletal muscle and may require a greater stimulus. To our knowledge, no other study has investigated mitochondrial quality

control in sarcopenic individuals in response to resistance exercise, but few studies have been conducted in healthy older adults. Mesquita et al.[74] found MFN1, MFN2, OPA1, and DRP1 protein content increased in middle to older adults following 10 weeks of resistance exercise. Zampieri et al.[166] demonstrated 9 weeks of leg press exercise was able to decrease OPA1 protein content in older adults but the intervention had no effect on MFN2 protein content. Although these studies found divergent results, they both observed changes in mitochondrial quality control protein expression in response to resistance exercise. While our intervention was modestly longer than the aforementioned studies, a 2021 meta-analysis on resistance training and sarcopenia found greater improvements in muscle mass in studies lasting more than 12 weeks[167]. Further, previous reports have suggested mitochondrial biogenesis may occur at a slower rate than muscular hypertrophy following resistance exercise training[168]. Thus, a longer intervention could be needed to observe alterations in muscle mass and mitochondrial quality control in sarcopenic individuals.

Interestingly, we did observe a numerical tendency for greater expression of fusion proteins following resistance training. As previously stated, mitochondria can fuse as a compensatory mechanism in order to mitigate mitochondrial dysfunction. The redistribution of matrix components, such as mtDNA, and the sharing of membrane potential via fusion can help maintain proper mitochondrial function[27,91]. Mitochondrial fusion also elongates the mitochondrial reticulum thus increasing interconnectivity and optimizing ATP production[169]. No study has evaluated mitochondrial quality control in conjunction with mitochondrial function following resistance exercise in older adults; however, Mesquita et al.[74] found MFN2 to be increased in older adults following 10 weeks of resistance exercise training. In a separate study, Mesquita et al.[170] reported greater antioxidant activity and reduced oxidative damage (as measured by 4-hydroxynonenal) in older males following 6 weeks of resistance exercise training. Thus, we

hypothesize that any potential increase in mitochondrial fusion is an attempt to optimize mitochondrial function.

Limitations

Mitochondrial structure, in part, regulates function and as such mitochondrial quality control proteins are necessary to maintain the mitochondrial reticulum[17]. Our study, however, did not directly measure mitochondrial function. Previous studies have found increased protein expression and activity of electron transport chain complexes as well as increased antioxidants catalase and superoxide dismutase 1 expression in older adults following resistance exercise training[71,165,170]. Therefore, a limitation of this study is the potential for missed alterations to respiration or mitochondrial enzyme activity, which would have demonstrated mitochondrial functional changes. Additionally, there are Parkin-independent pathways that could be differentially regulated in sarcopenia that were not investigated in this study[141]. Future studies are needed on how other mitochondrial degradation pathways may differ between sarcopenic individual and healthy age matched controls. The lack of differences in mitophagy and other mitochondrial quality control proteins between sarcopenic and non-sarcopenic older adults in our study may be partially explained by our sarcopenia criteria. As previously mentioned, our classification of sarcopenia included older adults who were moderately sarcopenic. A stricter cutoff to include older adults with severe sarcopenia may have resulted in differences in quality control proteins.

Conclusions

Overall, our results suggest markers of mitochondrial content and quality control do not differ between non-sarcopenic older adults and those with moderate sarcopenia. Our study also

demonstrated short-term (12 weeks) resistance exercise training does not affect protein abundance of markers of mitochondria content or quality control proteins in sarcopenic skeletal muscle. Considering we saw a tendency for mitochondrial fusion protein expression (MFN2) to increase post-training, future studies should investigate whether a longer or higher intensity intervention can elicit changes in mitochondrial quality control markers in sarcopenic individuals and what the functional implications of this may be. Given the overall lack of differences, our results suggest mitochondrial quality control proteins may not be altered in sarcopenia.

Chapter 4: Mitochondrial and lipid droplet morphology is not impacted by resistance exercise training in sarcopenic older adults

Introduction

The aging-associated decline in skeletal muscle mass, referred to as sarcopenia, affects an estimated 10% of independently living older adults worldwide[171]. Sarcopenia can be debilitating, as it is accompanied by reductions in skeletal muscle strength and function. Sarcopenia is also a major risk factor for frailty, falls, and fractures. Despite the high prevalence of sarcopenia, the pathophysiology is not fully understood and treatments have not yet been discovered. In addition to the loss of skeletal muscle mass, several cellular changes contribute to the pathophysiology of sarcopenia including reduced satellite cell number and activation, increased oxidative damage, and increased mitochondrial dysfunction[6,7]. Mitochondria are of particular interest in the context of aging due to the mitochondria's essential role in producing energy in the form of adenosine triphosphate (ATP). Aside from energy production, mitochondria also play critical roles in regulating intracellular calcium, lipid oxidation, and apoptosis signaling[172]. Impairments to these processes result in decreased mitochondrial quality and efficiency, which are commonly seen in skeletal muscle from older adults. Whether mitochondrial content decreases with age is still controversial. Some studies have indicated mitochondrial number and volume decreases with age in humans and rodents[60,80], while others have reported no differences[59,77].

Further complicating the debates as to whether mitochondrial content declines with age is the potential for localized changes in mitochondrial subpopulations. Skeletal muscle mitochondria are classified into two subpopulations based on their location. Peripherally located (PL) mitochondria are located just below the sarcolemma often grouped near capillaries and nuclei,

while intermyofibrillar (IMF) mitochondria are located between the myofibrils. PL and IMF mitochondria exist in a dynamic reticulum that is altered to optimize energy production. While both subpopulations work in tandem to meet the energetic demands of the cell, structural differences in PL and IMF mitochondria have recently led researchers to believe the mitochondrial subpopulations may contribute to energy production differently[15,173]. Immunostaining revealed greater complex IV concentration around the periphery of muscle fibers, with greater complex V concentration dispersed throughout the fiber[15,173].This has led to the belief that PL mitochondria have a greater potential to generate membrane potential that is used by the IMF mitochondria to generate ATP for muscle contractions[17,173].

Several studies have isolated mitochondria to better understand the content and functional differences between PL and IMF mitochondria, although current methods are flawed[79,174]. Mitochondrial isolation breaks the reticulum and removes the mitochondria from its cellular environment. Despite these concerns, studies on isolated PL and IMF mitochondria have provided insight as to the potential role of each subpopulation. Consistent with microscopy data, mitochondrial subpopulation isolation has demonstrated that most intramuscular mitochondria are found in the IMF region[58,174,175]. Functional analyses have established IMF mitochondria have greater respiration and complex V activity, while PL mitochondria have a greater tendency to produce ROS[174–176]. PL mitochondria also respond more robustly to endurance exercise than IMF mitochondria[177,178]. Despite evidence of structural and functional differences between the mitochondrial subpopulations little is known regarding how they may be differently affected by age. The few studies performed to date indicate that PL and IMF mitochondrial content and morphology may differ with age[51,58,59,101]. For example, Halling et al.[58] reported PL and IMF mitochondrial volume did not differ between young and old mice, but IMF mitochondria were

more fragmented in old animals. Similarly, Scudese et al.[179] found IMF mitochondria were more spherical and less complex in skeletal muscle from older adults. Scudese et al.[179] also reported a reduction in IMF mitochondrial volume in older adults compared with young adults.

Impaired mitochondrial function with age is often accompanied by increased intramuscular fat. A small amount of fat is normally stored in skeletal muscle in the form of lipid droplets. However, as we age, intramuscular fat infiltration increases and is associated with a reduction in knee extensor strength[180,181]. Interestingly, the loss of skeletal muscle strength occurs at a faster rate than the loss of skeletal muscle mass in older adults[182]. This suggests a loss of skeletal muscle mass and quality (strength per unit of muscle mass) contribute to functional declines with age[182]. The fatty infiltration into skeletal muscle may contribute to declines in muscle function and quality as greater fat infiltration is also associated with greater risk for mobility issues in older adults[183]. Despite increased intramuscular adipose tissue, skeletal muscle from older adult have reduced lipid oxidation and lipid-mitochondrial contacts[11,60].

Intramuscular lipid droplets are found throughout muscle fibers but are more abundant in the IMF region. Lipid droplets in the IMF region are often found tethered to IMF mitochondria so that the mitochondria can provide rapid energy for fatty acid oxidation and storage[17,57,184]. With age, these lipid droplets become larger and more numerous[60,181], but the localized changes are poorly understood. Crane et al.[60] found lipid droplet content to increase in the PL region of older adult skeletal muscle, but IMF lipid droplets were not measured. To our knowledge, no study has evaluated mitochondrial and lipid droplet subpopulation content in sarcopenic individuals.

The skeletal muscle mitochondrial reticulum and lipid droplet content are modified by a variety of stimuli, including exercise. Aerobic exercise training is a strong stimulus for mitochondrial biogenesis and the expansion of the mitochondrial reticulum[13,50,185]. However,

aerobic exercise is not as effective at increasing skeletal muscle mass or strength, which is a goal for older individuals with sarcopenia. Resistance exercise training is recommended for older adults to improve skeletal muscle mass and strength, and it may also elicit positive impacts on mitochondrial content and function in older adults. Indeed, there is evidence that 10 to 24 weeks of resistance exercise training can decrease oxidative stress and increase electron transport chain content and activity in skeletal muscle of older adults[69,71,74,170]. Yet, to our knowledge only one study has determined the impact of resistance exercise training on specific mitochondria subpopulations. Miller et al.[76] resistance trained older men and women with advanced stage knee osteoarthritis and found via electron microscopy that IMF mitochondrial number and fractional area (total mitochondrial area/total area of the image) increased after the intervention. Conversely, PL mitochondrial fractional area, number, and average size did not change following resistance exercise training[76].

Given the substantial role mitochondria play in energy homeostasis, proper mitochondrial function is vital for healthy muscles, especially in older adults. Additionally, surplus lipid droplets impede skeletal muscle function and contribute to functional impairments with age. Resistance exercise training is a viable method to increase skeletal muscle mass and may also decrease mitochondrial dysfunction in older and sarcopenic adults. However, the effects of resistance exercise on mitochondrial subpopulations and lipid droplets in sarcopenic individuals are not known. Thus, we sought to determine the effects of 12 weeks of lower-body resistance exercise training on mitochondrial and lipid droplet subpopulation content in sarcopenic individuals. In order to preserve mitochondrial and lipid droplet morphology, transmission electron microscopy was used to visualize and measure intracellular structures. By preserving skeletal muscle

ultrastructure, we were able to visualize mitochondria and lipid droplet organization to better understand any structural adaptations to resistance exercise training.

Methods

Participants, Whole-Body Measures, and Intervention

Detailed description of participants, muscle sampling, and whole-body measures can be found in Chapter 3 (Page 38). Briefly, nine independently living older adults between the ages of 65-88 were included in this study. One participant used in Chapter 3 was excluded from this study due to an insufficient amount of muscle acquired during their baseline muscle biopsy. Participants were at least moderately sarcopenic based on skeletal muscle mass index, as measured by bioelectrical impedance analysis (men <10.75 kg/m^2; women <6.75 kg/m^2)[186]. Participant underwent 3 months of supervised lower-body resistance exercise. Resistance exercise protocol can be found in Chapter 3 (page 38). Before and after exercise training, participant body composition (Dual-energy X-ray absorptiometry), maximal aerobic capacity (indirect calorimetry), and knee extensor force (Biodex dynamometer) were assessed. All procedures were approved by the University of Maryland Institutional Review Board and conformed with the Declaration of Helsinki.

Muscle Sampling

Vastus lateralis muscle biopsies were taken before and after exercise training using a Bergstrom needle (Stille, Solna, Sweden), as previously described[148,149]. Biopsies were pulled from the anterolateral aspect of the right leg approximately 12-13 cm above the patella, except for one participant that requested their biopsies to be acquired from their left leg. Approximately 1 cubic

millimeter of each muscle sample was immediately fixed in 100mM cacodylate (Electron Microscopy Sciences, Hatfield, PA, USA) with 2% glutaraldehyde (Thermo Fisher Scientific, Heysham, UK) and stored for a maximum of 4-weeks at 4ºC until analysis. Biopsies were taken 24-48 hours after the exercise intervention to avoid potential transient effects of the last exercise bout.

Transmission Electron Microscopy

Following primary fixation in 100mM cacodylate (Electron Microscopy Sciences, Hatfield, PA, USA) with 2% glutaraldehyde (Thermo Fisher Scientific, Heysham, UK), samples were washed with 100mM cacodylate and fixed a second time with 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA, USA) for one hour. Samples were washed with 100mM cacodylate and incubated with 2% uranyl acetate (Electron Microscopy Sciences, Hatfield, PA, USA) for one hour before being dehydrated with progressively higher ethanol concentrations starting at 30% and ending at 100%. Propylene oxide (Electron Microscopy Sciences, Hatfield, PA, USA) was used as a transitional solvent to replace the ethanol due to propylene oxide's miscible properties with embedding medium. Equal parts Spurr resin (Electron Microscopy Sciences, Hatfield, PA, USA) and propylene oxide were added to samples for one hour to begin the infiltration process. An additional one-part Spurr resin was added to the mixture before leaving overnight to infiltrate (~17 hours). The following day, an additional one-part Spurr resin was added to the mixture for one hour before replacing the mixture with 100% Spurr resin. After one hour the resin was removed and replaced a second time with 100% Spurr resin. Samples were then cured at 70ºC for ~20 hours and sectioned using a Reichert-Jung Ultracut E (C. Reichert Optische Werke AG Wein, Austria) and Leica EM UC6 (Wetzlar, Germany). 70nm sections were embedded on 200 hex mesh copper grids (Electron Microscopy Sciences, Hatfield, PA, USA) and stained with

2% uranyl acetate (Electron Microscopy Sciences, Hatfield, PA, USA) and 1% lead citrate (Electron Microscopy Sciences, Hatfield, PA, USA). Samples were imaged using a Hitachi HT7700 (Chiyoda, Tokyo, Japan) transmission electron microscope at a magnification of x8000 (Peabody, MA, USA). All incubations and washes prior to curing were done under gentle agitation.

Three fibers were randomly selected for imaging for each sample. A total of six images were taken from each fiber. Three images were acquired from the subsarcolemmal region, and three images were acquired from the intermyofibrillar region. Thus, for each sample, a total of nine images were taken of the subsarcolemmal region and nine images were taken of the intermyofibrillar region. All subsarcolemmal images acquired from the same sample were analyzed and averaged together to serve as an individual datapoint. Intermyofibrillar images were also analyzed and averaged together as a single datapoint. The subsarcolemmal region was defined as the space between the sarcolemma and the first row of myofibrils. The intermyofibrillar region was defined as the area between the first and last row of myofibrils within a muscle fiber. Representative images demonstrating the analyzed portions for the subsarcolemmal and intermyofibrillar regions are shown in Figure 15. Images were manually analyzed using ImageJ software for the following mitochondrial and lipid droplet measures in both regions: Number: Count per micrograph, Area: Size in micrometers squared, Fractional Area: Sum of all areas as a percentage of total micrograph area, Maximum Feret's Diameter: Longest distance between two points, Minimum Feret's Diameter: Shortest distance between two points perpendicular to maximum Feret's Diameter, Aspect Ratio: Major axis divided by minor axis, Circularity: Calculated as $4\pi*(\text{area}/\text{perimeter}^2)$, where 1.0 would indicate a perfect circle, and Mitochondrial-Lipid Contacts: Percent of lipid droplets touching mitochondria.

Statistical Analysis

Statistical analyses were completed using dependent samples t-tests to compare mitochondrial and lipid droplet measure before and after exercise training. Subsarcolemmal and intermyofibrillar regions were independently analyzed. All statistical analyses were two-tailed and performed using GraphPad Prism 9 with statistical significance set at $P < 0.05$.

Results

Participant characteristics

Participant characteristics and responses to resistance exercise training are presented in Table 5. Participants' body fat, lean mass, appendicular lean mass, and VO_{2max} were unaffected by the exercise intervention. However, body weight ($P < 0.01$) and BMI ($P < 0.01$) decreased following training. Sarcopenic index (ALM/BMI) significantly increased following 12-weeks of resistance exercise indicating greater lean appendicular mass relative to body mass ($P = 0.03$); however, ALM did not increase after resistance training. Despite no changes in lean muscle mass, maximal knee extensor force production significantly increased 18% from pre-testing values ($P = 0.03$).

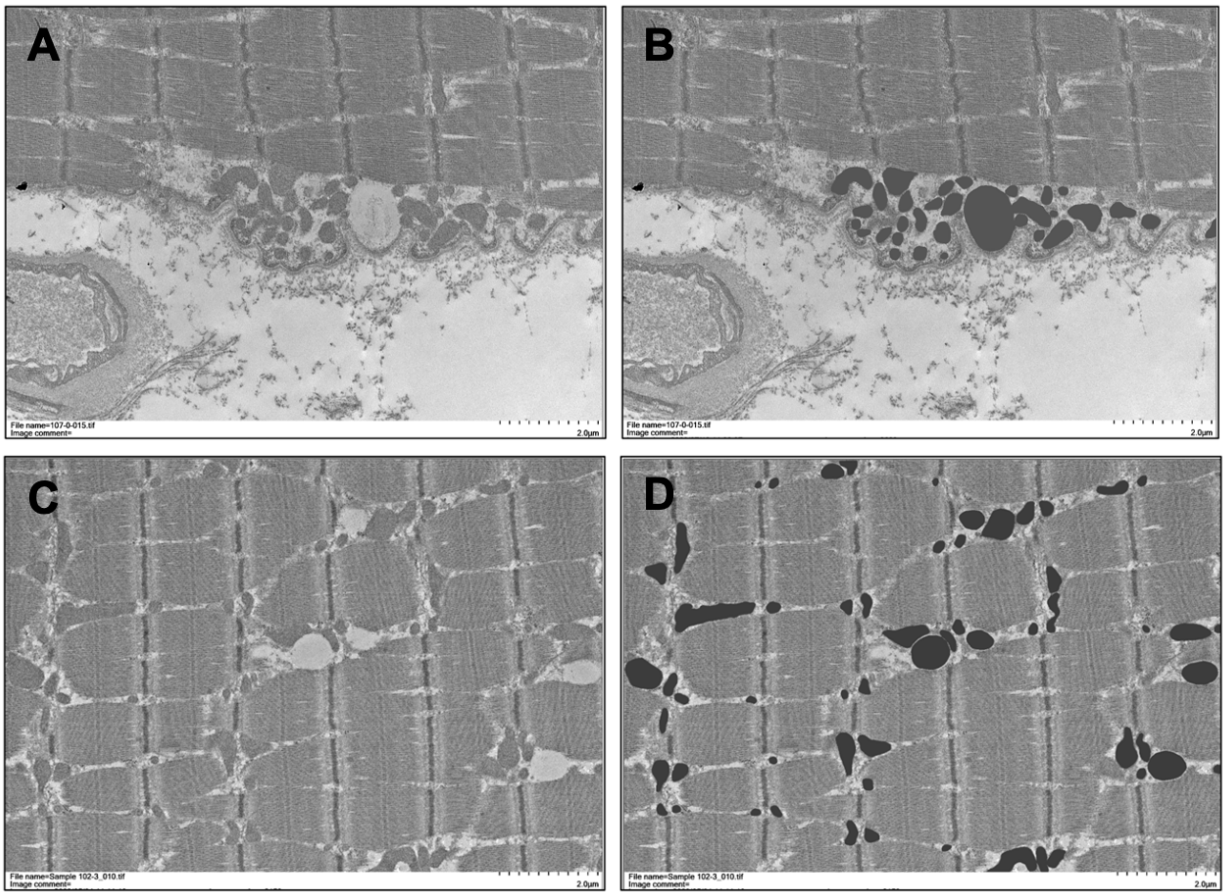

Figure 15. Representative transmission electron micrographs of subsarcolemmal and intermyofibrillar region. Figure 1A depicts subsarcolemmal region and 1C depict intermyofibrillar region. White-gray circular structures are lipid droplets and dark gray round and oblong shapes are mitochondria. Figures 1B and 1D demonstrate the analyzed portions including mitochondria and lipid droplets.

Peripherally-Located Mitochondria

PL mitochondrial number was unaffected by 12 weeks of resistance exercise training (Figure 16A). Similarly, PL mitochondria fractional area and average size were unaffected by 12 weeks of resistance exercise training (Figure 16B and C, respectively). PL mitochondrial morphology was also unchanged following the intervention (Figure 17).

Table 5. Participant Characteristics and Responses to 3 Months of Resistance Exercise Training

Variable	Baseline	3 Month Resistance Training
Age (years)	76.9 ± 1.9	
Sex	F = 6; M = 3	
Height (cm)	164.9 ± 3.3	
Skeletal Muscle Mass Index (kg/m^2)	F = 6.22 ± 0.22 M = 9.15 ± 0.31	
Weight (kg)	76.3 ± 4.5	74.6 ± 4.5*
BMI (kg/m^2)	27.9 ± 0.7	27.0 ± 0.7*
Body Fat (%)	36.9 ± 1.5	37.2 ± 1.6
Total Lean Mass (kg)	44.8 ± 3.6	44.6 ± 3.6
ALM (kg)	20.4 ± 1.9	20.3 ± 1.9
ALM/BMI	0.722 ± 0.052	0.746 ± 0.053*
VO_{2max} (L/min)	1.48 ± 0.17	1.53 ± 0.14
VO_{2max} (mL/kg/min)	19.4 ± 1.2	20.2 ± 1.1
Right Knee Extensor Force[#] (N*m)	107.3 ± 10.8[#]	126.2 ± 20.9*

BMI = body mass index, ALM = appendicular lean mass. Data are means ± SEM. *P < 0.05 from Pre-Training. [#]Includes force production from left leg of one participant.

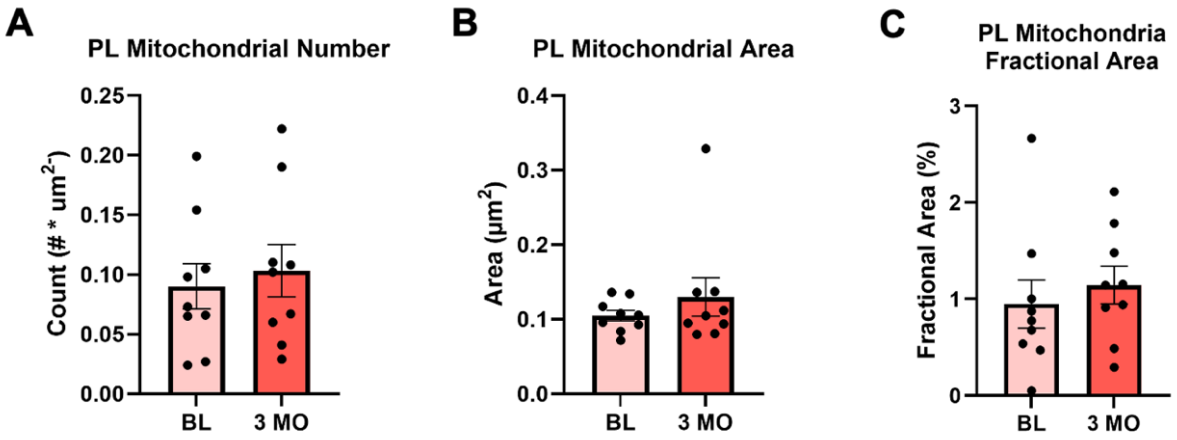

Figure 16. Content of peripherally located mitochondria is unaffected by resistance exercise training. Legend = PL, peripherally located; BL, baseline; 3 MO, post 3 months resistance exercise training. Data are presented as means ± SEM, n = 9 per group.

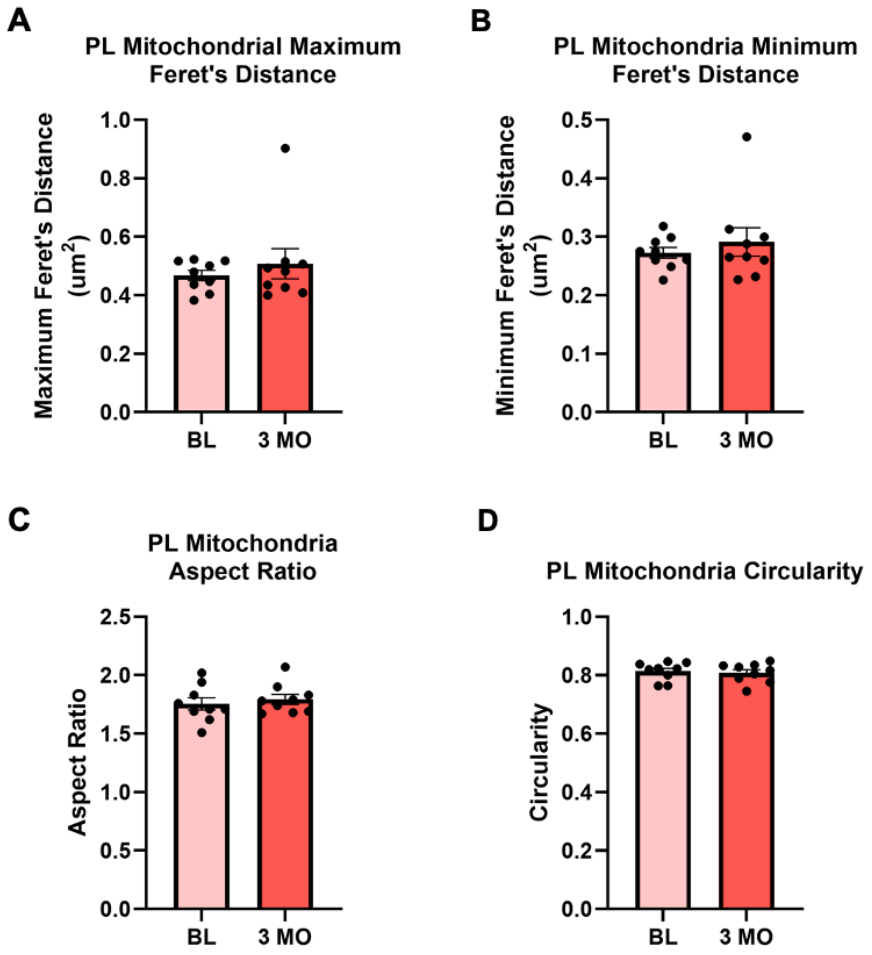

Figure 17. Resistance exercise training does not alter peripherally located mitochondrial morphology. Legend = PL, peripherally located; BL, baseline; 3 MO, post 3 months resistance exercise training. Data are presented as means ± SEM, n = 9 per group.

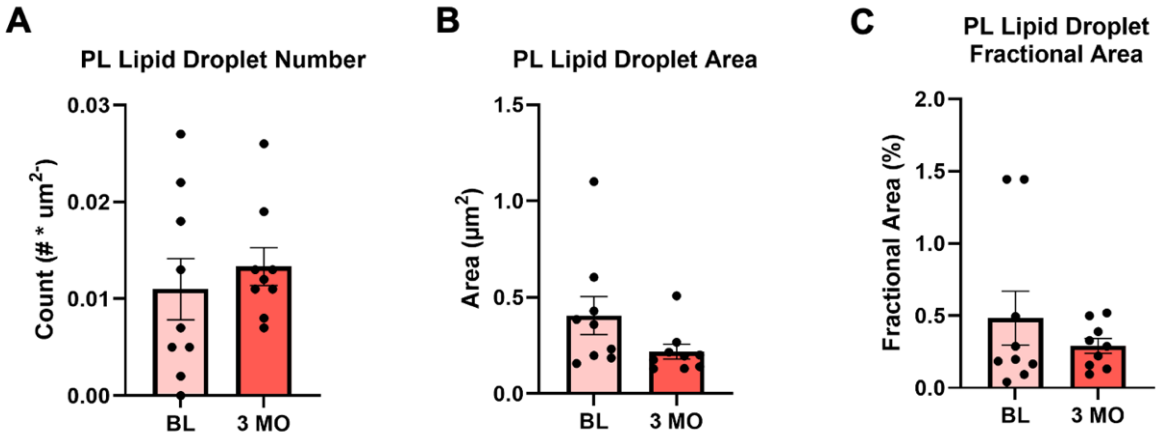

Figure 18. Content of peripherally located lipid droplet was similar before and after resistance exercise training. Legend = PL, peripherally located; BL, baseline; 3 MO, post 3 months resistance exercise training. Data are presented as means ± SEM, n = 9 per group.

Peripherally-Located Lipid Droplets

PL lipid droplet number and average size did not differ before and after training (Figure 18A and C, respectively). Total lipid droplet area was not altered by resistance exercise training (Figure 4B). However, PL lipid droplet aspect ratio increased post-training (Figure 19C; P = 0.04), suggesting elongation of lipid droplets. No other morphology measurement (e.g., Feret's distances or circularity) differed between baseline and post-training (Figure 19). Resistance exercise training did not change the percent of mitochondrial-lipid droplet contact in the PL region (Figure 19).

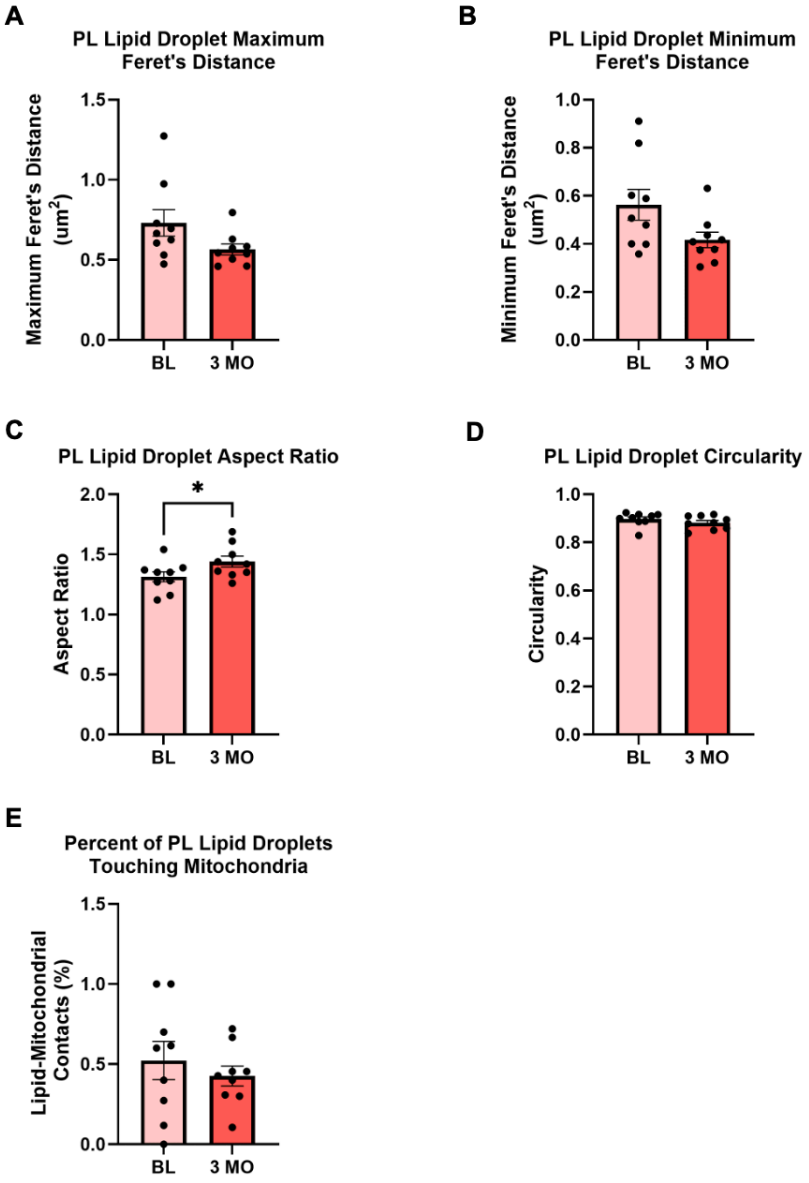

Figure 19. Peripherally located lipid droplets become elongated following resistance exercise training. Legend = PL, peripherally located; BL, baseline; 3 MO, post 3 months resistance exercise training. *Significant difference between groups ($P < 0.05$). Data are presented as means ± SEM, n = 9 per group.

Intermyofibrillar Mitochondria

In the IMF region of skeletal muscle fibers, mitochondrial number was unchanged post-training (Figure 20A). IMF mitochondrial fractional area and average size were also unaffected by the intervention (Figure 20B and C, respectively). Mitochondrial minimum Feret's distance in the IMF region tended to increase following resistance exercise training (Figure 21B; $P = 0.054$); however, maximum Feret's distance, circularity, and aspect ratio was unaffected following the intervention (Figure 21A, C, D, respectively). Change in minimum Feret's without concurrent changes to other morphological measures would indicate increased mitochondrial width, while retaining similar lengths and curvatures.

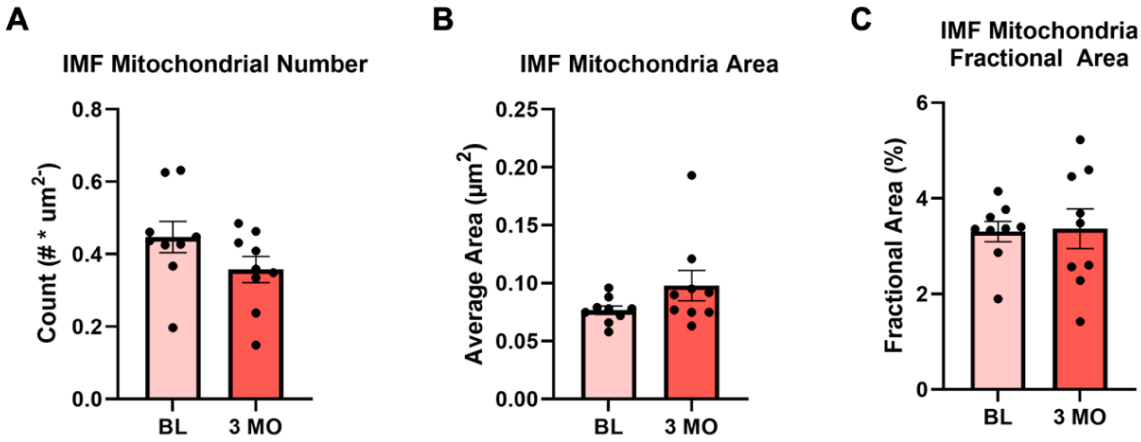

Figure 20. Intermyofibrillar mitochondrial content is unchanged by resistance exercise training. Legend = IMF, intermyofibrillar; BL, baseline; 3 MO, post 3 months resistance exercise training. Data are presented as means ± SEM, n = 9 per group.

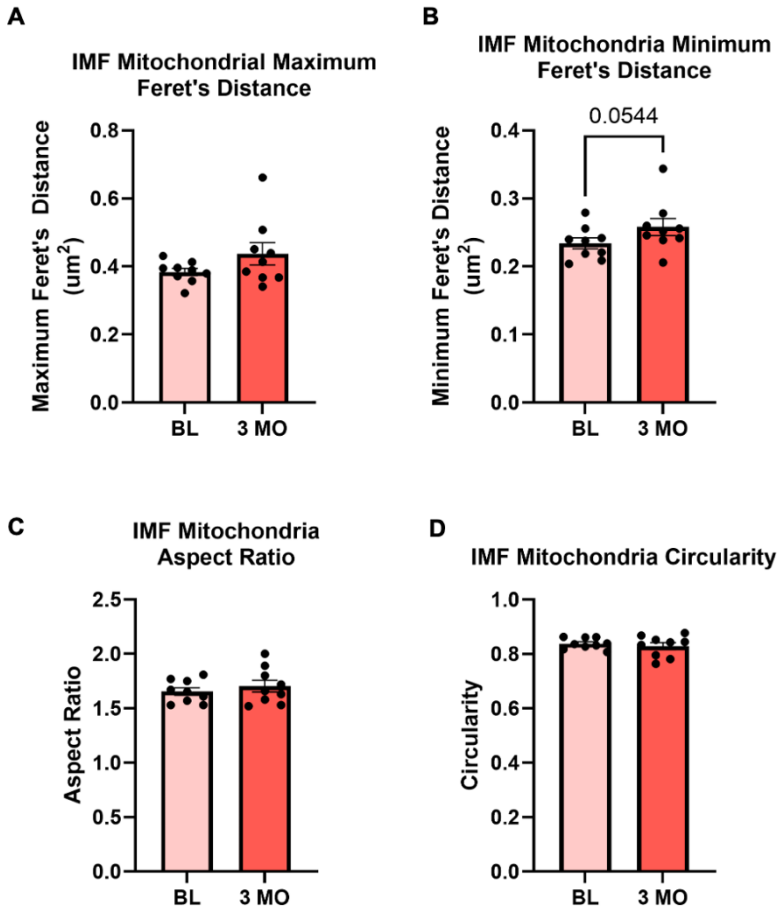

Figure 21. Resistance exercise training did not produce changes to intermyofibrillar mitochondrial morphology. Legend = IMF, intermyofibrillar; BL, baseline; 3 MO, post 3 months resistance exercise training. Data are presented as means ± SEM, n = 9 per group.

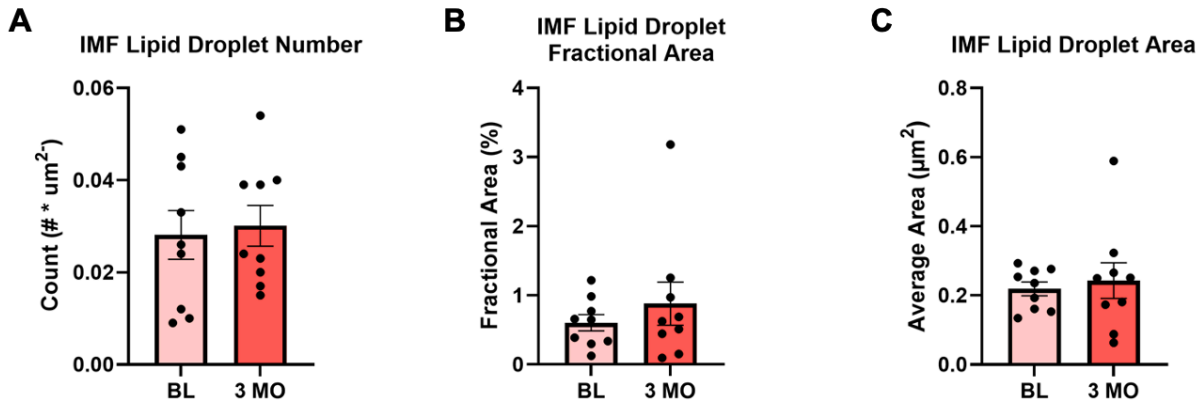

Figure 22. Lipid droplet content from the intermyofibrillar region is unaffected by resistance exercise training. Legend = IMF, intermyofibrillar; BL, baseline; 3 MO, post 3 months resistance exercise training. Data are presented as means ± SEM, n = 9 per group.

Intermyofibrillar Lipid Droplets

IMF lipid droplet number, average size, and fractional area was unaffected by 12 weeks of resistance exercise training (Figure 22). Similarly, IMF lipid droplet morphology was unchanged by resistance exercise training (Figure 23). The percent of IMF lipid droplets in contact with intermyofibrillar mitochondria was also unaltered post-training.

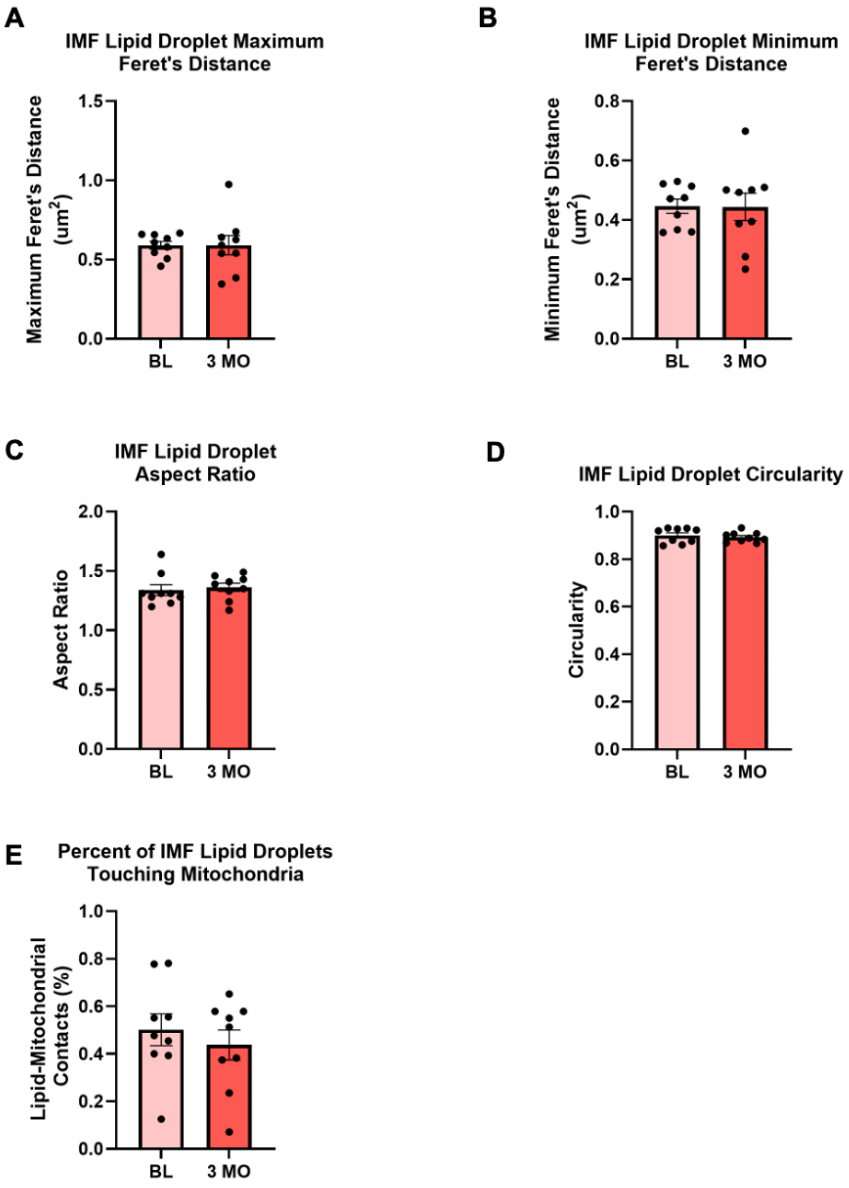

Figure 23. Morphology of intermyofibrillar lipid droplets remains unchanged by resistance exercise training. Legend = IMF, intermyofibrillar; BL, baseline; 3 MO, post 3 months resistance exercise training. Data are presented as means ± SEM, n = 9 per group.

Discussion

Twelve weeks of lower body resistance exercise training increased knee extensor force production, and reduced body weight and BMI in moderately sarcopenic older adults individuals. Resistance exercise training, however, did not change PL and IMF mitochondrial content or morphology in these sarcopenic individuals. While the majority of PL and IMF lipid droplet content and morphological measures were not altered by resistance exercise training, we found PL lipid droplet aspect ratio increased, suggesting elongation of lipid droplets, potentially to aid in mobilization of lipid droplets. Together, our results show 12 weeks of lower body resistance exercise training was largely ineffective at altering mitochondria and lipid droplet content and structure in sarcopenic older adults.

Our results indicate that 12 weeks of resistance exercise training may not be an adequate stimulus to induce changes to either PL or IMF mitochondrial content in sarcopenic individuals. This is consistent with our findings from Chapter 3 that CIV protein content from whole muscle homogenates did not change following 12 weeks of resistance exercise training in sarcopenic individuals. Our present findings, along with those from Chapter 3, are supported by the majority of the literature that has reported no changes in mitochondrial content in older adults following resistance exercise training[71,72,164,165,187]. Full body resistance exercise training lasting 8-14 weeks did not improve mitochondrial content in older adults across a variety of measures including, citrate synthase activity, total electron transport chain protein expression, and mitochondrial respiration normalized to muscle weight[71,72,164,187]. Parise et al.[165] resistance trained lower body only, but trained participants unilaterally on leg press and extension. Authors found citrate synthase and CIV activity did not differ following 12 weeks of resistance exercise in the trained leg[165]. Although these studies did not isolate mitochondrial subpopulations, they agree with our

findings that 12 weeks of resistance exercise training did not impact mitochondrial content in sarcopenic older adults.

While we are not aware of any other study that has specifically evaluated skeletal muscle mitochondrial subpopulation content in sarcopenic older adults, a few studies have been conducted in older adults with advanced-stage knee osteoarthritis, a condition that is a leading cause of disability in older adults due to cartilage loss and bone remodeling[188]. Using electron microscopy, Callahan et al.[189] found that older women with advanced-stage knee osteoarthritis had fewer PL mitochondria, but similar PL mitochondrial size compared to healthy age matched controls. Interestingly, PL mitochondrial number, area, and fractional area did not differ between osteoarthritic and healthy older men. Similarly, IMF mitochondrial number, area, and fractional area were similar between all participants regardless of osteoarthritis and sex[189]. These findings suggest that sex may differentially affect mitochondrial subpopulations in older adults. It is important to note that older adults with advanced-stage knee osteoarthritis participate in lower levels of leisure and household physical activity as well as have higher levels of inflammatory cytokines present in skeletal muscle than healthy older adults[190,191]. These factors may contribute to worsen skeletal muscle health and may negatively impact mitochondrial health.

Later work by the same group sought to determine the impact of 14 weeks of lower-body resistance exercise training on PL and IMF mitochondrial number, area, and fractional area in older men and women with advanced-stage knee osteoarthritis. They found that PL mitochondrial number, area, and fractional area did not change[76], which is in line with our finding of no differences in PL mitochondrial content before or after resistance training in sarcopenic older adults. These results are also in line with the aforementioned studies that have demonstrated 8 to 14 weeks of resistance exercise training does not impact markers of mitochondrial content

(electron transport chain complex content and citrate synthase activity) in older adults[72,164,165]. However, unlike the present study, Miller et al.[76] observed an increase in IMF content following resistance exercise training. One important difference to note is that although the participants studied by Miller et al.[76] were sedentary older adults, they were not sarcopenic. Further, the participants included by Miller et al.[76] had advanced-stage knee osteoarthritis, a condition that is accompanied by greater pain and reduced functional performance in comparison with healthy age matched controls which may indicate these individuals had worse skeletal muscle health than our participants[191]. Another difference to consider is the duration of the resistance exercise intervention between studies. Miller et al.[76] resistance trained participants for an additional two weeks compared to our present study (14 weeks versus 12 weeks). Although few studies have reported increased mitochondrial content in older adults following resistance exercise training, one such study resistance trained participants for 24 weeks[69]. That study also reported greater oxidative capacity following the resistance training, which may suggest a longer duration of training is needed to elicit changes to mitochondrial content and function in older sarcopenic adults.

As previously mentioned, PL mitochondria are more responsive to aerobic exercise training. We employed resistance exercise training to address the skeletal muscle declines in sarcopenic skeletal muscle. Thus, the lack of changes in PL mitochondria in our study may have been due to the stimuli (resistance exercise training) not having a large enough metabolic or vascular demand to incite mitochondrial changes in sarcopenic individuals. Confirming this, we found no changes in absolute or relative maximal oxygen consumption (VO_{2max}) before or after resistance exercise training.

In contrast to PL mitochondria, IMF mitochondria have a greater propensity to generate ATP for muscle contractions. As previously mentioned, isolated IMF mitochondria have greater

respiration and complex V activity[174,175,192]. This is consistent with confocal microscopy analysis of muscle fibers which has revealed greater complex V activity relative to complex IV in the IMF region[57,173]. In theory, one might expect increased IMF mitochondrial content due to larger energy demands as a result of resistance exercise training induced muscle hypertrophy. However, we did not observe any changes to IMF mitochondrial content or structure in response to resistance exercise training. This may have been due to the lack of improvements in ALM after 12 weeks of resistance exercise training. Considering our resistance exercise program only consisted of lower body resistance exercise, it is not completely surprising that ALM, which takes both upper and lower body mass into account, did not change after the intervention. Alternatively, sarcopenic skeletal muscle may have an impaired ability to regulate mitochondrial structure. This is consistent with our findings in Chapter 3 that proteins that regulate mitochondrial biogenesis and structure, such as PGC-1α, MFN2, and DRP1 were not impacted by 12-weeks of resistance exercise training in sarcopenic older adults.

While IMF lipid droplet content and morphology was unaffected by resistance exercise training, we found PL lipid droplet aspect ratio increased after the intervention The significance of lipid droplet morphology is currently poorly understood. It has been hypothesized that altering lipid droplet shape in favor of elongation aids in lipid droplet mobilization by increasing the surface area to volume ratio[193]. Lipid droplet content and morphology are impacted by endurance and high intensity interval training[185,193,194], but little is known regarding lipid droplet adaptations to resistance exercise training. Moro et al.[195] reported a decrease in intramuscular lipid droplet content in older adults following 12 weeks of whole-body resistance exercise training. Consistent with previous reports[196,197], Moro et al.[195] observed greater lipid droplet content in the PL region of oxidative muscle fibers prior to training, although no other morphological or content data were

reported. The lack of changes in intramuscular lipid droplet content in our study may stem from an inability of older, sarcopenic individuals to mobilize lipid droplets for lipolysis. Lipid droplets are surrounded by coating proteins and rely on the perilipin (PLIN) protein family for proper storage and mobilization[198]. PLIN2 is particularly important for intramuscular lipid droplets, as PLIN2 aids in intramuscular lipid droplet storage and the prevention of lipolysis[198]. PLIN2 is upregulated with age and is associated with greater levels of ubiquitin ligases and lower skeletal muscle thickness and strength[199–201]. We hypothesize that our resistance exercise training may not have been effective at decreasing PLIN2 levels, resulting in largely similar lipid droplet content after training.

In our study, all electron microscopy images were oriented longitudinally to myofibrils which is consistent with several prior studies[53,60,61,76,185]. However, it is important to acknowledge that Leduc-Gaudet et al.[51] reported differences in IMF mitochondrial morphology between old and young mice that were dependent on image orientation. IMF mitochondria from old animals had larger perimeters, aspect ratios, and form factors (a measure of shape complexity) when imaged longitudinally and transversely. However, the authors also reported significant changes in circularity and Feret's diameter only in the longitudinal orientation[51]. Therefore, a limitation of our study is the possibility for missed subpopulation morphological changes based on image orientation. Future studies aimed at characterizing skeletal muscle ultrastructure may require more advanced techniques, such as 3D renderings to better understand structural changes with age[118,179]. Another important consideration was our classification and quantification of all peripherally located mitochondria. Recent work by Parry et al.[202] has demonstrated peripherally located mitochondria may have distinct properties depending on their specific location (i.e., capillary adjacent, nucleus adjacent, or subsarcolemmal and not adjacent to either). However, to our

knowledge, we are the first study to use electron microscopy to evaluate mitochondrial and lipid droplet content and morphology in sarcopenic individuals. Furthermore, we are the first to compare mitochondrial subpopulations in sarcopenic individuals in response to resistance exercise training.

In conclusion, our study suggests 12 weeks of resistance exercise training did not cause robust changes to mitochondrial content or morphology in moderately sarcopenic older adults. These findings expand upon previous work by demonstrating neither PL nor IMF mitochondrial content nor morphology are impacted by resistance exercise in sarcopenic individuals. We further found that PL and IMF lipid droplets were also unaffected by resistance exercise training in sarcopenic older adults. While resistance exercise has beneficial effects to the skeletal muscle of sarcopenic individuals (i.e. increased muscle mass and strength, improved insulin resistance, decreased fatigue)[146], alternative exercise modalities may be needed to induce beneficial changes in PL and IMF mitochondrial content and morphology in sarcopenic individuals. In this sense, a combination of aerobic and resistance exercise training may be optimal to improve skeletal muscle health and reverse the effects of sarcopenia.

Chapter 5: Conclusion

Summary of Findings

The purpose of this dissertation was to understand whether mitochondrial structural dynamics are altered by age and age-related sarcopenia. We also sought to answer whether sex mediates age-related changes in mitochondrial quality control proteins, and whether changes are unique to skeletal muscle or are also present in cardiac muscle. To address these questions, we performed two separate studies, one in rats and the other in humans. In the first study (Chapter Two), we investigated young and old, male and female rats in order to determine the effects of age and sex on markers of mitochondrial quality control. Because mitochondrial structure, in part, regulates its function, we measured protein markers of mitochondrial fusion, fission, mitophagy, and biogenesis. In the second study (Chapter Three), we compared older adults with and without sarcopenia. In order to evaluate potential differences in mitochondrial structure that may contribute to worsening skeletal muscle health with sarcopenia, we quantified protein markers of mitochondrial fusion, fission, mitophagy, and biogenesis. We then determined the efficacy of resistance exercise training to improve levels of mitochondrial quality control markers in sarcopenic individuals. Lastly, we determined morphological changes in the specific mitochondrial subpopulations and lipid droplets before and after resistance exercise training (Chapter Four).

In Chapter Two we evaluated mitochondrial quality control proteins in young and old, male and female rats in order to identify whether age impacts mitochondrial structural dynamics. While the overwhelming majority of the previous literature has used exclusively male rodents or grouped male and female rodents together, Triolo et al.[47] demonstrated markers of mitophagy may be

impacted by sex and age. Thus, we compared male and female rats to determine if sex-specific responses existed in other mitochondrial quality control markers. We found TAs from old rats had lower mitochondrial content, but greater expression of OMM fusion and mitophagy proteins compared to young rats. One marker of fission (DRP1) was lower in the old TAs, while the other (FIS1) was significantly impacted by age and sex; on average FIS1 tended to decrease with age in the females but increase with age in the males. When analyzed relative to mitochondrial content, markers of OMM fusion and mitophagy were still greater in old versus young TAs. However, when fission proteins were expressed relative to mitochondrial content, no differences between groups were observed, suggesting the differences in fission proteins were due to reductions in mitochondrial content. We also found greater expression of one mitophagy marker (Pink1) in old compared to young and male compared to female rats when expression was normalized to mitochondrial content. Since we did not observe these differences prior to the secondary normalization, we concluded that this sex and age difference was due to differences in mitochondrial content.

We further analyzed hearts from the same young and old, male and female rats to determine if mitochondrial quality control was impacted in highly oxidative, cardiac muscle. Unlike in the TAs, mitochondrial content did not differ in the hearts. Markers of IMM and OMM fusion, as well as mitophagy, were greater in the hearts from old animals. No differences, however, were observed in either fission protein with age. The same pattern persisted when these proteins were expressed relative to mitochondrial content. No sex-specific changes in any of the proteins measured were found in cardiac muscles.

Together, our findings suggest mitochondrial dysfunction is increased in aged skeletal and cardiac muscle. Fusion and mitophagy are both induced in response to mitochondrial dysfunction;

Fusion can occur as a compensatory mechanism to dilute mutated mtDNA while mitophagy is triggered by a loss in membrane potential[87,203]. Mitochondrial damage leading to mitophagy and mitochondrial degradation can decrease mitochondrial content[204]. Consistent with this, we found mitochondrial content (CIV) to be lower in aged skeletal muscle. We did not observe this decrease in mitochondrial content in cardiac muscle, which may be explained by mitochondrial biogenesis (as measured by PGC-1α) negating any deficits in mitochondrial content. While we are not certain of the implications of the sex-specific changes in fission and mitophagy proteins in skeletal muscle, it does nonetheless warrant further investigation as it appears females may have a greater reduction in fission and mitophagy with age.

Given the age-related changes we observed in mitochondrial quality control proteins in rat skeletal muscle, we next investigated whether these proteins were altered in skeletal muscle of older adults with sarcopenia. In Chapter Three we determined expression of markers of mitochondrial fusion, fission, mitophagy, and biogenesis proteins in sarcopenic compared with non-sarcopenic individuals that were matched for age, sex, and BMI. There were no differences in protein expression of any markers of mitochondrial content, fusion, fission, mitophagy, nor biogenesis between sarcopenic participants and non-sarcopenic older adults. These results were unexcepted given that mitochondrial dysfunction has been implicated in the pathophysiology of sarcopenia. We did, however, find that markers of fusion were inversely correlated with total lean mass, ALM, and sarcopenia index (ALM/BMI), suggesting greater mitochondrial fusion in individuals with lower skeletal muscle mass. Considering mitochondrial fusion can occur as a compensatory response to stress and age in order to share matrix components, we hypothesize that a higher abundance of fusion proteins with lower skeletal muscle mass are a result of mitochondrial dysfunction with age.

An interesting trend is appearing in the literature where alterations in mitochondrial quality control with age are frequently reported in rodent studies, but these changes are not mirrored in human studies. While a few studies have reported alterations in mitochondrial quality control proteins in aged older adults, the majority do not. It is puzzling as to why these inconsistences exists, but they may stem from the variability in human research due to the inability to control for human lifestyle choices. Regardless, the overall lack of differences in fusion, fission, and mitophagy proteins between sarcopenic and non-sarcopenic older adults on average indicate mitochondrial quality control proteins may not significantly contribute to sarcopenia. Further, mitochondrial quality control proteins may be more sensitive to changes in age, as we demonstrated in Chapter Two, rather than sarcopenia.

We subsequently determined whether resistance exercise training could elicit positive benefits to whole-body measures as well as mitochondrial quality control proteins in the sarcopenic individuals. We found that 12 weeks of lower body resistance exercise training improved lean mass relative to body mass (decreased body weight and BMI, increased ALM/BMI) and increased strength (knee extensor force). These improvements were not reflected in the mitochondrial measures as neither mitochondrial content nor quality control proteins were altered in response to 12 weeks of resistance exercise training. This held true when we expressed the mitochondrial quality control proteins relative to mitochondrial content. While others have reported improved mitochondrial content and function in older adults in response to resistance exercise training, our exercise intervention was ineffective at producing differences in mitochondrial quality control proteins in moderately sarcopenic individuals. There are a number of possible explanations as to why we did not observe differences including inadequate stimuli (i.e. training duration was too short) or a blunted mitochondrial response from sarcopenic skeletal muscle. Additional studies are

needed to decipher whether training duration is a determinant of mitochondrial structural adaptations in sarcopenic skeletal muscle or whether intrinsic properties of sarcopenic skeletal muscle may be inhibiting mitochondrial structural adaptations.

Since current methods to analyze fusion, fission, and mitophagy *in vitro* and *in vivo* are challenging and are continually developing, we quantified the expression of proteins that regulate these mitochondrial quality control processes. To further analyze potential morphological changes to the mitochondrial reticulum, in Chapter Four we used electron microscopy to visualize and measure mitochondrial morphology in sarcopenic individuals before and after resistance exercise training. The use of electron microscopy allowed for the independent analysis of mitochondrial subpopulations which have specific roles in energy production. Since fat infiltration has been shown to increase in sedentary individuals with age and can negatively impact skeletal muscle function, we also investigated whether lipid droplet content and morphology were impacted by resistance exercise training. Both PL and IMF mitochondrial number, area, and fractional area were unchanged by 12 weeks of lower body resistance exercise training. Similarly, PL and IMF lipid droplet number, area, and fractional area were unaffected by the resistance exercise training. While PL and IMF mitochondrial morphology remained unchanged after the intervention, there was a numerical trend for IMF mitochondrial minimal Feret's distance (shortest distance perpendicular to maximal Feret's distance) to increase. Given that maximal Feret's distance (longest distance between two points) was similar before and after resistance exercise training, a trend towards increased minimal Feret's distance would indicate slightly wider mitochondria in the IMF region. These wider mitochondria would occupy more space but would have increased volume that could aid in energy production. PL and IMF lipid droplet content and morphology

were similarly unchanged following 12 weeks of resistance exercise training, with the exception of increased PL lipid droplet aspect ratio (height over width).

Similar to our findings in Chapter Three, our results from Chapter Four indicate mitochondrial subpopulations from sarcopenic individuals may not be sensitive to resistance exercise training. Alternative interventions (aerobic, high intensity interval) may be better suited to elicit positive adaptations in PL and IMF mitochondrial content and morphology in sarcopenic individuals as they have been shown to increase mitochondrial content and decrease intramuscular lipid droplets[60,193]. Further, the lack of robust changes in lipid droplet content and morphology in either region may stem from the inability of sarcopenic skeletal muscle to mobilize lipid droplets for lipolysis. The current literature on sarcopenia lacks a comprehensive understanding of which alterations that are strictly due to sarcopenia and not age. Here we demonstrated mitochondrial quality control proteins are similar between moderately sarcopenic and non-sarcopenic older adults, and that resistance exercise training did not produce changes to mitochondrial content and structure in sarcopenic individuals. However, the inclusion of a non-sarcopenic exercise group would help address whether the lack of differences following training were due to the intervention or the presence of sarcopenia.

Limitations and Future Directions

While this dissertation provides many novel contributions, there are several limitations to our studies that should be addressed for future investigations. A weakness of this dissertation was that we did not measure mitochondrial function to corroborate our structural changes (or lack thereof). Previous studies have demonstrated increased mitochondrial dysfunction in skeletal muscle from older adults and rodents including increased oxidative damage, mitochondrial

uncoupling, and greater mtDNA deletions and mutations. However, future studies on mitochondrial function in sarcopenic older adults in comparison to non-sarcopenic older adults are needed. Functional analyses would not only help determine which aspects of mitochondrial function is disrupted in sarcopenia (i.e., respiration, ROS production) but would help confirm or reject our hypotheses as to why greater fusion was correlated with lower muscle mass in older adults. Similarly, we did not quantify phosphorylated forms of mitochondrial quality control proteins. While the majority of the literature only quantifies non-phosphorylated mitochondrial quality control proteins, multiple proteins are activated via phosphorylation and measuring the phosphorylated proteins could further solidify the presence of these proteins are leading to greater activity and alterations in mitochondrial structure.

Another limitation of Chapters Three and Four is that we did not compare men and women. We recruited men and women but did not stratify enrollment by sex which led to a limited number of male participants. As demonstrated in Chapter Two, limited sex differences may exist in the expression of fission and mitophagy proteins. Markers of fission appear to be differently affected in old male skeletal muscle where one marker (DRP1) was lower and the other (FIS1) on average was higher than young counterparts. Similarly, one mitophagy marker (Pink1) was also higher in male TAs when normalized to mitochondrial content. Thus, we may have masked potential sex-specific changes in our study by grouping both sexes together. Additional studies are needed to compare mitochondrial quality control in sarcopenic and non-sarcopenic men and women. Since previous studies have reported greater ROS production in skeletal muscle in older men[111], future studies should include functional measures when analyzing mitochondrial quality control proteins in sarcopenic men and women separately.

Considering our sarcopenic individuals were moderately sarcopenic, they may not have had as pronounced mitochondrial changes as severely sarcopenic older adults. We elected to include older adults who were at least moderately sarcopenic so that we could assess a relative healthy population that would not be inhibited by mobility issues. In doing so, we may have missed potential alterations in mitochondrial structural dynamics that could exist with greater skeletal muscle atrophy observed in severely sarcopenic older adults. Additionally, we only measured mitochondrial quality control proteins and morphology at two timepoints (before and after resistance exercise training), which only provided us with a momentary snapshot of the muscle status. While our goal was to determine the impacts of 12 weeks of resistance exercise training, a longer longitudinal study with more than two time points would be preferred to track mitochondrial structural dynamics over time. Future studies focused on markers of mitochondrial quality control and morphology and its effect on mitochondrial function should determine the time course of mitochondrial changes with sarcopenia.

Conclusions

Cumulatively, the findings presented in this dissertation demonstrate differences in mitochondrial structural dynamics in skeletal muscle as a result of age but not sarcopenia. We showed mitochondrial structure regulating proteins did not differ between moderately sarcopenic and non-sarcopenic older adults. Additionally, to our knowledge, we are the first to compare and demonstrate mitochondrial morphology and structure regulating proteins do not differ before and after resistance exercise training in sarcopenic individuals. These results were unexpected considering mitochondrial function is regulated, in part, by its structure and mitochondrial dysfunction has been heavily implicated in the onset and progression of sarcopenia. Resistance

exercise training has also been shown to elicit positive effects on mitochondrial function in previously sedentary older adults. Our findings suggest mitochondrial quality control may not be robustly altered in moderately sarcopenic older adults. Instead, other regulators of mitochondrial function (i.e. ROS, mitochondrial permeability transition pore, and mtDNA) may be involved in the pathophysiology of sarcopenia to a greater degree.

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