HUMAN SKELETAL MUSCLE MITOCHONDRIA: CHANGES WITH AGING AND ROLE IN SARCOPENIA

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HUMAN SKELETAL MUSCLE MITOCHONDRIA:

CHANGES WITH AGING AND ROLE IN SARCOPENIA

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Sarcopenia, the age-related progressive loss of muscle mass, strength and physical function, is a well-established risk factor for several negative health-related conditions. While cellular, systemic and lifestyle factors have shown to be associated with the onset and development of sarcopenia, the primary mechanisms contributing to this process are still uncertain, especially in humans. Among the cellular factors hypothesized to play a role on muscle aging is mitochondrial function. Several questions, however, remain. The purpose of study 1 of this dissertation was to investigate the association between mitochondrial capacity and chronological aging. Percutaneous biopsies of the vastus lateralis were obtained from 68 healthy men and women with a wide range of age (20-88yrs), body mass index (BMI, 19-47kg/m^2) and cardiorespiratory fitness (VO_2max: 1.08-5.04L/min). Mitochondrial capacity was evaluated through mitochondrial respiration in

permeabilized myofibers and expression of proteins that mediate mitochondrial fusion, fission and autophagy. Mitochondrial respiration and expression of mitochondrial quality control proteins were elevated in young physically active individuals, but were similar among sedentary young, middle-aged and older subjects. The findings of this study suggest that mitochondrial capacity is not influenced by chronological age *per* se, but is closely related to BMI and cardiorespiratory fitness. Study 2 was performed to investigate the association between mitochondrial function and sarcopenia in very old adults. Specifically, the association between mitochondrial respiration and myofiber cross-sectional area, intramyocellular lipid content, and physical function were evaluated. Percutaneous biopsies from vastus lateralis were collected from 41 very old men and women (85-95yrs). Myofiber cross-sectional area and intramyocellular lipid content were evaluated histologically. Grip strength was tested by a handheld dynamometer, and knee extension torque was evaluated by an isokinetic dynamometer. Physical function was evaluated by physical performance tests including the multiple chair stand, standing balance, and gait speed. Mitochondrial respiration explained a significant amount of variation in grip strength and knee extension peak torque, and was associated with preferred gait speed. The findings of this study suggest that mitochondrial capacity plays a role in sarcopenia among the very old, but that other factors secondary to aging, including decreased physical activity and higher adiposity may influence this association.

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PREFACE

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1.0 INTRODUCTION

1.1 STATEMENT OF THE PROBLEM

Skeletal muscle aging is characterized by a progressive loss of muscle mass, strength, and physical function, known as sarcopenia (1-3). These declines are often associated with impaired mobility, greater tendency to fall, loss of independence, and functional limitations in the elderly population (4-8). Global life expectancy is increasing, and the healthcare costs with sarcopenia are also expected to rise. Therefore, there is a clinical, social and economic need to determine factors underlying reduced skeletal muscle mass and function in older subjects.

For several decades, mitochondria have been hypothesized to play a major role in muscle aging (9-11). However, there is still confusion as to the precise contribution of mitochondrial capacity to aging muscle. Despite a great number of studies that demonstrate decreases in mitochondrial function with aging (11-14), several have failed to observe this association (15-18). These contradictory results could be partially explained by the use of different approaches and techniques to evaluate mitochondria. In addition to methodological factors, some studies of mitochondrial capacity have not controlled for other physiological factors that have been shown to affect mitochondria, such as adiposity and physical activity (15-18). Careful consideration of the methods and participant characteristics are needed when investigating age-related declines in mitochondrial capacity.

Recent data from *in vitro* and animal studies have strongly suggested that there may be a mechanistic link between mitochondria and the loss of muscle mass that occurs with aging (19-21). In addition to changes in muscle mass, recent evidence has shown that mitochondrial capacity may be associated with physical function in older adults (14, 22). Human studies further investigating the relationship between mitochondrial capacity and muscle mass, muscle strength and physical performance in old subjects is needed. A better understanding of the role of mitochondria on skeletal muscle aging and sarcopenia will assist on the development of approaches to prevent and treat the decreases in muscle mass and function observed in the old population.

1.2 BACKGROUND

Sarcopenia represents a major risk factor for several negative health-related conditions. The development of sarcopenia is associated with a higher predisposition to injuries, loss of independence, functional limitations in activities of daily living, and reduced quality of life; these declines may ultimately lead to disability, institutionalization, and mortality (23). Several factors are known to influence the pathogenesis of sarcopenia, including lifestyle habits, systemic factors, local environment perturbations, and intramuscular specific processes (24). Among the intramuscular factors, mitochondrial capacity has been hypothesized to be a major contributor to the age-related loss of muscle mass and function (24). A thorough understanding of the mitochondrial processes underlying muscle aging and the pathogenesis of sarcopenia is needed.

1.2.1 Why study mitochondria?

Mitochondria have been a topic of investigation for several decades. For the most part, the biochemistry of mitochondria has been examined in isolated systems, and its relationship with the rest of the cell is often not fully considered. In the past decades, the analysis of mitochondria as an independent and isolated organelle has changed as the development of new methodologies has allowed the examination of mitochondria within the cell environment (25). Additionally, the mitochondrion is now recognized as a dynamic organelle in which morphology is regulated constantly to assure proper function (26, 27).

Mitochondrial structure is characterized by a double-membrane that separates the organelle into four distinct compartments (Outer membrane, intermembrane space, inner membrane and matrix). The inner membrane is folded into cristae, and stores the electron transport chain (ETC). Through ETC, mitochondria perform one of their major functions- the consumption of oxygen and substrates to produce adenosine triphosphate (ATP). In addition to ATP production, mitochondria participate in a wide range of cellular processes, including thermogenesis, regulation of the cell cycle, signal transduction, oxidative stress, calcium handling, heme synthesis and apoptosis. They are also a major source of reactive oxygen species (ROS) (28-30).

Due to the essential role of mitochondria on cell function, it is not surprising that dysfunction or damage of mitochondria is hypothesized to underlie a diverse range of human diseases, such as neurodegenerative conditions (31), cardiac dysfunction (32), liver disease (33), type 2 diabetes (34), and cancer (35). Furthermore, mitochondria have been hypothesized to play a major role on the aging process, including the decreases in skeletal muscle mass and function that occurs with aging (14, 19, 20, 22, 36, 37). The wide impact of mitochondria in several diseases makes them an important topic for study as well as a target for therapy.

1.2.2 Is skeletal muscle mitochondrial capacity associated with muscle aging?

The influence of mitochondrial capacity on age-related changes in skeletal muscle has been extensively investigated as a means to understand the cause of sarcopenia. The free radical theory of aging, which was first proposed in 1956 by Harman (9), proposes that mitochondrial free radicals cause oxidative damage that may ultimately lead to aging. Cells constantly generate ROS, and the majority of this production is generated by the mitochondria as byproducts of oxygen consumption in the electron transport chain (ETC) (38, 39). While ROS are fundamental for proper cell signaling and homeostasis, a healthy balance between ROS production and consumption is required (28, 40). To manage the physiological ROS production, mitochondria possess a defense network comprising of detoxifying enzymes and non-enzymatic antioxidants. If mitochondrial antioxidant defenses are working properly and electron leakage occurs within the physiological range, oxidative damage is almost completely prevented. However, if ROS production exceeds the capacity of the antioxidant defense system, oxidative damage to mitochondrial DNA may occur (40). Mutations in mitochondrial DNA can lead to the synthesis of defective respiratory chain components, which may result in impairment of oxidative phosphorylation, decreased ATP production and further ROS generation (39). The increased ROS production will result in further mitochondrial damage. This vicious cycle of oxidative stress and mitochondrial dysfunction has been proposed as a mechanism by which muscle aging is accelerated.

Several studies have been conducted to investigate whether mitochondria play a role on skeletal muscle aging, and a significant number of them have shown decreases in mitochondrial function with chronological aging (11-13, 41-44). Rat and mouse studies performed in isolated skeletal muscle mitochondria demonstrated age-related decreases in mitochondrial content (45, 46), ATP production (43, 44), activity of electron transport chain complexes (44), state 3 (47) and

state 4 (46) respiration, and mitochondrial affinity for ADP (45). Additionally, increases in hydrogen peroxide production (44), mitochondrial DNA oxidative damage (44), mitochondrial apoptotic susceptibility (48), ROS production (48) and mitochondrial swelling (47) has also been observed. Similarly, *in vivo* measurement of mitochondrial oxidative phosphorylation to contraction-induced increase in ATP demand has been shown to be reduced with aging in rat muscle (45).

Human studies performed with muscle biopsies have confirmed the findings of animal studies demonstrating decreases in mitochondrial capacity with aging. Morphological changes include declines in mitochondrial content, expressed by reduced number, density and/or size of mitochondria (49-51), reduced mitochondrial DNA and mRNA (41, 52), and decreased mitochondrial protein expression (41, 52, 53). Mitochondrial functional changes observed with aging include reduced ATP production rate (41, 52), decreased oxidative capacity (50, 53, 54), reduced enzymatic activity (11, 13, 49, 52-56), and lower levels of mitochondrial respiration (11-13, 55). Additionally, *in vivo* human studies that utilized magnetic resonance spectroscopy have demonstrated reduced maximal ATP flux with aging in the gastrocnemius (57), vastus lateralis (50, 58) and soleus (59) muscles.

Despite a wide number of studies describing age-related changes in mitochondrial capacity, several studies have failed to observe these alterations (15-17, 60, 61). Animal studies performed in isolated mitochondria from rat muscles have not observed age trends related to enzyme activity (48), state 3 respiration (46, 48), state 4 respiration (48) , and ROS production (43). Human studies have also reported no differences in mitochondrial capacity with aging, including mitochondrial content (60, 62), enzymatic activity (15), ATP synthesis (16, 60), mitochondrial respiration (16, 17, 62), activity of electron transport chain complexes (15, 16, 60), and ROS production (55, 62).

Furthermore, *in vivo* studies have failed to demonstrate changes in maximal ATP flux with aging (63-66).

It is still not clear whether there is a decrease in mitochondrial capacity with aging, particularly in humans. The disparity in the literature regarding the association of aging and muscle mitochondrial function in humans may be explained by two main factors: study methodology and participants' characteristics. Study methodology such as the muscle analyzed, technique utilized to evaluate mitochondrial properties (*ex vivo* or *in vivo*), functional output being evaluated, and age-range being investigated are factors that can affect study results and need to be considered when comparing the outcome of different studies. Additionally, several recent studies have supported the influence of subject characteristics and physiological parameters, such as sex, race, physical fitness, body fat content/distribution, and insulin sensitivity on mitochondrial function, suggesting that these characteristics should also be considered when evaluating the association between mitochondrial capacity and aging (17, 49, 60, 67).

1.2.3 Possible confounders of the relationship between aging and mitochondrial capacity

While several studies have failed to demonstrate an association between age and mitochondrial capacity, many have suggested that declining mitochondrial capacity in older age can be attributed to participants' characteristics and physiological parameters. Possible confounders of the relationship between aging and mitochondrial capacity include sex, race, body fat, and physical activity levels.

Mitochondrial respiration of permeabilized muscle fibers was found to be higher in men when compared to age-matched women, suggesting that men have slightly greater quality or the quantity of skeletal muscle mitochondria (17). Additionally, accumulation of damaged proteins

and lipofuscin was shown to be more pronounced in men than in women, which means that the accumulation of molecular damage in skeletal muscle may differ between sexes (17). Similarly, high-resolution respirometry of permeabilized muscle fibers from Caucasian and African-American women showed decreased mitochondrial respiration, content, and oxidative capacity in African-American women, when compared to Caucasian women (67). These findings suggest that sex and race may influence mitochondrial capacity and, therefore, should be considered when evaluating the association between aging and mitochondria.

Body fat is another factor that has been suggested to play a role in mitochondrial function. While mitochondrial respiratory capacity was observed to be fully preserved with aging in a study performed with permeabilized human skeletal muscle, a significant negative correlation between mitochondrial respiration and the percentage body fat was found (17). Similarly, studies have shown that obese subjects present reduced capacity for lipid oxidation, and lowered activity of mitochondrial enzymes (68, 69).

Lipids have an essential impact on several cellular processes, and are key mitochondrial fuels (70). A balance between lipid production and oxidation or storage needs to occur for a healthy cellular state. Increased circulating levels of lipids have shown to result in metabolic alterations in fatty acids utilization and intracellular signaling, process also known as lipotoxicity (71). Cells can protect themselves from lipotoxicity by oxidizing fatty acids or storing them as triacylglycerol within lipid droplets (72-74). Triacylglycerol catabolism will later release fatty acids that will be used for β-oxidation and ATP synthesis in the mitochondria (70). Excess free fat acids can promote diverse effects on the mitochondria, and can act as both uncouplers and inhibitors of mitochondrial respiration (75).

Excessive lipid droplet accumulation is a characteristic of several metabolic diseases including obesity. There is considerable debate in the literature whether lipid oversupply and mitochondrial dysfunction play a role in the development of insulin resistance, and a number of studies have supported this lipotoxicity hypothesis (76-80). However, it is still unclear whether there is a direct relationship between lipids, mitochondria dysfunction and insulin resistance, and if changes in mitochondrial capacity are a cause or consequence of insulin resistance. In any case, it is well known that lipids have a considerable impact on mitochondria. Lipids have been shown to affect mitochondrial function, both are associated with obesity, and body fat increases with aging, thus strongly supporting the inclusion of body fat as one of the covariates of this study.

In addition to changes in body composition, aging is generally associated with decreased levels of physical activity. The ability of exercise to increase mitochondrial content and function is well documented (81, 82). Exercise training, including endurance and resistance exercise, stimulates mitochondrial biogenesis through increases in the peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (83, 84). Furthermore, recent studies have suggested that exercise can improve function/efficiency of mitochondria through remodeling of the mitochondrial network (fusion, fission, and autophagy) (85-88). Additional research should be performed to better understand the effects of exercise training on mitochondrial quality control processes, especially in the aging context. In contrast with the beneficial effects of exercise, decreases in physical activity, such as with bed rest, have shown to result in lower mitochondrial DNA content and lowered activity of mitochondrial enzyme (89). These findings strongly suggest that physical activity influences mitochondria and therefore should also be considered when evaluating the relationship between aging and mitochondrial capacity.

Several studies have shown that mitochondrial function is not affected by chronological aging, but rather by decreases in physical activity that normally occurs with aging (15, 16). Barrientos et al. (1996) and Brierley et al. (1996) observed that the association between age and mitochondrial capacity disappeared after controlling for physical activity levels (15, 16). Similarly, no differences in mitochondrial content and respiration was observed between young and old subjects matched for physical activity, both engaged in moderate to vigorous-intensity exercise training (62).

Studies that included a group of old trained subjects have been performed in an attempt to investigate whether maintenance of physical activity levels during aging can prevent decreases in skeletal muscle capacity. It has been shown that while old sedentary subjects present a reduced mitochondrial oxidative capacity when compared to young subjects, mitochondrial content, biogenesis, ETC function and antioxidant capacity is preserved in skeletal muscle of active older individuals (18). Similarly, muscle biopsies from well-trained seniors who exercised regularly in the previous 30 years demonstrated that lifelong physical exercise delays age-associated skeletal muscle declines (90). These well-trained seniors presented better mitochondria organization, including preserved fiber morphology and ultrastructure of intracellular organelles involved in Ca^{2+} handling and ATP production, lowered expression of genes related to autophagy and reactive oxygen species, in comparison with health matched sedentary senior. Likewise, age-related declines in oxidative capacity (54), mitochondrial ATP production (52), citrate synthase activity (52) have been observed in sedentary subjects, but not in endurance-trained subjects.

Taken together, these studies suggest that mitochondrial function is influenced by subject's characteristics, such as sex and race, and by physiological parameters, such as body fat and physical activity levels. In the present study the association between mitochondrial capacity and

aging was investigated. While studies have suggested the influence of sex, race, BMI, and cardiorespiratory fitness on mitochondrial capacity, there is still no consensus on whether mitochondrial capacity decreases with aging or not. None of the cited studies had performed a comprehensive evaluation of age-related changes in mitochondrial capacity when controlling for all these factors. In this dissertation study, the specific contribution of these covariates were evaluated using a sample of 68 muscle biopsies, from men and women with a wide range of age, BMI and cardiorespiratory fitness.

1.2.4 Methods to evaluate mitochondrial capacity

Several measurements, each with distinct advantages and limitations, are available to examine skeletal muscle mitochondrial capacity. The selection of the technique to be used is usually based on the study question and instrumentation availability. *In vivo* assessment of mitochondrial function can be achieved with the use of magnetic resonance spectroscopy (50, 64). *Ex vivo* assessment of mitochondrial capacity is possible with the collection of a skeletal muscle biopsy (22). *Ex vivo* approaches include measurement of mitochondrial content through electron microscopy, quantification of mitochondrial DNA, mitochondrial enzyme activity and protein expression of electron transport chain complexes (50, 53, 91, 92). Common measurements of mitochondrial function include ATP production (52, 61), mitochondrial enzyme activity (53, 93) , mitochondrial respiration (22) and ROS production (94).

Magnetic resonance spectroscopy (MRS) allows an *in vivo* and non-invasive assessment of muscle oxidative capacity through measurement of ATP synthesis rate (50, 64). The ability to assess mitochondrial function under conditions where circulatory and regulatory systems are intact is the strength of this technique. However, this *in vivo* measurement needs to be complemented by

an ex vivo analysis, such as mitochondria density, to really confirm the presence of a mitochondrial dysfunction.

Measurement of mitochondrial number, density and size can be achieved with the use of electron microscopy, which is the gold standard for analysis of mitochondrial content and morphology. Other analysis of mitochondrial content include measurement of cardiolipin content, an exclusive component of the inner mitochondrial membrane, measurement of mtDNA copy number, mitochondrial enzyme activities, such as citrate synthase, and expression of electron transport chain proteins through western blot (50, 53, 91).

Analysis of mitochondrial enzymes, such as citrate synthase, succinate dehydrogenase and cytocrome c oxidase have been widely used as a measured of mitochondrial oxidative capacity (53). Due to requirement of small amounts of frozen tissue, the spectrophometric-based enzyme activity assays are a reasonable method in studying mitochondria characteristics when the quantities of tissue are limited. However, it is very unlikely that a single enzyme can reflect the collective function of mitochondria.

Respiratory measurements of oxygen consumption of isolated mitochondria using polarographic oxygen electrodes has long been used to assess function of fresh isolated mitochondria (11). A downside of this method is that the conventional polarographic system requires large quantities of tissue and the sensitivity of measurement is low. A novel perspective of mitochondrial respiratory physiology emerges from a series of studies based on high-resolution respirometry (17, 95, 96). This method allow the determination of many parameters of mitochondrial function using small sample of biological material, which makes the analysis of human tissues feasible.

Although studies conducted with isolated mitochondria have the advantage to exclude all potentially confounding factors from the cellular environment, it does not preserve the complex structural arrangement of the organelle and it eliminates other constituents of the cell that could affect mitochondrial response. Additionally, the isolation procedure can cause damage to the mitochondria (60) and promote changes that are not representative of *in vivo* characteristics. A more recent approach utilized to examine skeletal muscle mitochondrial function is the dissection and permeabilization of the muscle sarcolemma yielding permeabilized myofiber bundles (17). This technique preserves mitochondrial structural cell interactions and morphology (25).

A study specifically designed to compare mitochondrial respiration in aged muscles between isolated mitochondria and permeabilized muscle fiber bundles demonstrated that the mitochondrial isolation procedure exaggerated functional age-related impairments in sarcopenic skeletal muscle (97). Similarly, contradictory results were found with the use of isolated mitochondria *vs* permeabilized muscle fibers. The authors suggested that the discrepancy could be explained by a biased harvest of "healthy" mitochondria and/or disruption of structural components during the process of isolation (55).

A considerable number of previous studies investigating the association between mitochondrial capacity and aging were performed in isolated mitochondria. In this dissertation study, mitochondrial function was evaluated by analysis of mitochondrial respiration in permeabilized muscle fibers using high-resolution respirometry. We opted for using this methodology to preserve the complex structural arrangement of the organelle and its interactions with the cell. We also assessed mitochondrial content through western blot technique, which give us information on the protein expression of each one of the five electron transport chain complexes.

1.2.5 Mitochondrial capacity may be associated with mitochondrial dynamics

The view of mitochondria as a dynamic organelle has increased in the recent years. It is recognized that mitochondria structure is constantly remodeled to assure proper function. Examination of mitochondrial quality control processes can provide essential information regarding mitochondrial capacity in aging and disease, and complement standard analysis of mitochondrial morphology, content and function.

Mitochondria structural and functional integrity relies on the efficiency of quality control processes. These processes include the mitochondrial oxidant scavenging systems, protein repair and degradation pathways, and mitochondrial dynamics and turnover (24, 98). ROS scavenging systems can prevent oxidative damage to the mtDNA, however when damage has occurred, several mechanisms to repair the mitochondria will be activated. Mitochondria that has suffered irreversible damage can be removed by the mitochondrial proteolytic system and replaced by newly synthetized proteins (99), while the ones that are partially damaged can be restored through the fusion with a neighboring intact mitochondria (98) or segregated from the vital mitochondrial network through fission and eventually eliminated by autophagy (98).

Mitochondrial morphology is regulated by continuous fusion and fission, and the balance between them is controlled by complex mitochondrial dynamics machinery and changes in metabolism. Mitochondrial fusion results in the formation of a network that enables the mitochondria to mix their contents, redistribute metabolites, proteins, mtDNA, and prevent the accumulation of abnormal organelles (100). A few main proteins are involved in the fusion process: dynamin-related GTPases mitofusion 1 and 2 (Mfn1 and Mfn 2) are responsible for fusion of the outer mitochondrial membrane (101), while the optic atrophy protein 1 (OPA1) is responsible for fusion of the inner mitochondrial membrane (102). The other quality control

process used by the mitochondria is fission. This process segregates components of the mitochondria that are irreversibly damaged or unnecessary, allowing for their autophagic removal (103). Mitochondrial fission is regulated by the dynamin-related protein 1 (Drp1) (104) and fission protein 1 (Fis 1) (105). A functional link exists between mitochondrial dynamics and autophagy. The segregation of damaged mitochondria by fission and inhibition of their fusion are prerequisites for their autophagic degradation (98).

Autophagy is a "self-eating" process through which cells degrade their own components (damaged or unnecessary cellular proteins and organelles) allowing for the recycling and reuse of the structures (106). Briefly, autophagy occurs through the sequestration of damaged organelles into a double-membrane structure known as autophagosome (106). Autophagosomes then fuse with lysosomes to form autolysosomes, in which the enveloped content is degraded. Autophagy can be specifically directed towards the degradation of mitochondria, known as mitophagy. Mitophagy is responsible for regulating the total number of mitochondria to match metabolic demands and serves as a quality control mechanism to remove damaged organelles (107).

While the orchestration of fusion and fission are important for maintaining mitochondrial integrity, few animal and human studies have examined mitochondrial quality control process in muscle aging, and the results are contradictory. Lower levels of mitochondrial fusion protein Mfn2, and increased expression of fission protein FIS1 and DRP1 were reported in skeletal muscle of aged rats when compared to young controls (108). A recent human study performed with young and old subjects observed no age-related changes in fusion and fission proteins (109). Conversely, a decreased expression of the fusion marker OPA-1 has been reported in low-functioning elderly subjects when compared to young and high-functioning elderly individuals (14), while no changes in fission were observed. Decreased Mfn2 gene expression was also observed in old individuals

when compared to young individuals (49). Similarly, studies investigating changes in autophagy are also contradictory and have shown both increases and decreases with aging (20, 110-112).

In this study, we proposed to explore the association between expression of fusion, fission and autophagy proteins with aging, using a group of young, middle-aged and old subjects. We hypothesized a decrease in both mitochondrial fusion (reduced expression of OPA1 and Mfn2 proteins) and fission (reduced expression of Fis1 and DRP1 proteins) in the old group when compared to young and middle-aged groups, figure 1. Additionally, we hypothesize that with reduced fission damaged components of dysfunctional mitochondria are not segregated for subsequent elimination by autophagy (reduced expression of Beclin1, LC3 and BNIP3), figure 1. While we believe that mitochondrial fusion, fission and autophagy are decreased with aging, we hypothesize that these changes are associated with age-related changes in BMI and cardiorespiratory fitness.

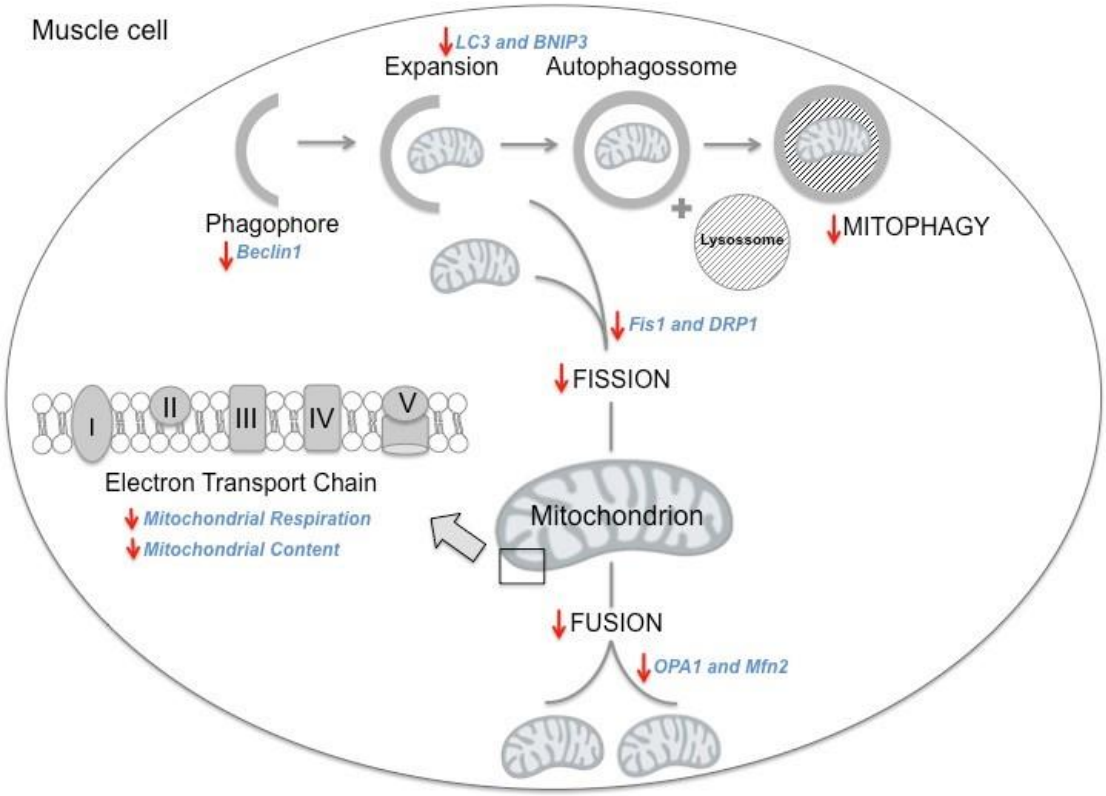

Figure 1. Hypothesis schematic for age-related decreases in mitochondrial capacity.

* Blue headings indicate mitochondrial measures of study 1; Red arrows indicate direction of changes hypothesized to occur with aging.

1.2.6 Potential links between mitochondrial capacity and sarcopenia

Given the central role of mitochondria in cellular quality control and cell death pathways, it is not surprising that impaired mitochondrial function is hypothesized to play a major role in the age-related loss of muscle mass and function. Changes in mitochondrial respiratory capacity, imbalance between mitochondrial fusion and fission, and failure of mitochondrial turnover resulting from insufficient biogenesis and/or defective autophagic removal of dysfunctional mitochondria are all factors that may be involved in the loss of muscle mass during aging.

Specifically, it is hypothesized that mitochondrial dysfunction and increased ROS production stimulates catabolic signaling pathways, and muscle atrophy by activating the two major proteolytic systems: the ubiquitin proteasome and the autophagy lysosome (113, 114).

Several *in vitro* and animal studies have provided information on the molecular pathways involved in these processes (19, 21, 113, 115). Forkhead Box O (FoxO) transcription factors have been identified as the main coordinators of the two proteolytic pathways by stimulating several autophagy-related genes (such as LC3 and BNIP3) as well as ubiquitin ligases (atrogin-1 and MuRF-1). Excessive activation of autophagy had been shown to aggravate muscle wasting by removing a portion of cytoplasm, proteins, and organelles (115). In contrast, inhibition of autophagy has been shown to result in muscle degeneration and weakness (19). The exact role of these processes in regulating muscle mass still remains poorly understood in humans.

Despite several lines of evidence indicate an association between mitochondrial capacity and sarcopenia, limited human studies directly investigated the relationship between mitochondrial capacity with muscle function and physical performance in very old adults. Recent evidence has shown that lower mitochondrial capacity and efficiency is associated with reduced physical performance in older adults (22). Similarly, high-functioning elderly individuals have been shown to maintain muscle mass and mitochondrial capacity, while low-functioning elderly individuals demonstrate decreased muscle mass and mitochondrial function in comparison to young individuals (14). These results suggest an association of mitochondria with physical function. In the present study, the association between mitochondrial respiratory capacity and myofiber cross-sectional area as a measure of muscle size, intramyocellular lipid content, and physical function was investigated. The findings of this study complement the previous findings by using a group of very old individuals (85-95yrs), in which decreases in muscle mass and physical function are

pronounced and the associations more likely to be established, by investigating the association between mitochondrial capacity and muscle size at the cellular level, and by comprehensively investigating the association between mitochondrial capacity and physical function.

1.3 SIGNIFICANCE

According to the U.S. Census Bureau, the elderly population will more than double by 2040 when compared to 2000 (116, 117). The current challenge for researchers and clinicians is how to expand active life expectancy, free of disability. The development of sarcopenia is commonly associated with reduced quality of life, and may ultimately lead to disability, institutionalization and mortality. Despite the clinical and social relevance of sarcopenia, the exact biochemical and molecular mechanisms involved in this process are not completely understood, especially in humans. In this ancillary study we proposed not only to investigate biochemical factors that may play a role in human sarcopenia, but also to investigate how these biological characteristics translate to muscle function and physical performance. This translational research initiative is an essential step to establish the possible role of mitochondria on age-related decreases in muscle mass and function, and it is the basis for the development of preventive approaches and effective rehabilitation for sarcopenia.

In Study 1 of this dissertation we investigated the association between mitochondrial capacity and chronological aging. While this has been a topic of investigation of several other studies, there is still confusion as to the precise contribution of mitochondrial capacity to aging muscle. Study 1 was conducted to elucidate issues still debated in the literature. First, measurements of mitochondrial function were investigated in permeabilized muscle fibers using

high-resolution respirometry, the gold standard method for the analysis of mitochondrial respiration. Second, we explored the specific contribution of potential confounders such as sex, race, BMI and cardiorespiratory fitness on the association between mitochondrial capacity and aging. Third, few human studies have investigated changes in mitochondrial dynamics with aging. In addition to investigate the association between proteins that mediate mitochondrial dynamics with aging, this study investigated the influence of BMI and cardiorespiratory fitness on mitochondrial quality control proteins.

In Study 2 of this dissertation, we investigated the association between mitochondrial capacity and sarcopenia. Despite *in vitro* and animal studies strongly suggest that mitochondria play a major role in the loss of muscle mass (19-21), human studies designed to investigate whether mitochondrial capacity associates with muscle mass, muscle fat, muscle strength and physical performance in very old subjects are lacking. A cohort of very old subjects (85-95yrs) was selected to participate in this study because at this age decreases in muscle mass and physical function are pronounced and associations with mitochondria more likely to be established.

The results of this dissertation study provide a better understanding of: 1. the association between mitochondrial capacity and aging; 2. the influence of adiposity and cardiorespiratory fitness on the association between mitochondrial capacity and aging; 3. the influence of mitochondrial fusion, fission and autophagy on muscle aging; and 3. the association of mitochondrial capacity with muscle size and physical function in an old population.

1.4 INNOVATION

The relationship between mitochondrial capacity and aging and sarcopenia was investigated in 108 skeletal muscle biopsies, including a group of very old (85-95yrs) subjects. Subjects participating in this study present a wide range of age, BMI, and cardiorespiratory fitness, which gives us the unique opportunity to investigate the association between mitochondria and aging, when controlled for body fat and physical fitness. While other *ex vivo* studies have examined the relationship between mitochondrial capacity and age, this is the first time, to our knowledge, that muscle biopsies are collected from a cohort of 41 very old individuals (85yrs and older).

This study utilized innovative techniques to investigate mitochondrial respiration. Several studies have demonstrated significant age-related alterations in mitochondrial capacity (11, 41, 49, 52). The majority of these studies were performed in isolated muscle mitochondria, a widely employed method when examining mitochondrial function in aging. However, this method does not preserve the complex structural arrangement of mitochondria, and there is a possibility that the isolation procedure exposes vulnerabilities in aged mitochondria that are not evident *in vivo* (97). A more recent approach utilized to examine skeletal muscle mitochondrial function is the dissection and permeabilization of the muscle sarcolemma yielding permeabilized myofiber bundles. This technique preserves mitochondrial interactions and morphology (25). A study specifically designed to compare mitochondrial respiration in aged muscles between isolated mitochondria and permeabilized muscle fiber bundles demonstrated that the mitochondrial isolation procedure exaggerated functional age-related impairments in sarcopenic skeletal muscle (97). In the present study, saponin-permeabilized fiber bundles were used to assess mitochondrial respiratory capacity.

Using human samples, we examined novel biochemical markers of mitochondrial capacity. Moreover, this study used comprehensive methods to give a broad understanding of mitochondrial capacity on the aging process. The importance of efficiency of mitochondrial quality control process for proper muscle capacity has been extensively highlighted in the recent years (24, 118). Using human samples, this study examined associations between quality control proteins, i.e. proteins responsible for mitochondrial fusion, fission and autophagy, and muscle aging. Additionally, this study explored the influence of BMI and cardiorespiratory fitness on these processes.

We investigated whether mitochondrial capacity associates with myofiber area and lipid content, muscle strength and physical performance in very old individuals. It is well established that aging is characterized by pronounced muscle atrophy and declines in functional capacity (1, 2). However, muscle mass and strength do not decline in the same proportion (119, 120), suggesting that the decrease in muscle mass does not alone explain decreases in muscle strength and physical function. Other physiological factors, such as neurological, vascular, and hormonal changes may also contribute to these declines (121). In this study, we aimed to investigate the contribution of mitochondrial capacity to declines in muscle strength and physical performance.

1.5 AIMS AND HYPOTHESIS

The overall purpose of this dissertation study was to determine the association of mitochondrial capacity with chronological age and sarcopenia. We hypothesized that mitochondrial capacity is associated with age, but this association is attenuated when controlled for body mass index and cardiorespiratory fitness. Furthermore, we hypothesized that lower mitochondrial capacity is

associated with increased intramyocellular lipid content, and decreased myofiber cross-sectional area, muscle strength and physical performance.

1.5.1 Specific aims and hypotheses of study 1

Determine the association between chronological age and mitochondrial capacity (respiration, content, and expression of quality control proteins) using a group of young (20-30yrs), middle-aged (31-60yrs) and older (61 and older) subjects, and to examine if there is an association between age and mitochondrial capacity after controlling for body mass index and cardiorespiratory fitness.

Hypothesis 1a: Mitochondrial respiration and content will be lower in the middle-aged and old groups when compared to young, and lower in the old group when compared to middle-aged group.

Hypothesis 1b: Mitochondrial fusion, fission and autophagy will be reduced in the middle-aged and old groups when compared to young, and reduced in the old group when compared to middle-aged group.

Hypothesis 1c: When controlled for body mass index and cardiorespiratory fitness the association between mitochondrial capacity and chronological age will be attenuated.

1.5.2 Specific aim and hypothesis of study 2

Determine the association between mitochondrial respiratory capacity and myofiber cross-sectional area, intramyocellular lipid content, and physical function in a group of very old subjects (85yrs and older).

Hypothesis: Mitochondrial respiratory capacity will be negatively associated with intramyocellular lipid content, and positively associated with myofiber cross-sectional area and physical function.

2.0 CARDIORESPIRATORY FITNESS AND ADIPOSITY, BUT NOT CHRONOLOGICAL AGE, INFLUENCE SKELETAL MUSCLE MITOCHONDRIAL RESPIRATION AND QUALITY CONTROL PROTEINS IN YOUNG, MIDDLE-AGED AND OLDER ADULTS

2.1 SUMMARY

Considerable debate continues to surround the concept of mitochondrial dysfunction in aging muscle. We tested the overall hypothesis that cardiorespiratory fitness (VO_2max) and adiposity (BMI) more strongly correlate with mitochondrial function and markers of mitochondria quality control, i.e., expression of fusion, fission and autophagy proteins, than age *per se*. **Methods:** Percutaneous biopsies of the vastus lateralis were obtained from sedentary young (n=14, 24±3yrs), middle-aged (n=24, 41±9yrs) and older adults (n=20, 78±5yrs). A physically active group of young adults (n=10, 27±5yrs) was studied as a control. Mitochondrial respiration was determined in saponin permeabilized fiber bundles. Fusion, fission and autophagy protein expression was determined by western blot. Cardiorespiratory fitness was determined by a graded exercise test. **Results:** Mitochondrial respiratory capacity and expression of fusion (OPA1 and MFN2) and fission (FIS1) proteins were not different among sedentary groups despite a wide age range (21 to 88yrs). Mitochondrial respiratory capacity and fusion and fission proteins were, however, negatively associated with BMI, and mitochondrial respiratory capacity was positively associated with cardiorespiratory fitness. The young active group had higher respiration, complex I and II respiratory control ratios, and expression of fusion and fission proteins. Finally, the expression of fusion, fission, and autophagy proteins were linked with mitochondrial respiration. **Conclusions:**

Mitochondrial respiration and markers of mitochondrial dynamics (fusion and fission) are not associated with chronological age *per se*, but rather are more strongly associated with BMI and cardiorespiratory fitness.

2.2 INTRODUCTION

Age-associated decline in skeletal muscle mitochondrial capacity has been extensively studied as an underlying factor for sarcopenia (14, 62), a condition characterized by a progressive loss of muscle mass and strength (2), and slower walking speed (22) and fatigability (122). However, while numerous cross-sectional human studies have demonstrated decreases in mitochondrial capacity with chronological age (11, 13, 41, 52, 55, 123), several others have failed to observe these changes (17, 61, 62, 124, 125). The inconsistent results may be partially due to the various definitions of the term "mitochondrial function" and the different approaches employed to assess mitochondrial function, including: respiration (22), ATP production (52, 61), mitochondrial permeability transition pore function, (62) and H_2O_2 emission (94). Furthermore, several of these investigations were performed in isolated mitochondria (11, 13, 41), which has been shown to exaggerate the observed deficit in mitochondrial function, when compared to measurements conducted on permeabilized myofibers (97). Moreover, most studies of mitochondrial respiration have not controlled for important covariates such as participant physical activity levels (13), adiposity (41), which could confound the relationship between mitochondrial capacity and age (17, 18, 52, 54). Collectively, these studies suggest that careful consideration of the methods and participant characteristics are needed when investigating age-related declines in mitochondrial function.

The mitochondrial reticulum is dynamic and undergoes constant remodeling through fusion, fission (126) and recycling by autophagy (127). Emerging evidence indicates that these processes are essential for the maintenance of a healthy mitochondrial pool (118). Mitochondrial fusion is regulated by proteins mitofusion-1 and -2 (MFN1 and MFN2) and optic atrophy-1 (OPA1). Fusion allows the components of the mitochondria to be exchanged and diluted, which is thought to prevent mutations in mitochondrial DNA caused by respiratory dysfunction (128). Mitochondrial fission is regulated by proteins fission-1 (FIS1) and dynamin-related protein 1 (DRP1). Fission segregates damaged portions of the mitochondria for removal by mitophagy (mitochondrial specific autophagy) (129). While the orchestration of fusion, fission, and autophagy are important for maintaining mitochondrial integrity, few human studies have examined mitochondrial quality control in aging and how they relate to other mitochondrial functions (14, 109).

The purpose of this study was to comprehensively assess mitochondrial respiratory characteristics across a wide age range using permeabilized myofibers, while controlling for participant cardiorespiratory fitness (VO_2max), and body mass index (BMI). We also sought to determine whether the expression of mitochondrial quality control proteins is affected by age. We hypothesized that mitochondrial respiration and expression of quality control proteins would be unaffected by chronological age *per se*, but would be more closely associated with age-related changes in BMI and cardiorespiratory fitness.

2.3 METHOD

2.3.1 Study design

This cross-sectional ancillary study utilized data from three cross-sectional research studies conducted at the University of Pittsburgh's Endocrinology and Metabolism Research Center. The first study was designed to investigate mechanisms associated with lipid-induced insulin resistence. Subjects were recruited through print advertisements in the Pittsburgh (PA) area, and were medically screened at the University of Pittsburgh's Clinical and Translational Research Center (CTRC). A total of 30 men and women (30-35yrs), consisting of 13 sedentary lean, 14 sedentary obese, and 14 young active participated in the parent study. Subjects in the young active group performed less than 1 structured physical activity session per week, while the young active group engaged in 3-5 structured physical activity sessions per week as determined by self-report. The active young control group predominantly engaged in endurance cycling and running. The second study was undertaken in middle-aged subjects to examine markers of nutritional stress in obesity and type 2 diabetes mellitus (T2DM). Subjects were recruited through print advertisements in the Pittsburgh (PA) area, and were medically screened at the University of Pittsburgh's CTRC. A total of 26 sedentary men and women (30-55yrs), consisting of 10 lean, 9 obese non-diabetic, and 7 obese with T2DM were included in the study. Only the subjects without T2DM participated in this ancillary study. The third study was conducted to examine the relationships between muscle function and physical performance with measurements of mitochondrial function in a cohort of older adults. A total of 21 men and 16 women with BMI between 21-31kg/m^2 were recruited. Subjects were included in the study if they were able to walk without the assistance of a device or another person; were free of basic activities of daily living (ADL) disability (defined as no

difficulty getting in and out of bed or chairs, and no difficulty walking across a small room). Specific exclusion criteria for old subjects include history of hip fracture, heart attack, angioplasty, or heart surgery within the past 3 months; cerebral hemorrhage within the past 6 months; stroke within the past 12 months; chest pain during walking in the past 30 days; symptomatic cardiovascular or pulmonary disease; regular pain, aching, or stiffness in the legs, hips, knees, feet, or ankles when walking; bilateral difficulty bending or straightening fully the knees; regularly taking Coumadin, Plavix, Aggrenox, Ticlid, or Agrylin/Xagrid.

2.3.2 Subjects

Sixty-eight men and women were included in this ancillary study. Subjects were eligible if they were between the ages of 20-90 years, were weight stable (+/-3kg in preceding 3 months), and in good general health. Subjects were excluded if they were participating in another interventional research study, had a chronic medical condition (such as heart disease, renal insufficiency, liver disease/cirrhosis, cancer, or any disease that requires anticoagulants and steroids), and were pregnant or breast-feeding. All subjects provided written informed consent and the study was approved by the University of Pittsburgh Institutional Review Board.

2.3.3 Groups

Subjects were recruited into one of the following groups: young sedentary (YS, 21-30yrs), middle-aged sedentary (MAS, 31-55yrs), older sedentary (OS, 70-88yrs), and a young active control group (YA, 21-33yrs) based on age and physical activity levels. The sedentary subjects performed ≤1 structured physical activity session per week of <20min, while the young active group engaged in

3-5 structured physical activity sessions per week as determined by self-report. The active young control group predominantly engaged in endurance cycling and running.

2.3.4 Clinical measures

Participants attended an assessment visit where they provided general demographics including date of birth, gender, and race. Additionally, data on subjects' body weight and height were collected and body mass index (BMI) of each participant were calculated (individual's body mass divided by the square of their height, kg/m^2) and used as an indication of body fat and obesity. Cardiorespiratory fitness (VO_2max) was determined as peak aerobic capacity measured using a graded exercise protocol, as previously described (22, 130). Briefly, subjects breathed through a mouthpiece connected to a two way breathing valve (Hans Rudolph, Kansas City, MO) during the test, and expired air was collected via open-circuit spirometry (AEI Technologies, Pittsburgh, PA) to determine oxygen consumption (VO_2) and carbon dioxide production (VCO_2). Heart rate, blood pressure, and ECG were recorded before, during, and immediately after the test. The test was terminated as per the criteria outlined in the American College of Sports Medicine exercise testing guidelines (131).

2.3.5 Skeletal muscle biopsy procedure

Percutaneous muscle biopsies were obtained at the University of Pittsburgh's Clinical Translational Research Center on a morning after an overnight fast. Participants were instructed not to perform physical exercise 48 hours prior to the biopsy procedure. Biopsy samples were obtained from the middle region of the vastus lateralis under local anesthesia (2% buffered

lidocaine) as described previously (132). A portion of the biopsy specimen (~10mg) was placed in ice-cold preservation buffer (BIOPS) (22) for analysis of mitochondrial respiration. The remaining muscle tissue were processed for histochemistry (~30 mg) or frozen in liquid nitrogen (~50mg) and stored at −80°C.

2.3.6 Mitochondrial respiratory capacity

Immediately after the biopsy procedure, permeabilized myofiber bundles were prepared as previously described (22). Briefly, myofiber bundles were gently teased apart in a petri dish containing ice-cold BIOPS solution using 2 sharp tweezers and a dissecting microscope (Leica Microsystems, Heerbrugg, Switzerland). The myofiber bundles were then permeabilized with saponin (2 mL of 50 ug/mL saponin in BIOPS solution) for 20 minutes at 4°C on an orbital shaker, and then washed twice for 10 minutes at 4°C with MIR05 respiration medium (22).

Mitochondrial respiratory capacity in permeabilized myofibers was evaluated by high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) through the sequential addition of substrates, inhibitor and uncoupler (figure 2). Measurements were performed in duplicate, at 37°C, in the range of 230-150 nmol O_2/ml. LEAK (L_I) respiration was determined through the addition of pyruvate (5mM), malate (2mM) and glutamate (10mM). ADP (5mM) was added to elicit complex I supported oxidative phosphorylation (OXPHOS) (P_I). Cytochrome c (10μM) was added to check the quality of the muscle fiber preparation and assess the integrity of the outer mitochondrial membrane. Any sample that showed an increase in respiration of more than 15% with the addition of cytochrome c was not included in the final analysis. Succinate (10mM) was then added to elicit complex I+II supported OXPHOS (P_{I+II}). FCCP (1uM) was added to determine electron transfer system (ETS) capacity or maximal

uncoupled respiration. Finally, rotenone (1µM) was added to inhibit complex I supported OXPHOS respiration, and the remaining respiration revealed the maximal ETS capacity with complex II substrates only (E_{II}). Following the assay, myofiber bundles were recovered, dried, and weighted on an analytical balance (Mettler Toledo, XS105). Steady state O_2 flux for each respiratory state was determined and normalized to myofiber bundle dry weight using Datlab 4 software (Oroboros Instruments, Innsbruck, Austria).

Respiratory control ratios/factors were calculated to investigate intrinsic mitochondrial respiratory capacity. Since the different respiratory states were evaluated in the same myofiber bundle, and therefore same population of mitochondria, this approach is a valuable method to estimate mitochondrial function/quality independent of mitochondrial content, quality of myofiber bundle preparation, and unintentional variation in assay conditions (123). Respiratory acceptor control ratios were calculated as P_I/L_I and P_{I+II}/L_I. Considering maximal uncoupled respiration (E_{I+II}) as an internal measure of the ETS capacity, flux control ratios were calculated for leak respiration (L_I/E_{I+II}), complex I supported OXPHOS respiration (P_I/E_{I+II}), and complex I+II supported OXPHOS respiration (P_{I+II}/E_{I+II}). The results from the flux control ratios offer an estimation of the leak and OXPHOS capacity within the ETS capacity. Complex I (CI) and complex II (CII) control factors were calculated to investigate the relative change of O_2 flux in response to a transition of substrate availability in a defined coupling state. CI control factor corresponds to the difference between complex I+II supported OXPHOS respiration and E_{II} respiration. CII control factor corresponds to the difference between complex I+II supported OXPHOS respiration and complex I only supported OXPHOS respiration.

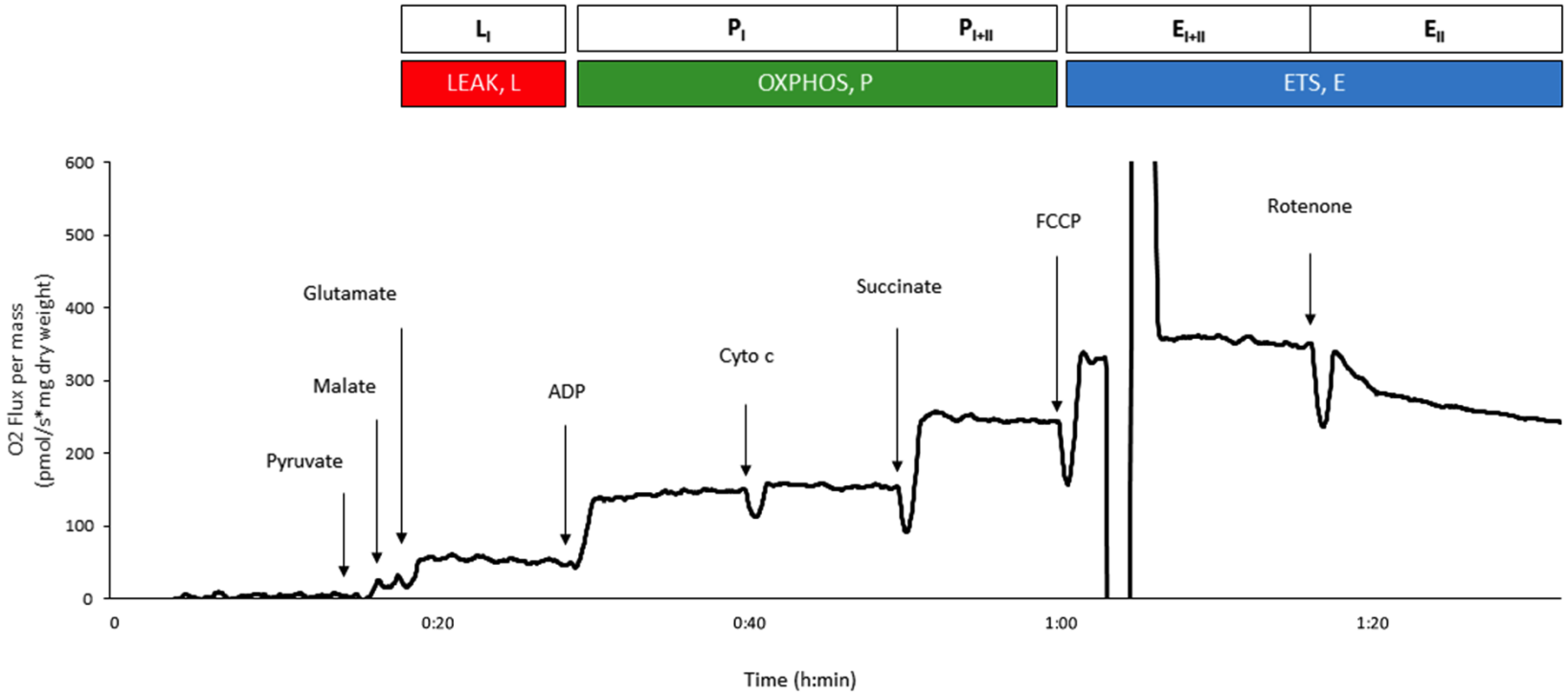

Figure 2. Representative oxygraph generated from the titration protocol.

2.3.7 Myofiber distribution and cross-sectional area

Histochemical analyses were performed on serial sections using methods previously described (22). Briefly, biopsy samples were sectioned (10 μm) on a cryostat (Cryotome E; Thermo Shandon, Pittsburgh, PA) at -20 °C and placed on individual pre-cleaned glass slides (Fisherfinest, Fischer Scientific, Pittsburgh, PA). Sections were incubated with primary antibodies for anti-human myosin heavy chain (MYH)-7 (type I myocytes), and MYH-2 (type IIa myocytes) (Santa Cruz Biotechnologies, Santa Cruz, CA) overnight at room temperature. Samples were then incubated with rhodamine (type I myocytes) and fluorescein (FITC) (type IIa myocytes) conjugated secondary antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA). Type IIx fibers remained unstained. Images were visualized using a Leica microscope (Leica DM 4000B; Leica Microsystems, Bannockburn, IL), digitally captured (Retiga 2000R camera; Q Imaging, Surrey, Canada), and analyzed using specialized software (Northern Eclipse, v6.0; Empix Imaging, Cheektowaga, NY).

2.3.8 Mitochondrial content and quality control proteins

Muscle homogenates were prepared as previously described (133). Proteins were separated by gel electrophoresis using a 4-20% gel (Bio-Rad, Mini-PROTEAN® TGX™ Precast Gel) and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in 5% non-fat milk, and incubated with the following primary antibodies overnight: DRP1 and OPA1 (BD Biosciences, San Jose, CA, 1:500), Fis1 (Enzo Life Sciences, Farmingdale, NY, 1:1000), Mfn2 and BNIP3 (Sigma Aldrich, St. Louis, MO, 1:500, 1:1000),

beclin-1 and LC3A/B (Cell Signaling, Danvers, MA, 1:1000), OXPHOS (MitoSciences, Eugene, OR, 1:1000) and anti-α tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, 1:1000). Membranes were then incubated in appropriate species-specific HRP-conjugated secondary antibodies (Cell Signaling, Danvers, MA). Protein bands were visualized using a chemiluminescence detection kit (Bio-Rad Laboratories, Hercules, CA) and gel documentation system (ChemiDoc XRS+; Bio-Rad, Hercules, CA). Protein bands were quantified by densitometry using the software ImageJ (National Institutes of Health, Bethesda, MD). Protein loading was controlled by normalizing bands of interest to α-Tubulin expression. Gel-to-gel variation was controlled for by using a standardized sample on each gel.

2.3.9 Power calculation

Hypotheses 1a and 1b: The primary outcome of this study was complex I+II supported OXPHOS respiration (P_{I+II}) and was the basis for power calculation. A previous study performed in our laboratory found that a group of old subjects present a mean ± SD value of 174 ± 68pmol/s*mg dry weight for this variable (22). Using these published values for P_{I+II} respiration, an alpha level of 0.05, 2-tailed test, and at least 20 subjects per age group, this study has 81% power to detect difference in P_{I+II} respiration between groups as small as 25%. Power calculation was performed using G*Power 3.1 software.

Hypothesis 1c*:* To test hypothesis 1c at an alpha level of 0.05, with a total sample size of 68 subjects, this study has 82% power to detect an R-square of 15% attributed to 4 independent variables (Sex, race, BMI and cardiorespiratory fitness) and aging accounting for an additional 10% of the variability. Power calculation was performed using SPSS SamplePower 3.0 software.

2.3.10 Statistical analysis

In order to test group differences Shapiro-Wilk and Levene's tests were initially performed to assess normality of data and equality of variances, respectively. If assumptions of normality and homogeneity of variances were met, a One-way ANOVA followed by post-hoc Tukey tests were performed to compare differences across groups. If assumptions were not met, comparisons between groups were performed using the non-parametric Kruskal-Wallis test, followed by Mann-Whitney U tests. The distribution of sex and race across groups were determined by Chi-squared test. Multiple linear regressions were performed to test whether BMI and cardiorespiratory fitness play a role on the association between mitochondrial capacity and age. Sex and race were also tested as potential covariates. Bivariate correlations were first tested to investigate relationships between mitochondrial variables and age, with the covariates. Each of the covariates were controlled in the regression model if they significantly associated with the dependent and independent variable. Assumptions for the use of multiple linear regression were checked. Analyses were performed using IBM SPSS v22.0 software (Armonk, NY). Statistical significance was established at $p \leq 0.05$.

2.4 RESULTS

2.4.1 Participant characteristics

The participant characteristics can be found in Table 1. A total of 68 individuals with a wide range of age (20-88yrs), BMI (19-47kg/m^2) and VO_2max (1.08-5.04L/min) participated in this study.

There were no baseline differences in sex and race among groups ($p>0.05$) tested by the chi-squared test. However, the ratio for gender and race were different between the groups. The YS and MAS groups had a higher weight and BMI than the YA and OS groups ($p<0.05$). Cardiorespiratory fitness was the highest in the YA group and lowest in the OS group ($p<0.05$).

Table 1. Subject characteristics of study 1

	Young Active (YA)	**Young Sedentary (YS)**	**Middle-aged Sedentary (MAS)**	**Older Sedentary (OS)**
N	10	14	24	20
Sex (ratio)	4M/6F	5M/9F	6M/18F	12M/8F
Race (ratio)	10C	9C/3AA/2O	15C/6AA/1A/2O	19C/1AA
Age (years)	27±5	24±3	41±9**	78±5***
Weight (kg)	62.3±7.4	85.1±28.5*	86.7±22.2*	70.6±12.4
BMI (kg/m^2)	21.4±1.2	29.2±8.0*	30.8±7.5*	25.9±3.0****
VO_2max (L/min)	3.50±0.93	2.59±0.79*	2.27±0.81*	1.47±0.45***

Values are Mean±SD, n=10-24 in each group.* P<0.05 vs. YA, ** P<0.05 vs. YA and YS, *** P<0.05 vs. YA, YS and MAS, **** P<0.05 vs. MAS. Abbreviations: M (male), F (female), C (caucasian), AA (african american), A (asian), O (other), and BMI (body mass index).

2.4.2 Myofiber type and cross-sectional area

The YA group had a higher percentage of type I fibers, and a lower percentage of type II fibers, compared to the YS, MAS and OS groups ($p<0.05$, Table 2). Additionally, the YA and OS groups presented a lower percentage of type IIx fibers compared to the YS and MAS groups ($p<0.05$, Table 2). There were no significant differences in cross-sectional area for any fiber type among groups ($p>0.05$).

Table 2. Myofiber type distribution and cross-sectional area

	Young Active (YA)	Young Sedentary (YS)	Middle-aged Sedentary (MAS)	Older Sedentary (OS)
Fiber Type (%)				
Type I	60±9	46±10*	41±11*	47±16*
Type II	40±9	54±10*	59±11*	53±16*
Type IIa	37±10	37±8	41±8	47±14
Type IIx	4±3	17±10**	18±8**	7±7
Cross-Sectional Area (μm^2)				
Type I	4122±1110	4046±1202	4379±1304	3934±1291
Type II	3716±913	4092±1060	4248±1333	3141±1492
Type IIa	3586±785	3773±917	3990±1337	3201±1302
Type IIx	4023±1288	4484±1534	4506±1557	2931±1894

Values are Mean±SD, n=5-18 in each group. * P<0.05 vs. YA, ** P<0.05 vs. YA and OS.

2.4.3 Mitochondrial respiration

When examining only the sedentary groups, there were no age-related deficits in mitochondrial respiration (Figure 3A, p>0.05). The sedentary groups presented lower L_I, P_I, P_{I+II}, E_{I+II}, and E_{II} respiration compared to the YA group (Figure 3A; p<0.05). Respiratory acceptor control ratios were not different among the groups (p>0.05; P_I/L_I: 5.3±2.4, P_{I+II}/L_I: 9.7±4.3). While L_I/E_{I+II} and P_I/E_{I+II} flux control ratios did not differ across groups, we found that the OS group had higher P_{I+II}/E_{I+II} flux control ratio when compared to MAS group (Figure 3B; p<0.05). CI and CII control factors were both reduced in the sedentary groups compared to the YA group (Figure 3C; p<0.05).

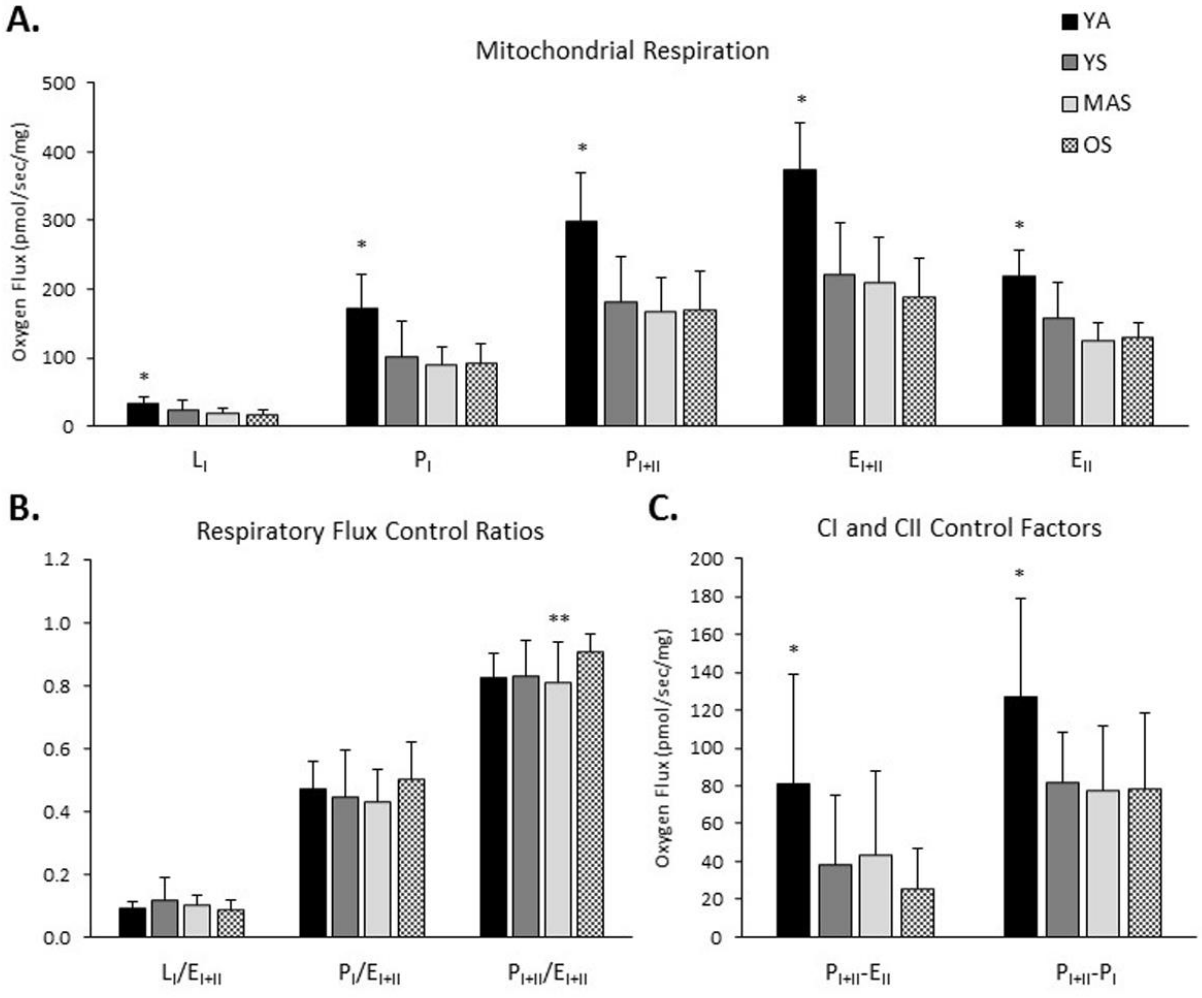

Figure 3. Mitochondrial respiratory capacity in young, middle-aged and older individuals.

(A) Mitochondrial respiration consisting of complex I supported Leak (L_I) respiration, complex I supported OXPHOS (P_I) respiration, complex I+II supported OXPHOS (P_{I+II}) respiration, maximal electron transfer system (ETS) capacity with substrates for complex I and II (E_{I+II}), and ETS with substrates for complex II (E_{II}). **(B)** Respiratory flux control ratios were determined as an estimation of leak and OXPHOS capacity within the ETS capacity. **(C)** Complex I (CI) control factor measured as P_{I+II} minus E_{II} respiration, and Complex II (CII) control factor measured as P_{I+II} minus P_I. Data presented as Mean and SD; n=9-20 per group. *p<0.05 vs. YA, **p<0.05 vs. OS.

While we observed that mitochondrial respiration was not influenced by sex (Figure 4), race (Figure 5) or fiber type percentage (Table 3) ($p>0.05$), we found that it was negatively associated with BMI and positively associated with cardiorespiratory fitness (VO_2max) (Table 4 and Figure 6; $p<0.05$). We then examined the association between mitochondrial respiration and age when controlled for these covariates (BMI and VO_2max) (Table 6). We found that BMI and VO_2max combined to explain 31-45% of the variance of mitochondrial respiration ($p<0.05$), depending on the respiratory state, with age explaining only an additional 1.4-6.8% of the variance in mitochondrial respiration (Table 6; $p<0.05$ for L_I and E_{I+II}, and $p>0.05$ for P_I and P_{I+II}). The YA subjects are likely driving these associations since they had higher VO_2max values and lower BMI when compared to the other groups (Table 1). We next examined the association between mitochondrial respiration and age when controlled for BMI and VO_2max in the sedentary subjects only. While BMI and VO_2max combined explained between 12-27% of the variance in mitochondrial respiration ($p<0.05$), we found that when these covariates are controlled for, age does not explain a significant percent of variance in mitochondrial respiration (Table 6, $p>0.05$). Collectively, these findings suggest that BMI and cardiorespiratory fitness, rather than chronological age *per se*, are more influential to mitochondrial respiratory capacity.

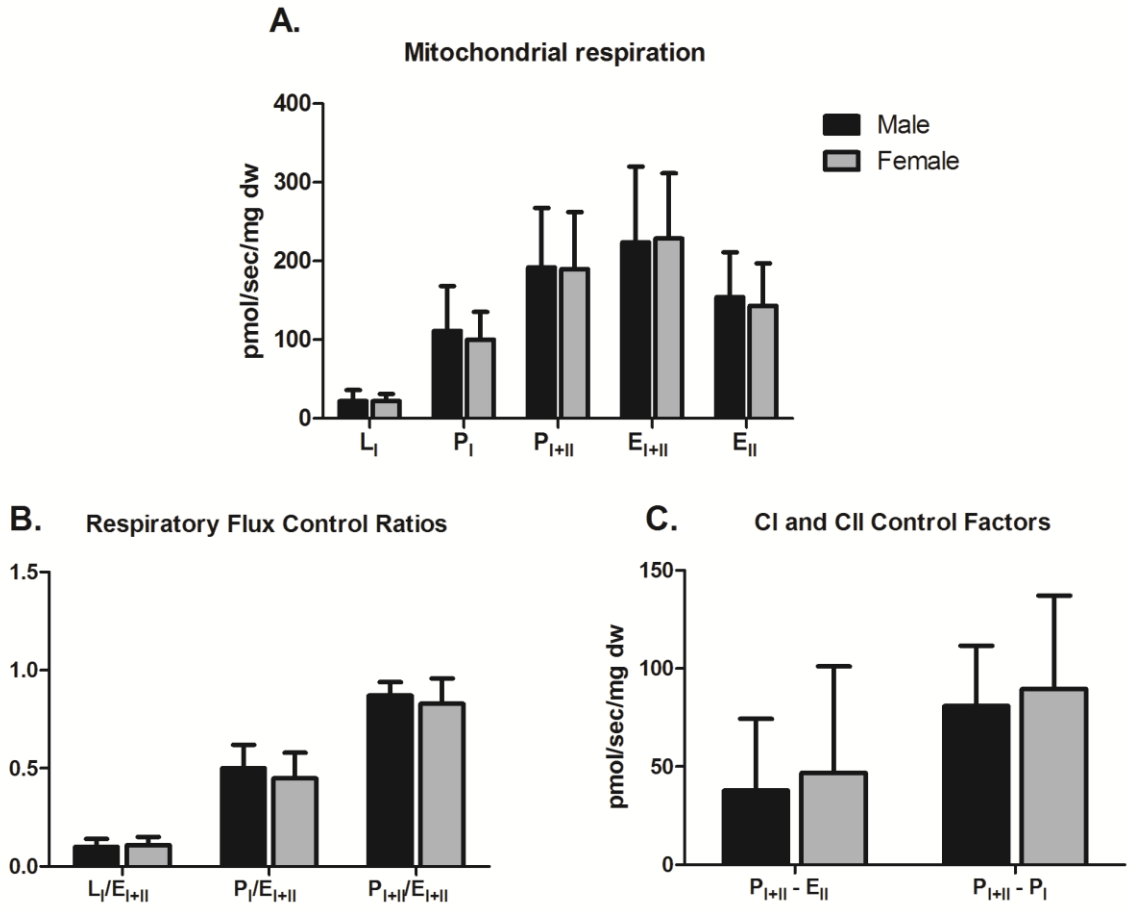

Figure 4. Mitochondrial respiratory capacity in males and females.

(A) Mitochondrial respiration consisting of complex I supported Leak (L_I) respiration, complex I supported OXPHOS (P_I) respiration, complex I+II supported OXPHOS (P_{I+II}) respiration, maximal electron transfer system (ETS) capacity with substrates for complex I and II (E_{I+II}), and ETS with substrates for complex II (E_{II}). **(B)** Respiratory flux control ratios were determined as an estimation of leak and OXPHOS capacity within the ETS capacity. **(C)** Complex I (CI) control factor measured as P_{I+II} minus E_{II} respiration, and Complex II (CII) control factor measured as P_{I+II} minus P_I. Data presented as Mean and SD; n=25 for male and n=34 for female.

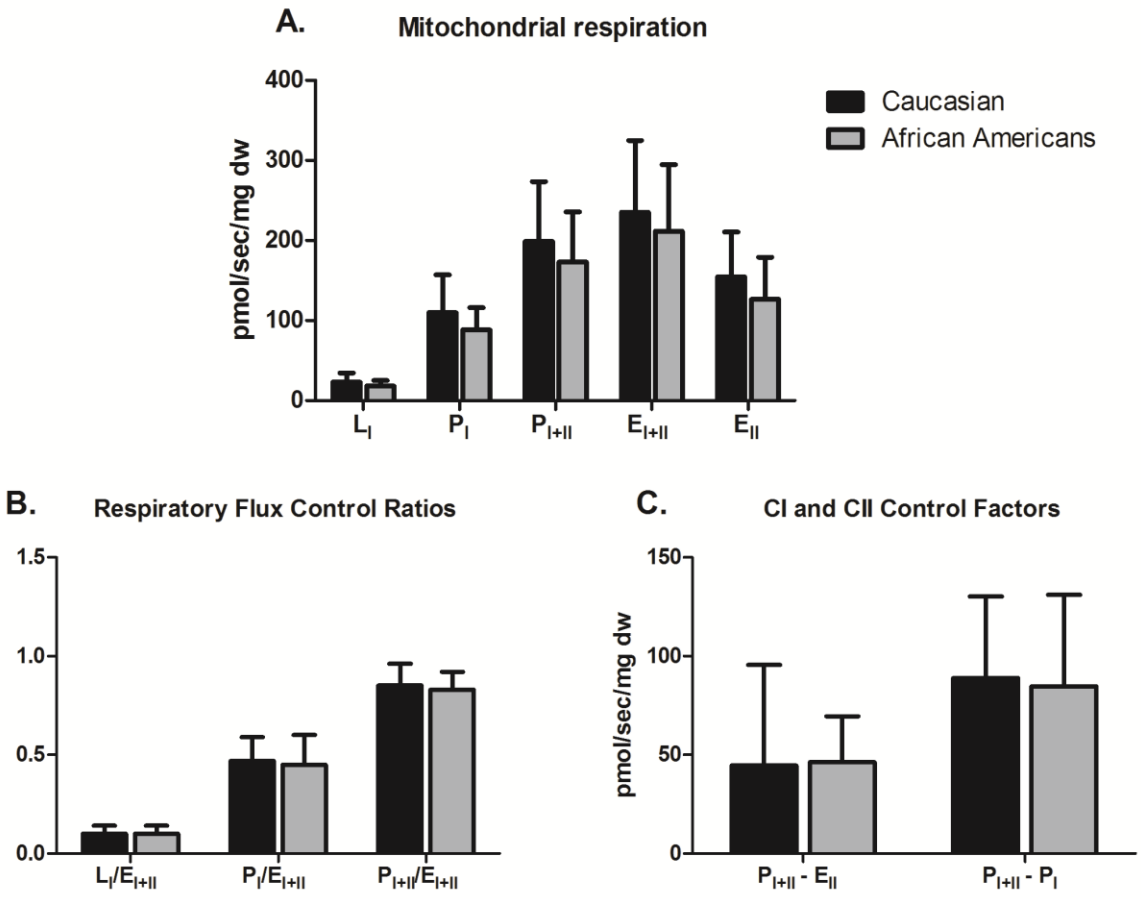

Figure 5. Mitochondrial respiratory capacity in Caucasians and African Americans.

(A) Mitochondrial respiration consisting of complex I supported Leak (L_I) respiration, complex I supported OXPHOS (P_I) respiration, complex I+II supported OXPHOS (P_{I+II}) respiration, maximal electron transfer system (ETS) capacity with substrates for complex I and II (E_{I+II}), and ETS with substrates for complex II (E_{II}). **(B)** Respiratory flux control ratios were determined as an estimation of leak and OXPHOS capacity within the ETS capacity. **(C)** Complex I (CI) control factor measured as P_{I+II} minus E_{II} respiration, and Complex II (CII) control factor measured as P_{I+II} minus P_I. Data presented as Mean and SD; n=47 for Caucasian and n=8 for African Americans.

Table 3. Bivariate correlation of mitochondrial respiration and fiber type distribution

	L_I	P_I	P_{I+II}	E_{I+II}	E_{II}
Type I	0.118	0.025	0.154	0.178	0.234*
Type IIa	-0.205	0.123	-0.055	-0.067	-0.098
Type IIx	0.070	-0.068	-0.095	-0.052	-0.123
Type II	-0.133	-0.017	-0.193	-0.194	-0.259

* $p<0.05$, n= 43. Abbreviations: L_I (complex I supported leak respiration), P_I (complex I supported OXPHOS respiration), P_{I+II} (complex I+II supported OXPHOS respiration), E_{I+II} (maximal electron transfer capacity with substrates for complex I and II), and E_{II} (maximal electron transfer capacity with complex II substrates only).

Table 4. Bivariate correlation of mitochondrial respiration with age, BMI and VO_2max

		L_I	P_I	P_{I+II}	E_{I+II}	E_{II}
With YA	**Age**	-0.279*	-0.198*	-0.227*	-0.305*	-0.195*
	BMI	-0.236*	-0.428*	-0.358*	-0.354*	-0.376*
	VO_2max	0.300*	0.274*	0.281*	0.311*	0.258*
Without YA	**Age**	-0.200*	-0.011	-0.092	-0.247	-0.030
	BMI	-0.100	-0.382*	-0.326*	-0.301*	-0.243*
	VO_2max	0.225*	0.142	0.211	0.287*	0.103

* $p<0.05$; n= 59 for analysis with YA and n=50 for analysis without YA. Abbreviations: YA (Young Active subjects), BMI (body mass index), L_I (complex I supported leak respiration), P_I (complex I supported OXPHOS respiration), P_{I+II} (complex I+II supported OXPHOS respiration), E_{I+II} (maximal electron transfer capacity with substrates for complex I and II), and E_{II} (maximal electron transfer capacity with complex II substrates only).

Table 5. Bivariate correlation of age, BMI and cardiorespiratory fitness

	Age	**BMI**	**VO_2max**
Age	1	0.057	-0.525*
BMI	-	1	-0.082
VO_2max	-	-	1

* $p<0.05$, n=67-68.

Table 6. Multiple linear regression investigating the association of BMI, VO_2max, and age on mitochondrial respiration

	Predictor	L_I		P_I		P_{I+II}		E_{I+II}	
		R^2	p	R^2	p	R^2	p	R^2	p
With YA	**BMI+VO_2max**	0.311	<0.001*	0.453	<0.001*	0.359	<0.001*	0.411	<0.001*
	Age after controlled for BMI+VO_2max	0.068	0.020*	0.003	0.540	0.014	0.289	0.052	0.029*
Without YA	**BMI+VO_2max**	0.271	0.001*	0.207	0.005*	0.122	0.05*	0.164	0.018*
	Age after controlled for BMI+VO_2max	0.046	0.093	0.003	0.669	0.002	0.751	0.052	0.093

*p<0.05, n= 59 for analysis with YA and n=50 for analysis without YA. Abbreviations: YA (Young active subjects), BMI (body mass index), L_I (complex I supported leak respiration), P_I (complex I supported OXPHOS respiration), P_{I+II} (complex I+II supported OXPHOS respiration), and E_{I+II} (maximal electron transfer capacity with substrates for complex I and II).

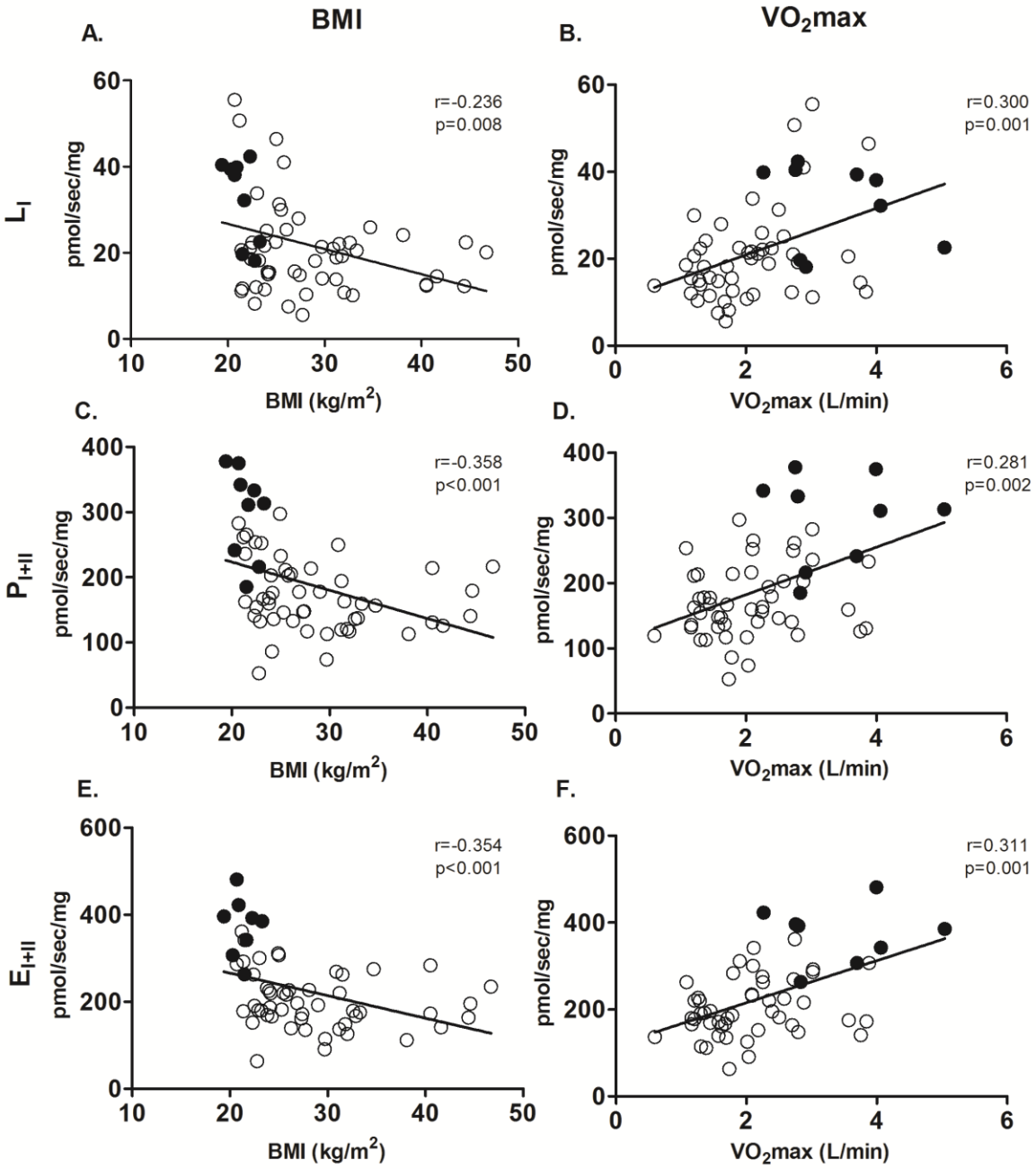

Figure 6. Association between mitochondrial respiration with BMI and VO_2max.

Bivariate correlation between complex I supported Leak (L_I) respiration with BMI **(A)** and VO_2max **(B),** complex I+II supported OXPHOS (P_{I+II}) respiration with BMI **(C)** and VO_2max **(D),** and maximal electron transfer capacity (E_{I+II}) with BMI **(E)** and **(F)** VO_2max. Black circles represent young active subjects and white circles represent sedentary subjects, n=58-59.

2.4.4 Protein expression

The sedentary groups had reduced levels of OPA1 compared to the YA group (Figure 7A; $p<0.05$). Additionally, the sedentary MAS and OS groups presented lower levels of MFN2, FIS1 and DRP1 protein expression compared to YA (Figures 7A, B; $p<0.05$). The MAS group had lower expression of beclin-1 compared to the YA (Figure 7C; $p<0.05$), while there were no differences in BNIP3 and LC3-II/LC3-I ratio between the groups (Figure 7C; $p>0.05$). When examining only the sedentary groups, there were no group differences for the proteins measured, except for DRP1. MAS group had a reduced level of DRP1 compared to YS (Figure 7B; $p<0.05$).

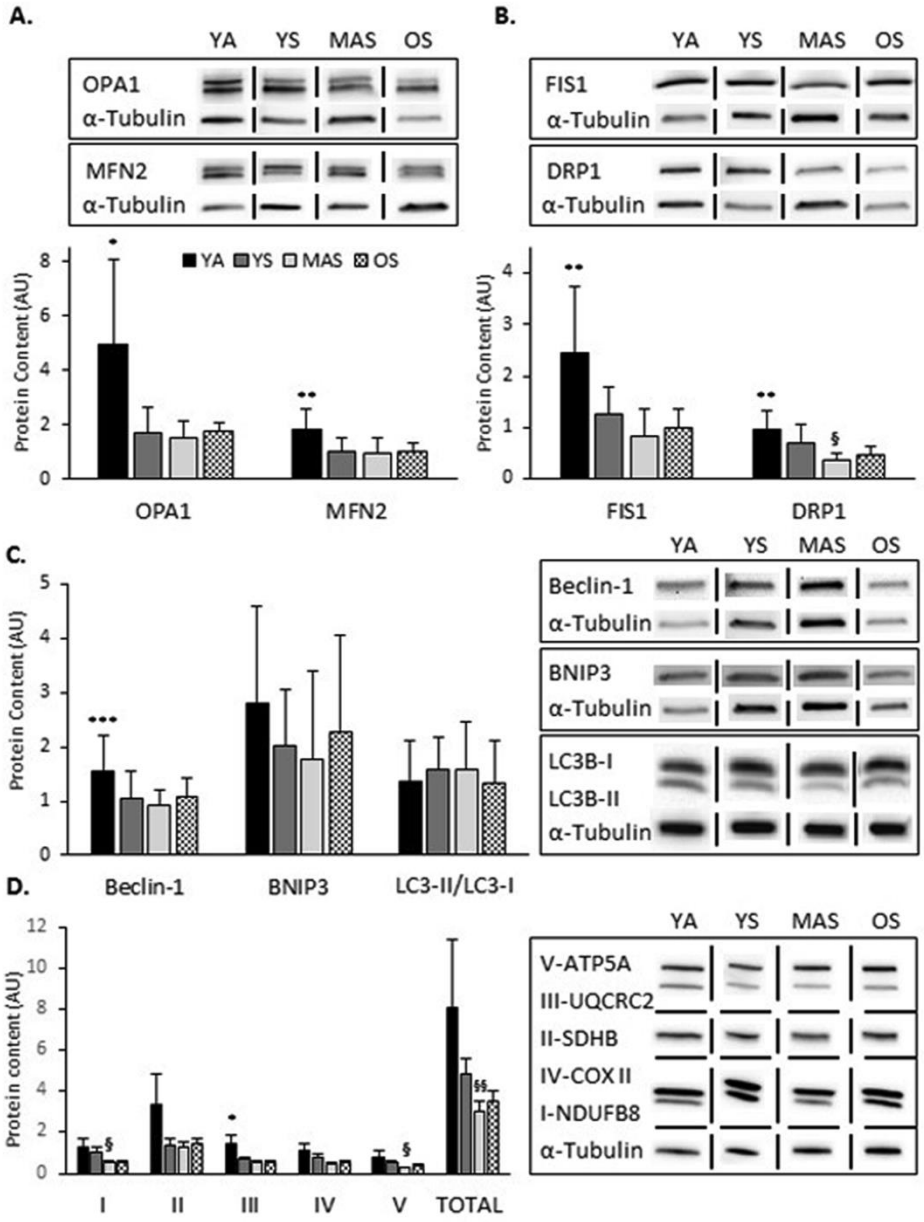

Figure 7. Expression of quality control proteins and OXPHOS complexes.

Western blot analysis of; **(A)** mitochondrial fusion proteins; **(B)** fission proteins; **(C)** autophagy proteins; and **(D)** mitochondrial content (OXPHOS, complexes I-V and Total). Values were normalized to α-tubulin and a loading control. Data are presented as Mean and SD for figures A, B, and C, and as Mean and SE for figure D. Results are expressed as arbitrary units (AU); Vertical and horizontal dividing lines were used in the western blot images to present lanes from the same gel that were reorganized for presentation purpose; n=5-17 per group. *p<0.05 vs. YS, MAS, and OS **p<0.05 vs. MAS and OS, ***p<0.05 vs. MAS, § p<0.05 vs. YS, §§ p<0.05 vs. YA and YS.

Quality control protein expression was not associated with age (Table 7). The expression of mitochondrial fusion proteins OPA1 and MFN2 (Figure 8A, B), fission proteins FIS1 and DRP1 (Figure 8C, D), and autophagy proteins Beclin-1 and BNIP3 correlated negatively with BMI (Table 7, $p<0.05$). Similarly, among the sedentary groups only, we found that OPA1, MFN2, FIS1 and Beclin-1 were also negatively correlated with BMI (Table 7, $p<0.05$). The findings of this study suggest that the capacity for mitochondrial fission, fusion and autophagy is preserved during chronological aging *per se*, but it is influenced by cardiorespiratory fitness and BMI. Interestingly, both fission and fusion proteins correlated with BMI, suggesting that increased BMI influences the capacity for both processes in sedentary individuals. Additionally, expression of fusion, fission, and autophagy proteins were correlated with several mitochondrial respiratory states (Table 8, $p<0.05$).

Table 7. Bivariate correlation of quality control proteins with age, BMI and VO_2max

		OPA1	MFN2	FIS1	DRP1	Beclin-1	BNIP3	LC3II/I
With YA	**Age**	-0.039	-0.111	-0.081	-0.223*	-0.058	-0.025	-0.060
	BMI	-0.360*	-0.378*	-0.320*	-0.288*	-0.352*	-0.186	-0.086
	VO_2max	0.068	0.136	0.117	0.135	0.078	-0.077	-0.087
Without YA	**Age**	0.147	0.007	0.021	-0.068	0.027	0.006	-0.108
	BMI	-0.264*	-0.310*	-0.242*	-0.166	-0.460*	-0.171	-0.200
	VO_2max	-0.169	-0.005	0.002	-0.068	-0.059	-0.162	-0.043

* $p<0.05$, n= 35-42 for analysis with YA and n=30-36 for analysis without YA. Abbreviations: YA (Young active subjects) and BMI (body mass index).

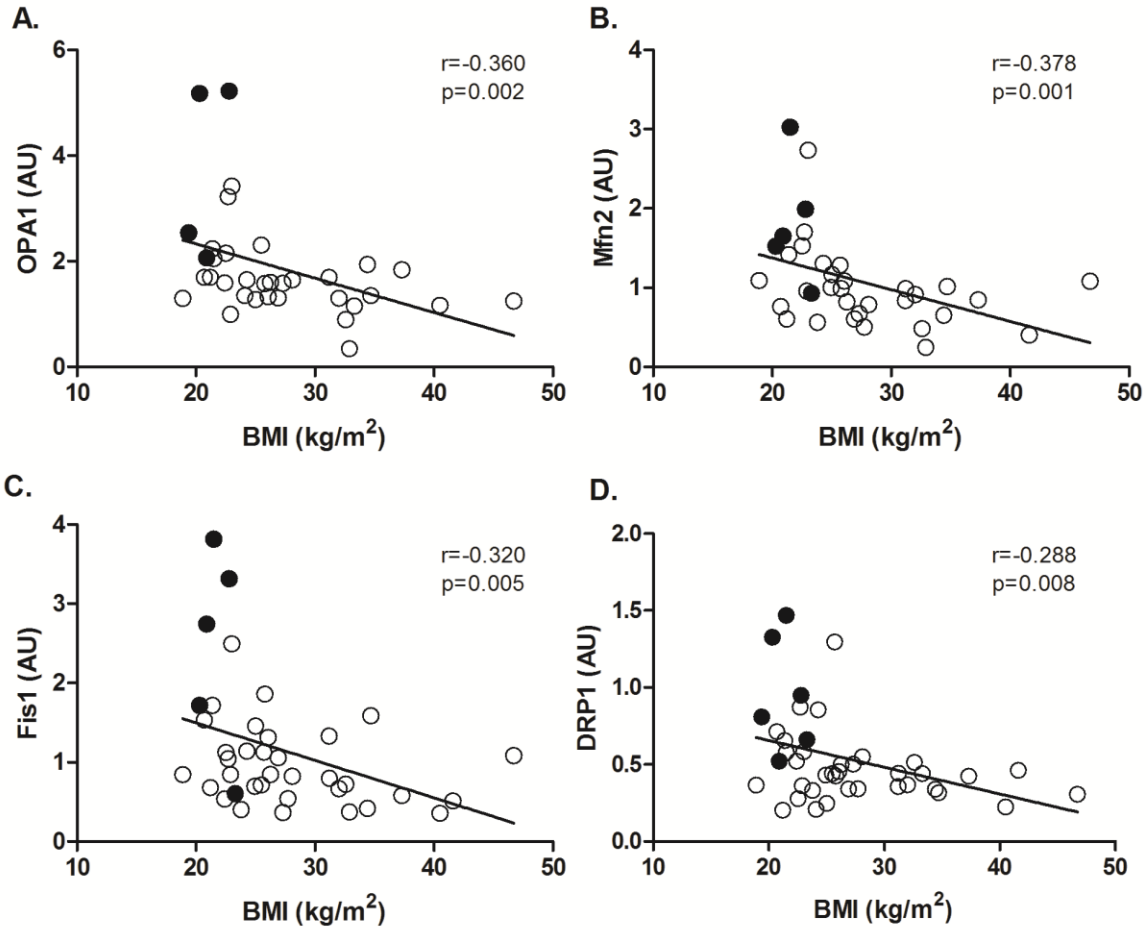

Figure 8. Association between mitochondrial fusion and fission proteins with BMI.

Bivariate correlation of mitochondrial proteins **(A)** OPA1, **(B)** MFN2, **(C)** FIS1 and **(D)** DRP1 with BMI. Results are expressed as arbitrary units (AU). Black circles represent young active subjects and white circles represent sedentary subjects n=34-41.

Table 8. Bivariate correlation of mitochondrial respiration and quality control proteins

	L_I	P_I	P_{I+II}	E_{I+II}	E_{II}
OPA1	0.232	0.421*	0.347*	0.305*	0.301*
Mfn2	0.261*	0.227	0.305*	0.265*	0.266*
Fis1	0.278*	0.302*	0.242	0.217	0.274*
DRP1	0.064	0.367*	0.306*	0.161	0.138
Beclin1	0.276*	0.384*	0.333*	0.314*	0.262*

* $p<0.05$, n=35-42. Abbreviations: L_I (complex I supported leak respiration), P_I (complex I supported OXPHOS respiration), P_{I+II} (complex I+II supported OXPHOS respiration), E_{I+II} (maximal electron transfer capacity with substrates for complex I and II), and E_{II} (electron transfer capacity with substrates for complex II only).

The expression of complex I and V protein was lower in the MAS compared to YS group (Figure 7D; $p<0.05$). Complex III protein was lower in the sedentary groups compared to YA (Figure 7D; $p<0.05$). Total OXPHOS protein content was lower in the MAS when compared to the YA and YS groups (Figure 7D; $p<0.05$).

2.5 DISCUSSION

Deeper interrogation into mitochondrial function in aging is crucial to better delineate mitochondria as a feasible therapeutic target for sarcopenia and loss of physical function. We measured several aspects of substrate-supported respiration and respiratory control parameters, along with markers of electron transport chain content and quality control in human muscle ranging from 21 to 88 years of age. Our main finding was that maximal rates of mitochondrial respiration, and capacity for fusion, fission, and autophagy are not altered with chronological age

per se, but that cardiorespiratory fitness and BMI play a more dominant role in explaining the apparent age associated changes.

There have been many reports describing lower mitochondrial oxidative capacity with aging (11, 13, 41, 52, 55, 123), however, the majority have not controlled for important covariates such as cardiorespiratory fitness (13, 123) and BMI (41, 55, 123). Many have also used isolated mitochondria (11, 13, 41). Tonkonogi et al. found lower mitochondrial respiration with age, when compared 8 young (22-31yrs) and 7 old (61-86yrs) sedentary subjects matched for physical activity levels (55). Despite matching groups for the degree of physical activity, the older subjects had lower cardiorespiratory fitness by VO_2max (55). However, no information on participants' body composition was presented, which could contribute and partially explain the lower mitochondrial respiration in the older group. Similarly, a recent study using permeabilized myofibers also reported decreased respiratory capacity and coupling control in older adults (62±8yrs, n=31) compared to young (28±7yrs, n=24) subjects (123). However, direct measurements of participants' cardiorespiratory fitness were not collected, and the groups were unbalanced for BMI with the older group presenting with increased body fat and higher BMI when compared to the young group. Without controlling for these covariates it is difficult to confirm whether the lower levels of mitochondrial respiration observed in the old group are due to age *per se,* or are related to differences in cardiorespiratory fitness and body composition.

A number of studies performed in permeabilized myofibers have failed to find age-related changes in mitochondrial respiration (17, 124, 125). Hutter et al. (2007) reported full preservation of mitochondrial respiration in older subjects when compared to young men and women (17). While cardiorespiratory fitness was not controlled for in the study, a significant correlation

between percent body fat and mitochondrial respiration was observed, suggesting that mitochondrial respiration is more related to changes in body composition rather than age. Similarly, Larsen et al. (2012) reported no differences in mitochondrial respiration between 10 young (23±3yrs) and 10 middle-aged (53±3yrs) subjects matched for VO_2max and BMI (124).

Our study is the first to investigate associations between mitochondrial respiration and quality control in a reasonably large number of subjects (n=68) with a wider range of age (21-88yrs) and BMI (19-47kg/m^2), and to examine the influence of cardiorespiratory fitness and BMI on these associations. We found that cardiorespiratory fitness (VO_2max) and adiposity (BMI) heavily influenced mitochondrial respiratory capacity, and that when controlled for these variables no age-related changes in mitochondrial respiration was observed in the sedentary groups. These observations are in line with and extend the findings that physical activity levels and cardiorespiratory fitness are confounders of the relationship between age and mitochondrial respiration (18, 52, 124). The influence of BMI is also supported by others that found reduced capacity for lipid oxidation and activity of mitochondrial enzymes in obese subjects (68, 69). The relationship between BMI and reduced mitochondrial capacity may be due to the interplay between adiposity and the development of insulin resistance and mitochondrial dysfunction (134) or simply to the lack of physical activity and/or other lifestyle factors. More research is needed to elucidate the intricacies of this relationship.

We also examined mitochondrial respiratory control ratios/factors. This approach permits a qualitative comparison of respiratory performance independent of mitochondrial content, methods used to prepare the mitochondria, and variations in assay conditions (135). We found higher CI and CII control factors in the YA when compared to the sedentary groups. These control factors express the relative change of O_2 flux in response to a transition of substrate availability in

a defined coupling state (136). CI control factor is experimentally induced when rotenone (complex I inhibitor) is added to CI+II-linked respiration and CII control factor when succinate is added to Complex I-linked respiration. The sedentary groups had a reduced CI and CII control factors, compared to the YA group, suggesting that regular physical activity is linked with oxygen flux through complex II and the individuals' ability to increase O_2 flux through complex II in response to a transition of substrate availability, independent of overall mitochondria content.

To determine the association between age and capacity for mitochondrial quality control, we measured several proteins that mediate mitochondrial fusion, fission, autophagy and mitophagy. The expression of these proteins were not influenced by age in the sedentary group while the young active individuals had elevated levels of fusion and fission proteins. These findings suggest that physical activity is linked with the capacity for mitochondrial turnover. Our results are in line with other studies that reported no age-related differences in mitochondrial fusion and fission (14, 137, 138) and autophagy proteins (139) suggesting that these processes are still intact in healthy sedentary individuals. Some studies also included an exercise intervention that reduced LC3BII/LC3BI in both old and young after an acute bout of resistance exercise (139) and reduced FIS1 and MFN after acute aerobic exercise (137). Additionally, aerobic exercise training resulted in an elevation of MFN1, MFN2 and FIS1 protein levels in both young and old subjects (138). Taken together, these findings suggest that mitochondrial quality control proteins are uninfluenced by age and respond to both acute aerobic and resistance exercise and chronic aerobic exercise training. Recent animal studies have expanded on the contribution of these processes in regulating exercise adaptations. Caffin et al. found OPA1 deficient mice have impaired exercise training induced mitochondrial biogenesis (140) and Lira et al. report that an increase in basal autophagy is required for metabolic adaptations induced by exercise training (141). These studies

indicate that mitochondrial quality control processes are likely involved and important for the remodeling of mitochondria in response to exercise. Interestingly, we also found significant associations between the expression of fusion, fission, and autophagy proteins with BMI and several mitochondrial respiratory states. These results suggest that alterations in mitochondrial quality likely play a significant role in maintaining mitochondrial function.

We acknowledge that multiple comparisons were performed in this study in order to investigate the association between chronological age and several respiratory states (total of 11) and mitochondrial proteins (7 proteins related to quality control and 5 complexes of mitochondrial content). However, we opted to not statistically control for multiple comparisons due to the exploratory nature of the study.

2.5.1 Limitations

While our results suggest that body mass index and VO_2max are more strongly associated with changes in mitochondrial capacity than age per se, we acknowledge that the use of a cross-sectional design limits the interpretation of the findings. Future longitudinal studies that investigate whether changes in body composition and cardiorespiratory fitness are concomitant with changes in mitochondrial capacity will be essential. Moreover, mitochondrial capacity was evaluated *ex vivo* through analysis of mitochondrial respiration, and expression of proteins related to mitochondrial dynamics and content. While analysis of mitochondrial respiratory capacity in permeabilized myofibers through high-resolution respiration is the gold standard method for investigating mitochondrial respiration, we acknowledge that use of an *ex vivo* approach may not completely represent the in vivo characteristics.

It is also important to highlight that BMI was used as an indicator of body fat and obesity, and it is not a direct measure of body fatness, and for an equivalent BMI, older subjects have a higher percentage body fat than young subjects (142). Future studies using direct measures of body fat, such as dual-energy x-ray absorptiometry (DEXA), would provide essential information on the influence of body fat, and its distribution to mitochondrial capacity in aging.

Another limitation of this study is the fact that the different age groups presented specific characteristics that were important for the parent study. First, the majority of the subjects were sedentary and only the young group included active subjects. The inclusion of active individuals in the middle-aged and older groups would provide important information on whether physical activity also influences the association between mitochondrial capacity and chronological age at these ages. Additionally, while the studies that included young and middle-aged subjects were specifically designed to include obese subjects, the old group only included two obese subjects. The old group only presented subjects in good general health and excluded individuals that had significant cardiovascular, neurological, and musculoskeletal conditions that would affect their lower limb extremity function. Future studies should also include older adults with a wide range of health, including subjects that are obese.

2.6 CONCLUSION

Mitochondrial respiratory capacity and expression of mitochondrial quality control proteins are elevated in young physically active individuals, but are similar among sedentary young, middle-aged and older subjects. Our findings suggest that mitochondrial capacity is not influenced by

chronological age *per* se, but is more closely related to BMI and cardiorespiratory fitness. Our data provide strong evidence that confounding factors of mitochondrial function, such as adiposity, cardiorespiratory fitness are critical phenotypic characteristics that should be considered in studies of aging muscle. This is essential if we are to provide clarity on the true nature of mitochondrial dysfunction with aging which in turn will allow us to more accurately develop mitochondrial targeted therapeutics in aging.

3.0 ASSOCIATION OF MITOCHONDRIAL RESPIRATION WITH MYOFIBER CROSS-SECTIONAL AREA, INTRAMYOCELLULAR LIPID CONTENT, AND PHYSICAL FUNCTION IN A GROUP OF VERY OLDER ADULTS

3.1 SUMMARY

While several factors have been associated with the onset and development of sarcopenia, the primary mechanisms contributing to this age-related decrease in muscle mass and strength remain to be clarified. This cross-sectional study sought to determine the influence of mitochondrial respiration on myofiber cross-sectional area as a measure of muscle size, intramyocellular lipid content, and physical function in a group of very old subjects. We hypothesized that mitochondrial respiratory capacity would be negatively associated with intramyocellular lipid content, and positively associated with myofiber cross-sectional area and physical function. **Methods:** Percutaneous biopsies of the vastus lateralis were obtained from 41 very old subjects (85-95yrs). Mitochondrial respiration was determined in saponin permeabilized myofiber bundles. Type I, IIa and IIx myofiber cross-sectional area and intramyocellular lipid content were evaluated histologically. Grip strength was tested by a handheld dynamometer, and knee extension torque was evaluated by an isokinetic dynamometer. Physical performance was evaluated by a series of physical function tests including the multiple chair stand, standing balance, 6-meter walk, and 20-meter walk tests. **Results:** Mitochondrial respiration were not associated with myofiber cross-

sectional area and intramyocellular lipid content. Complex I supported maximal OXPHOS respiration explained 19% of the variance in knee extensors peak torque, and mitochondrial respiration explained 11-14% percent of variation in grip strength, after controlling for age, sex, race, and BMI. Additionally, mitochondrial respiration was associated with preferred gait speed tested on the 6 and 20-meter walk tests. **Conclusions:** Skeletal muscle mitochondrial respiration is associated with measures of physical function in very older adults.

3.2 INTRODUCTION

Sarcopenia, the age-related decline in skeletal muscle mass and strength, is a well-established risk factor for several negative health-related conditions including disability, frailty, institutionalization, and mortality (23). While cellular, systemic and lifestyle factors have shown to be associated with the onset and development of sarcopenia, the primary mechanisms contributing to this process are still uncertain. Additionally, longitudinal studies have shown that the age-related changes in muscle mass do not correspond to the changes in muscle strength (119, 143). The dissociation between changes in muscle mass and strength suggests that other underlying factors such as cellular, neural, and metabolic features influence the age-related loss of muscle strength.

Among the cellular factors hypothesized to play a role on sarcopenia is mitochondrial function. Recent animal studies have provided strong evidence that there is a mechanistic link between loss of muscle mass and strength with mitochondrial capacity (19, 20, 36, 37, 144). These studies have shown that mitochondrial dysfunction plays a major role on sarcopenia through alterations in several processes such as apoptosis (144), and autophagy (19), and that mitochondrial

dysfunction is associated with age-related muscle atrophy (19, 20), and decreases in muscle strength (19) and performance (36, 37).

Despite the large number of animal studies supporting the influence of mitochondria on sarcopenia, few have provided evidence of this relationship in humans. A recent study performed by Gouspillou and colleagues have shown that mitochondrial-mediated apoptotic signaling is increased in muscle from older individuals (62). The authors suggest that accumulation of dysfunctional mitochondria with exaggerated apoptotic sensitivity is due to impaired mitophagy (62). Additionally, some recent cross-sectional human studies have shown an association between mitochondrial dysfunction and physical function in older adults. A study comparing 10 active and 10 sedentary older adults showed that the sedentary group presented a lower mitochondrial capacity as well as reduced fat-free mass, maximal isometric torque, and poorer performance on the walk test and stair-climb test when compared to active older adults (18). A previous study conducted by our research group has shown that mitochondrial efficiency is associated with preferred walking speed (22). Likewise, mitochondrial respiration have shown to be reduced in low-functioning elderly, determined by the short physical performance battery (SPPB) score, when compared to high-functioning elderly (14).

This study sought to determine the association between mitochondrial respiration with myofiber cross-sectional area as a measure of muscle size, intramyocellular lipid content and physical function in a group of 41 very old subjects (85 to 95yrs). We hypothesized that mitochondrial respiratory capacity would be negatively associated with intramyocellular lipid content, and positively associated with myofiber cross-sectional area and physical function.

The results of the present study will complement the previous findings by: 1. using a group of very old individuals (85-95yrs), in which decreases in muscle mass and physical function are

pronounced and the associations with sarcopenia more likely to be established, 2. investigating the association between mitochondrial capacity and muscle size at the cellular level, and 3. by comprehensively investigating the association between mitochondrial capacity and physical function, using several measures of muscle strength, balance and physical performance.

3.3 METHODS

3.3.1 Study design

This a**n**cillary cross-sectional study was designed to include a group of subjects participating at the Year 16 examination of the Dynamics of Health, Aging and Body Composition (Health ABC) study, Pittsburgh site. The Health ABC study is a longitudinal cohort study investigating factors that contribute to disability in the elderly, with special focus on body composition and weight-related health conditions. The study targeted to understanding the decline in function of healthier older persons, and the transition from vigor to frailty. The Health ABC study recruited 3,075 men and women with aged between 70-79yrs. All persons in the study were selected to be free of disability in activities of daily living and free of functional limitation (defined as any difficulty walking a quarter of a mile or any difficulty walking up 10 steps without resting) at baseline.

3.3.2 Subjects

A subset of forty-one older men and woman currently enrolled at The Health ABC study who were found to be eligible for the Year 16 examination and agreed to have a percutaneous muscle biopsy

performed were invited to be part of this sub-study. Subjects were included in the study if they had no known cognitive impairment, as defined by taking a medication for the treatment of dementia or scoring <20 on a phone telephone interview for cognitive status (TICS), live in the area, and do not reside in a nursing home. Additionally, a screening interview was done over the phone to determine if the participant was eligible for a percutaneous muscle biopsy.

Subjects were not tested for knee extension torque if they presented with any of the following conditions: history of cerebral aneurysm, cerebral bleeding within the past six months, blood pressure higher than 199/109mmHg, severe bilateral knee pain that would make the examination uncomfortable. Participants with severe unilateral knee pain, or that had previous unilateral total knee replacement, were tested on the opposite side. Similarly, exclusion from the performance tests was based on an individualized assessment of impairments and safety concerns. All subjects provided written informed consent and the study was approved by the University of Pittsburgh Institutional Review Board.

3.3.3 Outcome measures

Participants had two clinic visits for data collection. The first visit was at the University of Pittsburgh's Health Studies Research Center, where physical function was evaluated. The second visit was at the University of Pittsburgh's Clinical Translational Research Center, and a percutaneous muscle biopsy was collected for analysis of myofiber cross-sectional area, intramyocellular lipid (IMCL) content, and mitochondrial respiratory capacity.

3.3.4 Physical function

Physical function was evaluated through analysis of grip strength, knee extension torque, and a battery of tests including: multiple chair stands, standing balance, 6-meter balance walk, and 20-meter walk tests. All the performance based measurements were administered by a certified examiner, which demonstrated each maneuver for the participant before the test. Practice trial and rest between tasks were allowed if participants were fatigued during the assessments.

3.3.4.1 Grip strength

Handgrip strength (kg) was measured using a hand-held dynamometer (Jamar; TEC, Clifton, NJ), which has shown to be a valid and reliable approach (ICC=0.91-0.94) (145, 146). The test was performed with the participant in a seated position with the arm to be tested resting on a table and the elbow held at approximately a right angle. Subjects were instructed to squeeze the hand maximally. Grip size was adjusted for each participant, and one submaximal practice trial was performed to determine if the procedure was understood. Two trials were performed with a 15 to 20 seconds rest between trials. Average values for the right hand were used.

3.3.4.2 Isokinetic knee extension

Isokinetic knee extension was assessed using the Kin-Com 125 AP Isokinetic Dynamometer. After instrument calibration, subjects were shown how the task should be performed. Participants were seated in an upright position and were stabilized with straps across the pelvis and the distal thigh of the leg performing the task. The axis of the dynamometer was positioned so that it was aligned with the axis of rotation of the knee. To correct for gravity, participant's limb were weighed at

approximately 45°. Start and stop angles were set at 90° and 30°. Before testing begins, participants were asked to perform a maximum isometric effort to determine the starting force for the isokinetic dynamometry. The test starting force was set at half the maximum isometric effort. Participants were instructed to perform two good submaximal practice efforts before testing begin to ensure that the subject understood and was familiar with the task. For the data collection, subjects were instructed to perform a maximal effort with each contraction. Standard encouragement (verbal motivation and visual feedback) was given. Performance of knee extensors was tested at 60°/sec. Concentric efforts were repeated until three similar curves have been obtained, with a 20-second rest between each trial. The highest value obtained during the test was considered the knee extension peak torque and was used for analysis.

3.3.4.3 Multiple chair stand

Participants were instructed to stand up from a seated position and sit back down in the chair, without using their arms, five times as quickly as possible. The time subjects took to complete the task was recorded. The multiple chair stand test is a valid measure of dynamic balance and functional mobility, and has an excellent reliability (ICC=0.95) (147).

3.3.4.4 Standing balance

A series of timed, progressively more difficult, static balance tests was performed. The time (up to 30 seconds) the participant was able to hold each of the test positions (semi-tandem, tandem, and one-legged stands) were recorded. Subjects were allowed to try a second trial on the tandem stand and one-leg stand positions if they were not able to attain the positions, or attained the position for less than 30 seconds. The best performance in each one of the positions was used in the analysis.

3.3.4.5 Balance walk

Preferred gait speed was evaluated using the 6-meter walk test. In addition, gait speed was also evaluated with the subject walking along a narrowed path (20 cm). The narrowed walk path was designed to make the participant slow down during gait with a narrowed base of support, a situation that requires increased motor control. The test was performed up to three trials to obtain 2 valid times. The average of two trials were used.

3.3.4.6 20-meter walk test

Gait speed was also evaluated at the participant's usual and fast pace during a 20-meter walk test.

3.3.5 Skeletal muscle biopsy

Percutaneous muscle biopsies were obtained at the University of Pittsburgh Clinical Translational Research Center on a morning after an overnight fast. Participants were instructed not to perform physical exercise 48 hours prior to the biopsy procedure. Briefly, muscle biopsies were collected under local anesthesia (2 % buffered lidocaine) from the middle region of the vastus lateralis using a 5 mm muscle biopsy Bergström cannula (Stille Surgical instruments, Eskilstuna, Sweden), as previously described (132, 148). Immediately after the biopsy procedure the specimen was blotted dry and trimmed of visible adipose tissue using a standard dissecting microscope (Leica EZ4, Leica Microsystems, Switzerland). A portion of the specimen (~15mg) was placed in ice-cold BIOPS media (22) for analysis of mitochondrial respiratory capacity. A second portion was processed for histochemistry (30-40mg) and stored at -80 °C.

3.3.6 Myofiber cross-sectional area and intramyocellular lipid content

Histochemical analyses were performed on serial sections using methods previously described (93). Briefly, biopsy samples were sectioned (10 μm) on a cryostat (Cryotome E; Thermo Shandon, Pittsburgh, PA) at -20 °C and placed on individual pre-cleaned glass slides (Fisherfinest, Fischer Scientific, Pittsburgh, PA). Sections were stained in a filtered solution of Oil Red O (300mg/ml in 36% triethylphosphate) for 30 minutes at room temperature. Thereafter, sections were incubated with primary antibodies for anti-human myosin heavy chain (MYH)-7 (type I myocytes), and MYH-2 (type IIa myocytes) (Santa Cruz Biotechnologies, Santa Cruz, CA) overnight at room temperature. Samples were then incubated with rhodamine (type I myocytes) and fluorescein (FITC) (type IIa myocytes) conjugated secondary antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA). Type IIx fibers remained unstained. Images were visualized using a Leica microscope (Leica DM 4000B; Leica Microsystems, Bannockburn, IL), digitally captured (Retiga 2000R camera; Q Imaging, Surrey, Canada), and analyzed using specialized software (Northern Eclipse, v6.0; Empix Imaging, Cheektowaga, NY).

3.3.7 Mitochondrial respiration

Immediately after the biopsy procedure, permeabilized myofiber bundles were prepared. Briefly, myofiber bundles were gently teased apart in a petri dish containing ice-cold BIOPS solution using 2 sharp tweezers and a dissecting microscope (Leica Microsystems, Heerbrugg, Switzerland). The myofiber bundles were then permeabilized with saponin (2 mL of 50 ug/mL saponin in BIOPS solution) for 20 minutes at 4°C on an orbital shaker, and then washed twice for 10 minutes at 4°C with MIR05 respiration medium (22).

Mitochondrial respiratory capacity in permeabilized myofibers was evaluated by high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria), as shown on figure 2. Measurements were performed in duplicate, at 37°C, in the range of 230-150 nmol O_2/ml. LEAK (L_I) respiration was determined through the addition of pyruvate (5mM), malate (2mM) and glutamate (10mM). ADP (5mM) was added to elicit complex I supported OXPHOS (P_I) respiration. Cytochrome c (10 μM) was added to check the quality of the muscle fiber preparation and assess the integrity of the outer mitochondrial membrane. Any sample that showed an increase in respiration of more than 15% with the addition of cytochrome c was not included in the final analysis. Succinate (10mM) was then added to elicit complex I+II supported OXPHOS (P_{I+II}). FCCP (1uM) was added to determine electron transfer system (ETS) capacity or maximal uncoupled respiration. Finally, rotenone (1μM) was added to inhibit complex I supported OXPHOS respiration, and the remaining respiration revealed the maximal ETS capacity with complex II substrates only (E_{II}). Following the assay, myofiber bundles were recovered, dried, and weighted on an analytical balance (Mettler Toledo, XS105). Steady state O_2 flux for each respiratory state was determined and normalized to myofiber bundle dry weight using Datlab 4 software (Oroboros Instruments, Innsbruck, Austria).

Respiratory control ratios/factors were calculated to investigate intrinsic mitochondrial respiratory capacity. Since the different respiratory states were evaluated in the same myofiber bundle, and therefore same population of mitochondria, this approach is a valuable method to estimate mitochondrial function/quality independent of mitochondrial content, quality of myofiber bundle preparation and unintentional variation in assay conditions (123). Respiratory acceptor control ratios were calculated as P_I/L_I and P_{I+II}/L_I. Considering maximal uncoupled respiration (E_{I+II}) as an internal measure of the ETS capacity, flux control ratios were calculated for leak

respiration (L_I/E_{I+II}), complex I supported OXPHOS respiration (P_I/E_{I+II}), and complex I+II supported OXPHOS respiration (P_{I+II}/E_{I+II}). The results from the flux control ratios offer an estimation of the leak and OXPHOS capacity within the ETS capacity. Complex I (CI) and complex II (CII) control factors were calculated to investigate the relative change of O_2 flux in response to a transition of substrate availability in a defined coupling state. CI control factor corresponds to the difference between complex I+II supported OXPHOS respiration and E_{II} respiration. CII control factor corresponds to the difference between complex I+II supported OXPHOS respiration and complex I only supported OXPHOS respiration.

3.3.8 Statistical analysis

Multiple linear regressions were performed to investigate the association of mitochondrial respiration with sarcopenia. Myofiber cross-sectional area, intramyocellular lipid content, and measures of physical function were considered dependent variables. Separate multiple linear regression models were built for each dependent variable. Age, sex, race, and BMI were controlled in the models since these variables were associated with either the dependent or independent variables. Assumptions for the use of multiple linear regression were checked. Variables not normally distributed were transformed when necessary. The contribution of mitochondrial capacity to the outcome variables was assessed by the magnitude and significance of R^2 change. No adjustment was made for multiple comparisons because of the exploratory nature of the study. The association between the standing balance variables and mitochondrial respiration was tested by spearman partial correlation, adjusting for age, BMI, sex and race. We opted for analyzing the standing balance with spearman partial correlation, instead of multiple linear regression, because even after data transformations residuals were still not normally distributed.

In addition to using multiple linear regression to test the association between mitochondrial respiration and measures of sarcopenia, we further explored these associations by ranking the results of P_{I+II} respiration and splitting the scores into tertiles. Subjects in the lower (n=11) and higher (n=11) tertiles of mitochondrial respiration were compared for myofiber cross-sectional area, intramyocellular lipid content and physical function using independent t-test or Mann-Whitney U, as appropriate. Analyses were performed using SPSS v22.0 software (Armonk, NY). Statistical significance was established, a priori, at $p \leq 0.05$.

3.3.9 Power calculation

To test the study hypothesis at an alpha level of 0.05, with a sample size of 40 subjects, this study had 81% power to detect an effect size (r) of 0.42. Additionally, at an alpha level of 0.05, with a sample size of 40 subjects, this study had 80% power to detect an R-square of 22% attributed to the 4 covariates and mitochondrial respiration accounting for an additional 15% of the variability. Power calculation was performed using G*Power 3.1 and SPSS SamplePower 3.0 software.

3.4 RESULTS

3.4.1 Participant characteristics

A total of 216 potential participants were screened by telephone interview. Of those interviewed, 47 were not eligible for muscle tissue collection and 105 were not interested in participating in the study. Out of the 64 participants that were eligible to participate in this study, 41 had a muscle

biopsy collected (Figure 9). The participant characteristics can be found in table 9. A total of 16 men and 25 women, with age between 85 and 95 years participated in this cross-sectional study. The group had a wide range of BMI (16-36 Kg/m^2), including 1 subject that was underweight and 2 subjects that were severely obese. A wide range of physical performance was also presented (Table 9).

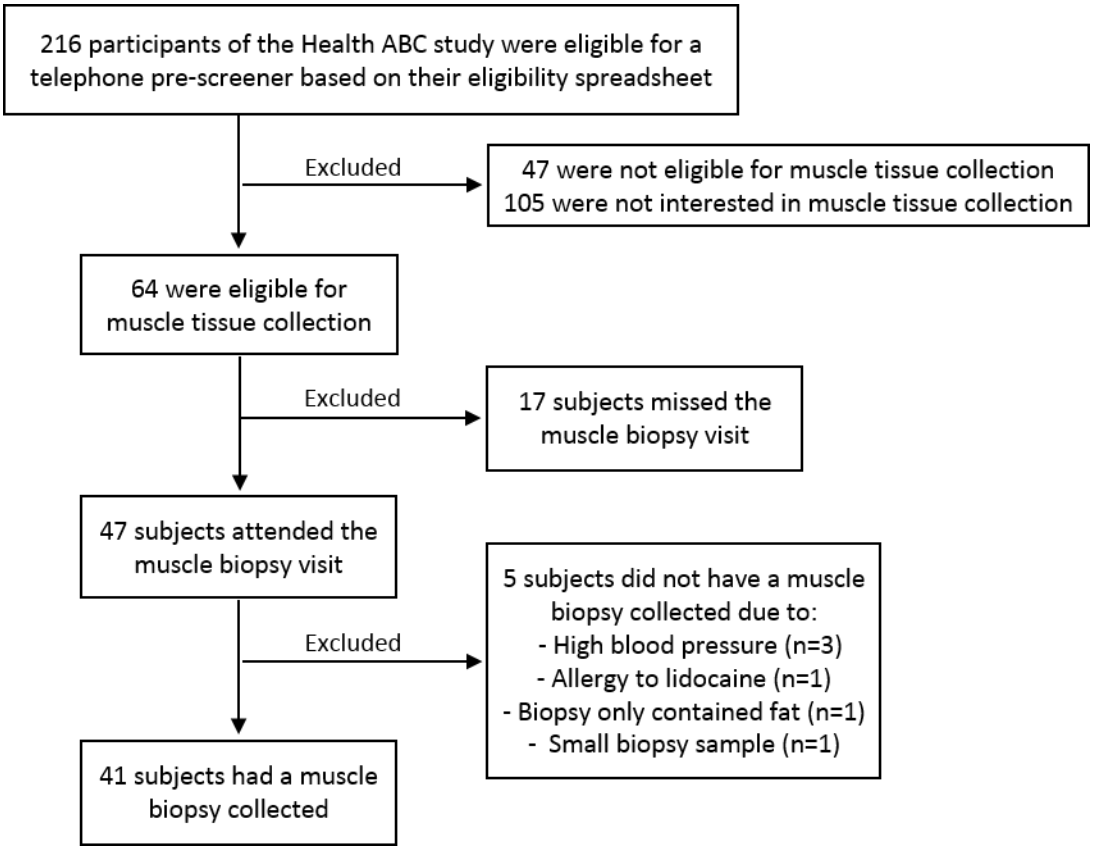

Figure 9. Participant flowchart

Table 9. Descriptive data for participants of study 2

Sex		Male	Female	p value
Age (yrs)		88.3 ± 2.7 (85-95)	88.5 ± 2.3 (85-93)	0.80
Weight (Kg)		75.2 ± 12.2 (62.7-110.4)	69.3 ± 13.3 (39-88.8)	0.16
BMI (Kg/m^2)		26.5 ± 3.0 (22.5-35.2)	28.1 ± 4.7 (16.5-36.5)	0.23
Race		C = 12, AA = 4	C = 15, AA = 10	-
Grip strength (kg)		28.9 ± 6.4 (18-45)	18.6 ± 5.7 (10-33)	<0.01*
Knee extension peak torque (Nm)		93.8 ± 13.5 (73-114), n=11	70.1 ± 23.8 (26-106), n=18	0.01*
Multiple chair stands (s)		13.1 ± 2.7 (9.5-19.6), n=13	14.4 ± 4.7 (7.1-24.8), n=21	0.38
Standing balance (s)				-
	Semi-tandem stand	25.7 ± 10.5 (0-30), n=15	23.4 ± 12.0 (0-30), n=25	0.55
	Tandem stand	28.9 ± 4.1 (15.25-30), n=13	17.6 ± 12.9 (0-30), n=22	<0.01*
	One leg stand	9.8 ± 10.9 (0-30), n=12	6.4 ± 7.3 (0-30), n=22	0.30
Gait speed on the 6-meter balance walk (m/s)				-
	Usual pace walk	0.95 ± 0.15 (0.82-1.28), n=13	0.90 ± 0.17 (0.54-1.27), n=20	0.44
	20 cm narrow walk	0.91 ± 0.17 (0.71-1.16), n=6	0.81 ± 0.27 (0.35-1.11), n=10	0.44
Gait speed on the 20-meter walk (m/s)				-
	Usual pace	1.05 ± 0.16 (0.78-1.43), n=14	1.01 ± 0.21 (0.68-1.46), n=25	0.49
	Fast pace	1.39 ± 0.20 (1.07-1.73), n=14	1.21 ± 0.27 (0.67-1.62), n=25	0.03*

Data presented as Mean ± SD (Min-Max). N=16 for male and N=25 for females, unless otherwise stated.

3.4.2 Association of mitochondrial respiration with myofiber area, intramyocellular lipid content, and physical function

Several multiple linear regression models were performed to investigate the association of mitochondrial respiration with myofiber cross-sectional area, intramyocellular lipid content, and physical function. Age, sex, race, and BMI were controlled in the models since they have demonstrated to be associated with either mitochondrial respiration or some of the dependent variables (Table 9 and 10, and Figures 10, 11, and 12). Mitochondrial respiratory capacity is described in table 11. Association between age, BMI and physical function measures are shown in table 12.

Table 10. Myofiber distribution, cross-sectional area and intramyocellular lipid content

	Male	Female	p value
Fiber Type (%)			
Type I	48 ± 18	43 ± 13	0.29
Type II	52 ± 18	57 ± 13	0.29
Type IIa	40 ± 16	47 ± 12	0.11
Type IIx	12 ± 11	10 ± 9	0.51
Cross-Sectional Area (μm^2)			
Type I	4677 ± 1273	3731 ± 1480	0.05*
Type II	3753 ± 1127	2006 ± 658	<0.01*
Type IIa	3730 ± 1105	2135 ± 748	<0.01*
Type IIx	3618 ± 1400	1551 ± 600	<0.01*
Intramyocellular lipid content (AU)			
Type I	5456 ± 1706	6081 ± 1541	0.25
Type II	3630 ± 1462	3312 ± 1189	0.46
Type IIa	3775 ± 1510	3454 ± 1329	0.49
Type IIx	2961 ± 1335	3160 ± 1198	0.66

Data presented as Mean ± SD, n=16 for male and n=22 for female.

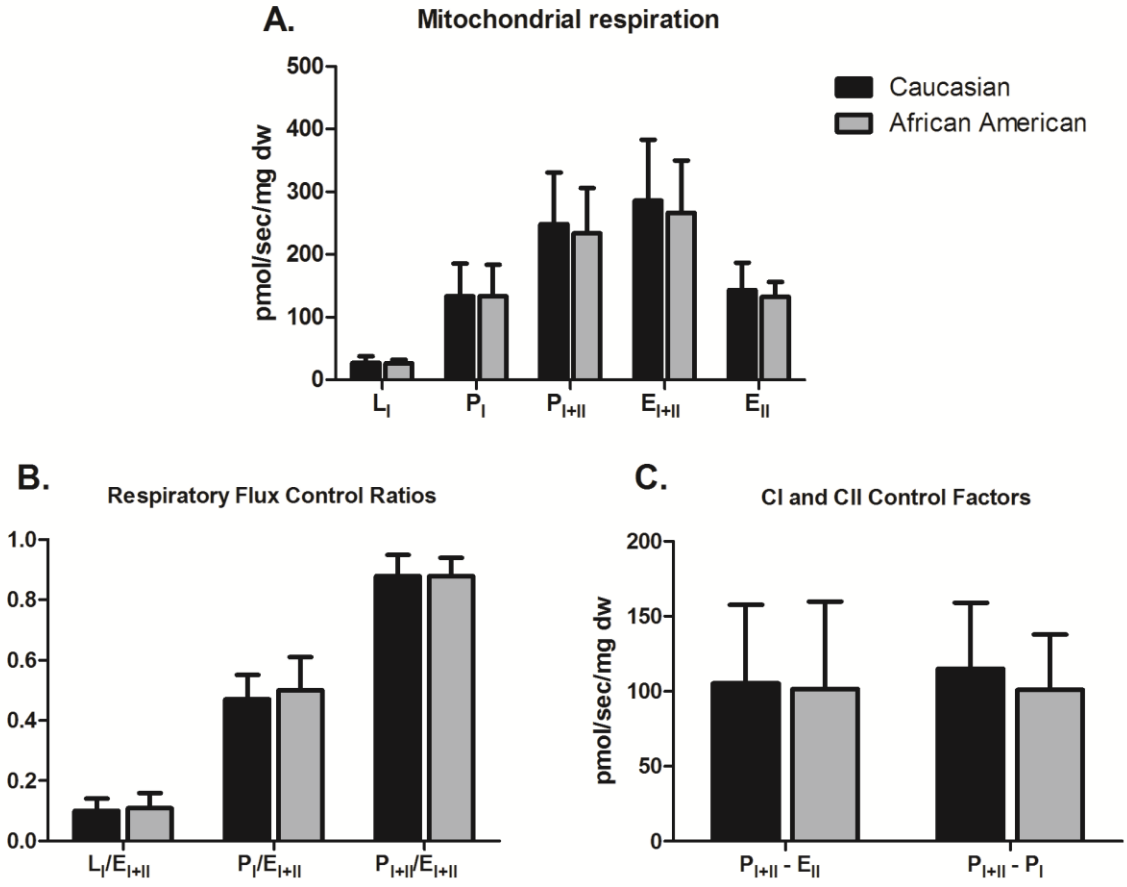

Figure 10. Mitochondrial respiratory capacity in Caucasians and African Americans.

(A) Mitochondrial respiration consisting of complex I supported Leak (L_I) respiration, complex I supported OXPHOS (P_I) respiration, complex I+II supported OXPHOS (P_{I+II}) respiration, maximal electron transfer system (ETS) capacity with substrates for complex I and II (E_{I+II}), and ETS with substrates for complex II (E_{II}). **(B)** Respiratory flux control ratios were determined as an estimation of leak and OXPHOS capacity within the ETS capacity. **(C)** Complex I (CI) control factor measured as P_{I+II} minus E_{II} respiration, and Complex II (CII) control factor measured as P_{I+II} minus P_I. Data presented as Mean and SD, n=22 for Caucasians and n=11 for African Americans.

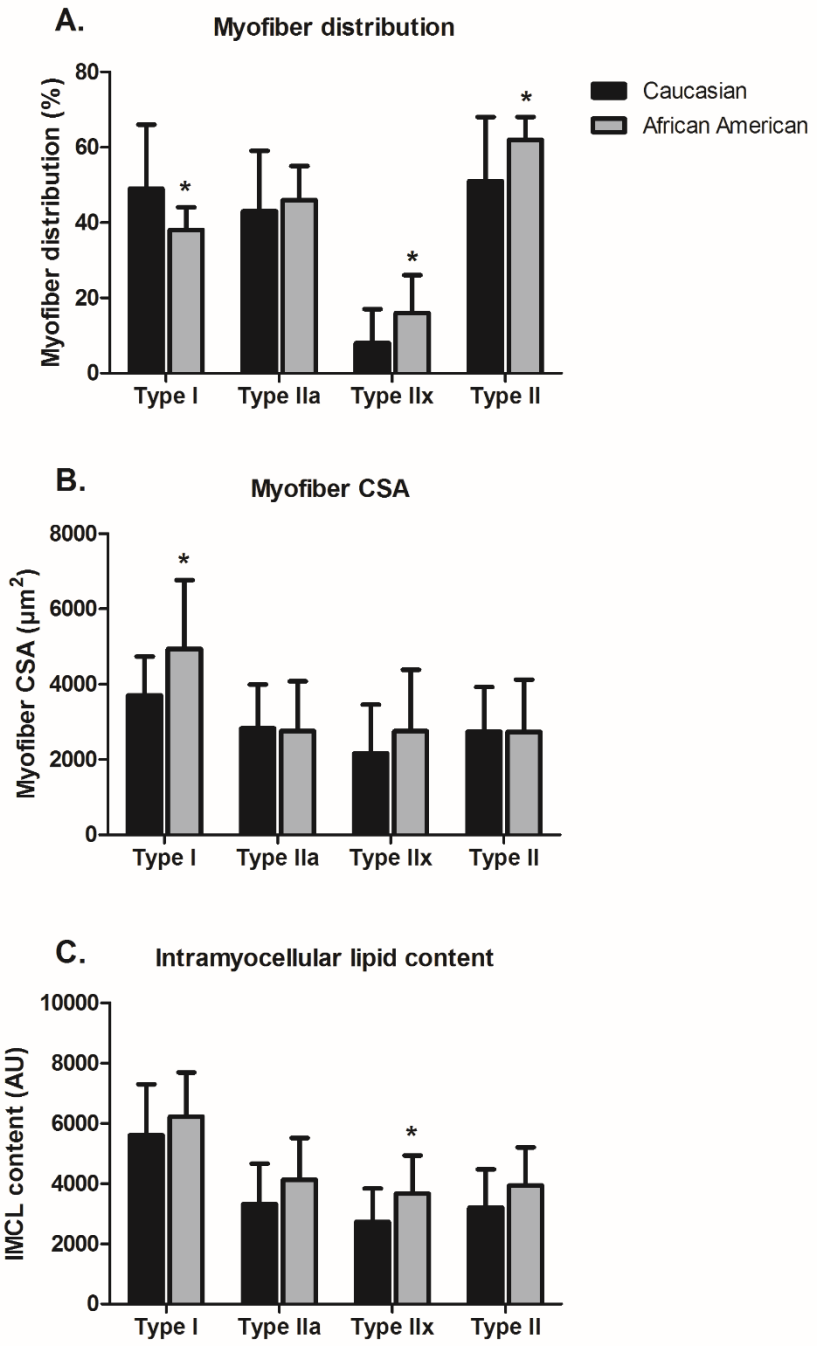

Figure 11. Myofiber distribution, cross-sectional area and intramyocellular lipid (IMCL) content in Caucasians and African Americans.

(A) Myofiber distribution. **(B)** Myofiber cross-sectional area (CSA). **(C)** Myofiber intramyocellular lipid content. Data presented as Mean and SD; * $p<0.05$, n=25 for Caucasian and n=13 for African American.

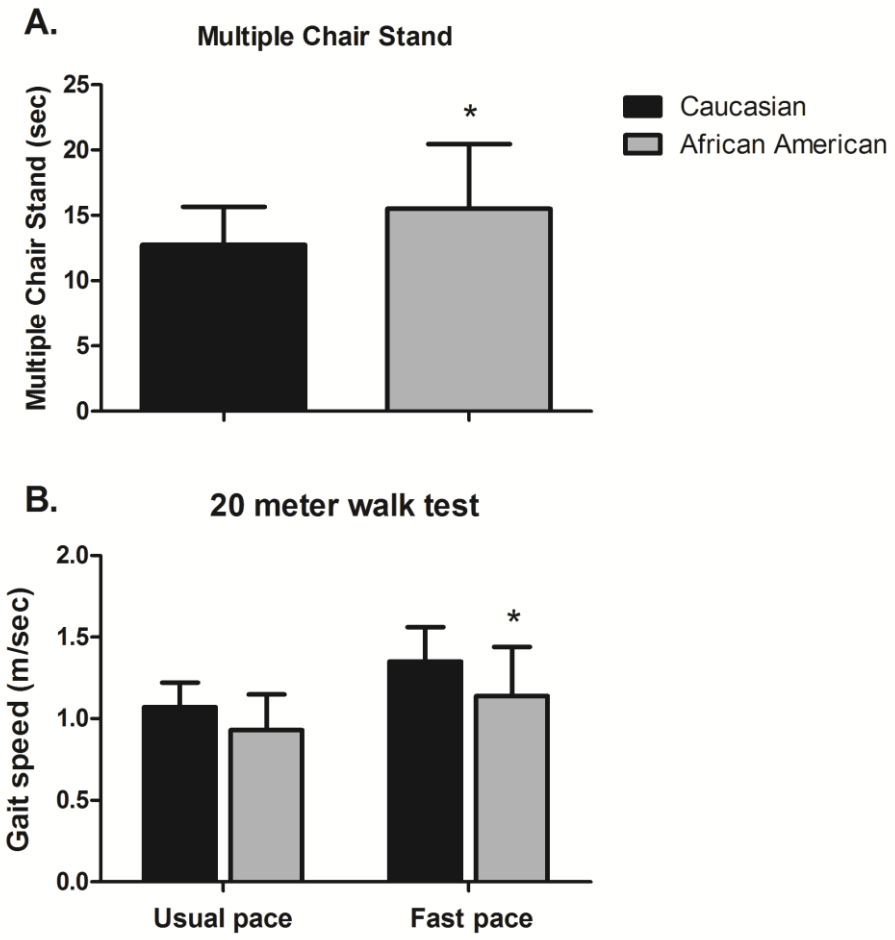

Figure 12. Multiple chair stand and gait speed in Caucasians and African Americans.

(A) Multiple chair stand (sec); n=20 for Caucasian and n=14 for African American. **(B)** Gait speed on the 20-meter walk test (m/sec); n=26 for Caucasian and n=14 for African American. Data presented as Mean and SD. * $p<0.05$.

Table 11. Mitochondrial respiratory capacity

	Male	Female	p value
Complex I supported leak respiration (L_I)	29 ± 8	26 ± 10	0.31
Complex I supported OXPHOS (P_I)	133 ± 53	133 ± 50	0.99
Complex I and II supported OXPHOS (P_{I+II})	244 ± 75	243 ± 82	0.98
Complex I and II supported electron transfer capacity (E_{I+II})	279 ± 94	280 ± 93	0.97
Complex II supported electron transfer capacity (E_{II})	144 ± 36	137 ± 40	0.59
L_I/E_{I+II} flux control ratio	0.11 ± 0.05	0.10 ± 0.04	0.24
P_I/E_{I+II} flux control ratio	0.47 ± 0.07	0.48 ± 0.10	0.84
P_{I+II}/E_{I+II} flux control ratio	0.89 ± 0.08	0.87 ± 0.06	0.51
Complex I control factor ($P_{I+II} – E_{II}$)	100 ± 57	107 ± 52	0.72
Complex II control factor ($P_{I+II} – P_I$)	111 ± 31	110 ± 48	0.96
P_I/L_I Respiratory control ratio	4.7 ± 2	5.3 ± 1.2	0.29
P_{I+II}/L_I Respiratory control ratio	8.7 ± 2.8	10.2 ± 3.1	0.19

Data presented as Mean ± SD, n = 13 for male and n=20 for female.

Table 12. Correlation matrix of age, BMI, and physical function measures

	Age (years)	BMI (kg/m^2)	Grip Strength (Kg)	Peak Torque (Nm)	Multiple Chair Stand (Sec)	Balance Semi-tandem (Sec)	Balance Tandem (Sec)	Balance One-leg stand (Sec)
Age	**1**	**-0.062**	**-0.036**	**-0.098**	**0.230**	**0.061**	**0.118**	**0.020**
BMI		**1**	**-0.004**	**0.185**	**0.161**	**0.124**	**-0.122**	**-0.211**
Grip Strength			**1**	**0.559***	**-0.231**	**0.375***	**0.717***	**0.304**
Peak Torque				**1**	**-0.141**	**0.066**	**0.285**	**0.349**
Chair Stand					**1**	**-0.061**	**-0.411***	**-0.044**
Semi-tandem						**1**	**0.368***	**0.132**
Tandem							**1**	**0.354***
One-leg stand								**1**
Gait speed[1]								
Gait speed[2]								
Gait speed[3]								
Gait speed[4]								

Table 12. (continued)

	Gait speed 1 6-meter walk **(m/sec)**	**Gait speed 2** 6-meter walk, narrow path **(m/sec)**	**Gait speed 3** 20-meter walk, usual pace **(m/sec)**	**Gait speed 4** 20-meter walk, fast pace **(m/sec)**
Age	**-0.088**	**0.439**	**-0.280**	**-0.260**
BMI	**0.019**	**-0.287**	**-0.224**	**-0.371***
Grip Strength	**0.294**	**0.349**	**0.182**	**0.366***
Peak Torque	**0.073**	**0.446**	**0.064**	**0.105**
Chair Stand	**-0.133**	**0.002**	**-0.326**	**-0.496***
Semi-tandem	**0.394***	**0.420**	**0.257**	**0.261**
Tandem	**0.205**	**0.641***	**0.140**	**0.208**
One-leg stand	**0.358**	**0.549***	**0.254**	**0.153**
Gait speed 1	**1**	**0.632***	**0.658***	**0.550***
Gait speed 2		**1**	**0.305**	**0.032**
Gait speed 3			**1**	**0.613***
Gait speed 4				**1**

3.4.2.1 Myofiber cross-sectional area

While age, sex, race, and BMI combined have shown to explain 39-64% of the variance in myofiber cross-sectional area ($p<0.05$, Table 13), mitochondrial respiration (L_I, P_I, P_{I+II}, E_{I+II}, and E_{II}) did not explain a significant additional amount of variation in type I, IIa, IIx, and II myofiber cross-sectional area ($p>0.05$, Table 13), after controlled for these covariates.

Table 13. Multiple linear regression investigating the association of mitochondrial respiration and myofiber cross-sectional area

		Myofiber cross-sectional area (μm^2)							
		Type I		Type IIa		Type IIx		Type II	
		R^2	p	R^2	p	R^2	p	R^2	p
	Age, sex, race, and BMI	0.394	<0.01*	0.492	<0.01*	0.644	<0.01*	0.558	<0.01*
L_I	After controlled for covariates	0.059	0.115	0.005	0.625	<0.001	0.907	0.003	0.686
P_I	After controlled for covariates	0.004	0.674	0.002	0.745	0.022	0.253	<0.001	0.998
P_{I+II}	After controlled for covariates	0.048	0.155	0.003	0.693	0.004	0.634	0.001	0.807
E_{I+II}	After controlled for covariates	0.035	0.228	0.004	0.644	0.005	0.603	0.002	0.766
E_{II}	After controlled for covariates	0.022	0.34	0.042	0.147	0.017	0.31	0.033	0.168

*p<0.05, n=27-31. Abbreviations: BMI (body mass index), L_I (complex I supported leak respiration), P_I (complex I supported OXPHOS respiration), P_{I+II} (complex I+II supported OXPHOS respiration), E_{I+II} (maximal electron transfer capacity with substrates for complex I and II), and E_{II} (electron transfer capacity with substrates for complex II only).

3.4.2.2 Intramyocellular lipid content

Mitochondrial respiration (L_I, P_I, P_{I+II}, E_{I+II}, and E_{II}) did not explain a significant amount of variation in type I, IIa, IIx, and II intramyocellular lipid content ($p>0.05$, Table 14), after controlling for age, sex, race, and BMI.

Table 14. Multiple linear regression investigating the association of mitochondrial respiration and intramyocellular lipid content

		Intramyocellular lipid content (AU)							
		Type I		Type IIa		Type IIx		Type II	
		R^2	p	R^2	p	R^2	p	R^2	p
	Age, sex, race, and BMI	0.072	0.735	0.174	0.272	0.232	0.196	0.161	0.317
L_I	After controlled for covariates	0.021	0.456	0.010	0.585	<0.001	0.975	0.019	0.459
P_I	After controlled for covariates	0.020	0.464	0.013	0.532	<0.001	0.940	0.002	0.826
P_{I+II}	After controlled for covariates	<0.001	0.972	0.002	0.824	0.026	0.404	0.009	0.609
E_{I+II}	After controlled for covariates	0.005	0.726	0.002	0.820	0.006	0.700	<0.001	0.955
E_{II}	After controlled for covariates	0.027	0.392	0.007	0.652	0.032	0.351	0.015	0.510

N=31. Abbreviations: BMI (body mass index), L_I (complex I supported leak respiration), P_I (complex I supported OXPHOS respiration), P_{I+II} (complex I+II supported OXPHOS respiration), E_{I+II} (maximal electron transfer capacity with substrates for complex I and II), and E_{II} (electron transfer capacity with substrates for complex II only).

3.4.2.3 Grip strength and knee extension torque

While age, sex, race, and BMI combined explained 50% of the variance in grip strength ($p<0.01$, Table 15), mitochondrial respiration (P_I, P_{I+II}, E_{I+II}, E_{II}) explained an additional 11-14% of this variance ($p<0.05$, Table 15). Age, sex, race, and BMI combined explained 31% of the variance in knee extension peak torque ($P=0.151$, Table 15), with P_I respiration explaining an additional 19% of this variance ($p=0.027$, Table 15).

Table 15. Multiple linear regression investigating the association of mitochondrial respiration on grip strength and knee extension torque

		Grip Strength (kg)		Knee extension torque (Nm)	
		R^2	p	R^2	p
	Age, sex, race, and BMI	0.500	<0.01*	0.313	0.151
L_I	After controlled for covariates	0.024	0.253	0.055	0.256
P_I	After controlled for covariates	0.110	0.010*	0.187	0.027*
P_{I+II}	After controlled for covariates	0.114	0.009*	0.071	0.195
E_{I+II}	After controlled for covariates	0.139	0.003*	0.064	0.218
E_{II}	After controlled for covariates	0.111	0.010*	0.008	0.661

*$p<0.05$, n=33 for grip strength and n=22 for knee extension peak torque. Abbreviations: BMI (body mass index), L_I (complex I supported leak respiration), P_I (complex I supported OXPHOS respiration), P_{I+II} (complex I+II supported OXPHOS respiration), E_{I+II} (maximal electron transfer capacity with substrates for complex I and II), and E_{II} (electron transfer capacity with substrates for complex II only).

3.4.2.4 Physical performance

Mitochondrial respiration (L_I, P_I, P_{I+II}, E_{I+II}, and E_{II}) did not explain a significant amount of variance in the results of the standing balance tests ($p>0.05$, Table 16), multiple chair stand ($p>0.05$, Table 17), and gait speed on the 6-meter walk test ($p>0.05$, Table 17) and 20-meter walk tests ($p>0.05$, Table 18), after controlling for age, sex, race, and BMI.

Table 16. Spearman partial correlation investigating the association of mitochondrial respiration and standing balance tests

	Semi-tandem stand (sec)	Tandem stand (sec)	One leg stand (sec)
L_I	0.2108	0.0545	-0.0402
P_I	0.2688	0.2209	0.1577
P_{I+II}	0.3335	0.1646	0.1728
E_{I+II}	0.2896	0.1944	0.1699
E_{II}	0.1778	0.0599	0.3271

Values represent spearman partial correlation after controlling for age, body mass index, sex and race, n=34-40. Abbreviations: BMI (body mass index), L_I (complex I supported leak respiration), P_I (complex I supported OXPHOS respiration), P_{I+II} (complex I+II supported OXPHOS respiration), E_{I+II} (maximal electron transfer capacity with substrates for complex I and II), and E_{II} (electron transfer capacity with substrates for complex II only)

Table 17. Multiple linear regression investigating the association of mitochondrial respiration on multiple chair stand and balance walk tests

		Multiple chair stand (sec)		6 meter walk Gait speed (m/sec)		6 meter walk (Narrow path) Gait speed (m/sec)	
		R^2	p	R^2	p	R^2	p
	Age, sex, race, and BMI	0.260	0.141	0.115	0.591	0.107	0.873
L_I	After controlled for covariates	0.003	0.768	0.003	0.795	0.005	0.825
P_I	After controlled for covariates	0.009	0.615	<0.001	0.989	0.050	0.486
P_{I+II}	After controlled for covariates	0.005	0.701	0.027	0.423	0.046	0.504
E_{I+II}	After controlled for covariates	0.003	0.766	0.014	0.567	0.018	0.667
E_{II}	After controlled for covariates	0.004	0.733	0.101	0.115	0.001	0.907

N=27 for the multiple chair stand, n=27 for the 6-meter walk test, and n=15 for the 6-meter walk test (Narrow path). Abbreviations: BMI (body mass index), L_I (complex I supported leak respiration), P_I (complex I supported OXPHOS respiration), P_{I+II} (complex I+II supported OXPHOS respiration), E_{I+II} (maximal electron transfer capacity with substrates for complex I and II), and E_{II} (electron transfer capacity with substrates for complex II only).

Table 18. Multiple linear regression investigating the association of mitochondrial respiration and gait speed on the 20-meter walk test

		Gait speed (m/sec) Usual pace		Gait speed (m/sec) Fast pace	
		R^2	p	R^2	p
	Age, sex, race, and BMI	0.245	0.097	0.339	0.021*
L_I	After controlled for covariates	0.004	0.727	<0.001	0.993
P_I	After controlled for covariates	0.003	0.760	<0.001	0.966
P_{I+II}	After controlled for covariates	0.029	0.314	0.005	0.652
E_{I+II}	After controlled for covariates	0.016	0.458	0.006	0.619
E_{II}	After controlled for covariates	0.015	0.478	0.020	0.379

*$p<0.05$, n=33. Abbreviations: BMI (body mass index), L_I (complex I supported leak respiration), P_I (complex I supported OXPHOS respiration), P_{I+II} (complex I+II supported OXPHOS respiration), E_{I+II} (maximal electron transfer capacity with substrates for complex I and II), and E_{II} (electron transfer capacity with substrates for complex II only).

3.4.3 Group comparison for high and low mitochondrial function

Our preliminary results showed that some of the dependent variables did not have a linear association with mitochondrial respiration. Moreover, although this study was designed to have 80% power to detect associations between measures of sarcopenia and mitochondrial respiration in 40 individuals, for many of the outcome variables these associations were tested in a lower number of subjects. Therefore, in addition to investigating the association between mitochondrial respiration and measures of sarcopenia using multiple linear regressions, we opted to further explore these associations using group comparisons. The results of mitochondrial respiration (P_{I+II}) were ranked and split into tertiles. Subjects in the lowest (n=11) and highest tertiles (n=11) were compared for myofiber cross-sectional area, intramyocellular lipid content, and physical function.

No differences in myofiber cross-sectional area ($p>0.05$, Figure 13A), intramyocellular lipid content ($p>0.05$, Figure 13B), and knee extension torque ($p>0.05$, Figure 14) were found between the low and high mitochondrial respiration groups. While we also observed no differences between groups for the multiple chair stand ($p>0.05$, Figure 15A) and standing balance tests ($p>0.05$, Figure 15B), we found that the high mitochondrial respiration group had a greater gait speed on both the 6-meter walk test ($p<0.05$, Figure 16) and the 20-meter walk test ($p<0.05$, Figure 17A). The group comparison also confirmed the results of the multiple linear regression that subjects with higher mitochondrial respiration have higher BMI ($p<0.05$, Figure 18 and 19).

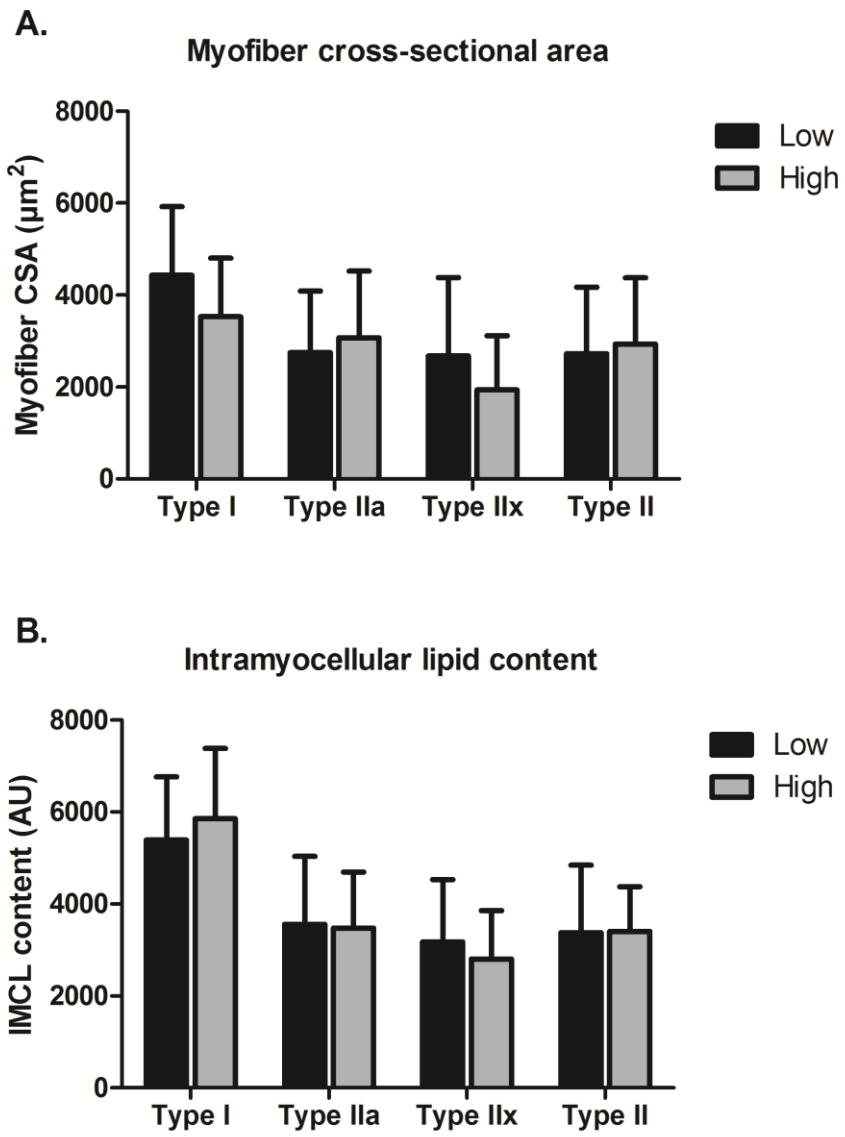

Figure 13. Myofiber cross-sectional and intramyocellular lipid content in Low and High mitochondrial respiration groups.

(A) Myofiber cross-sectional area. (B) Intramyocellular lipid content. Data presented as Mean and SD; n=11 per group.

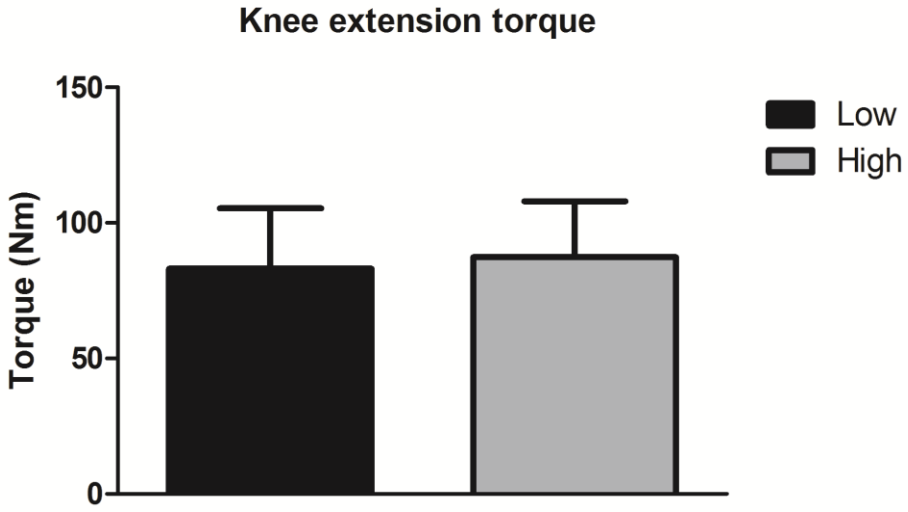

Figure 14. Knee extension torque in Low and High mitochondrial respiration groups.

Data presented as Mean and SD, n=8 per group.

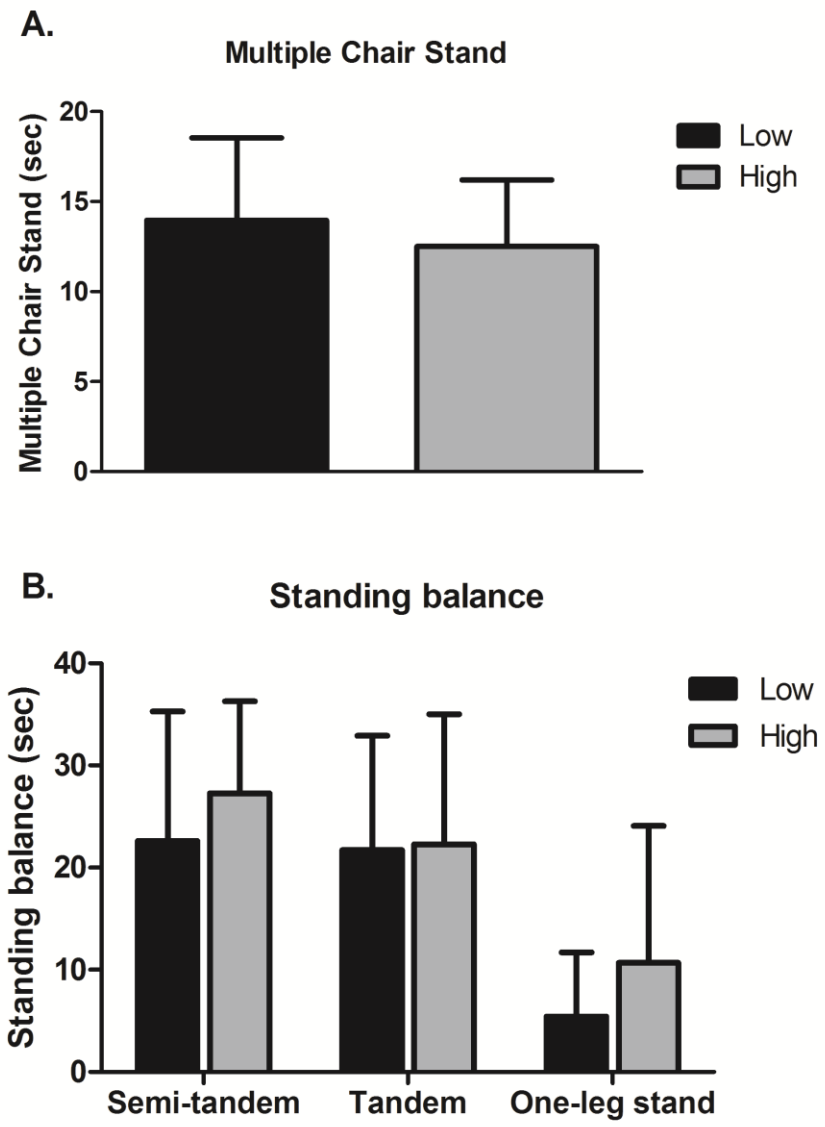

Figure 15. Multiple chair stand and standing balance tests in Low and High mitochondrial respiration groups.

(A) Time to complete the multiple chair stand test (sec), n=10 for the Low tertile group, and n=8 for the High tertile group. **(B)** Standing balance tests including semi-tandem, tandem and one-leg stand (sec), n=10-11 per group. Data presented as Mean and SD.

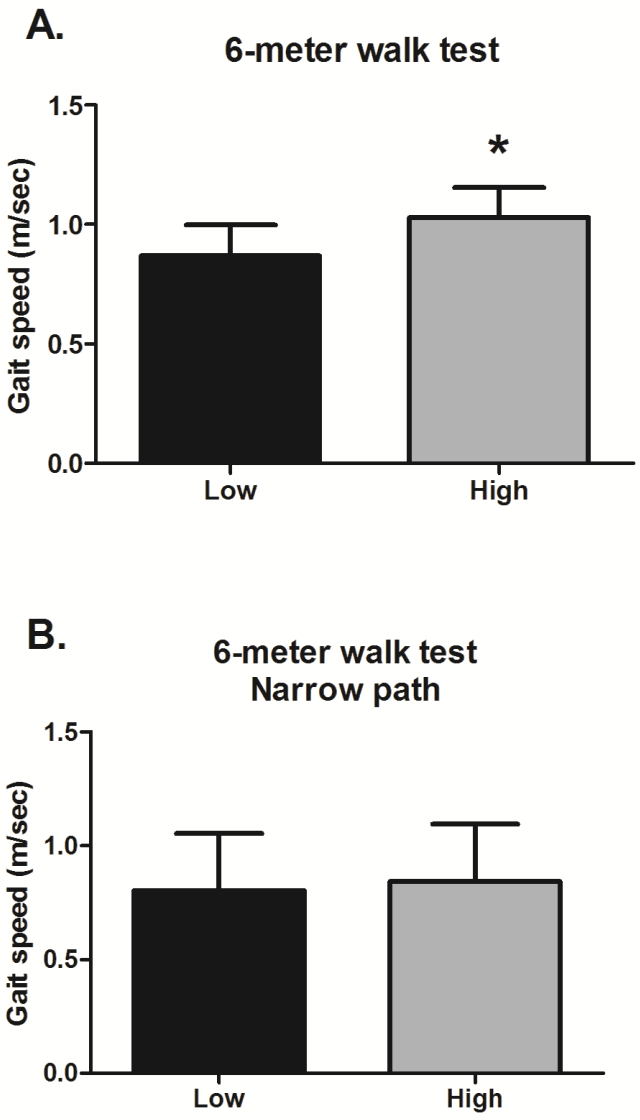

Figure 16. Gait speed on the 6-meter walk test in Low and High mitochondrial respiration groups.

(A) Gait speed on the 6-meter walk test; n=9 for Low tertile group, and n=10 for the High tertile group. **(B)** Gait speed on the 6-meter walk test; n=3 for Low tertile group, and n=6 for the High tertile group. Data presented as Mean and SD; *$p<0.05$.

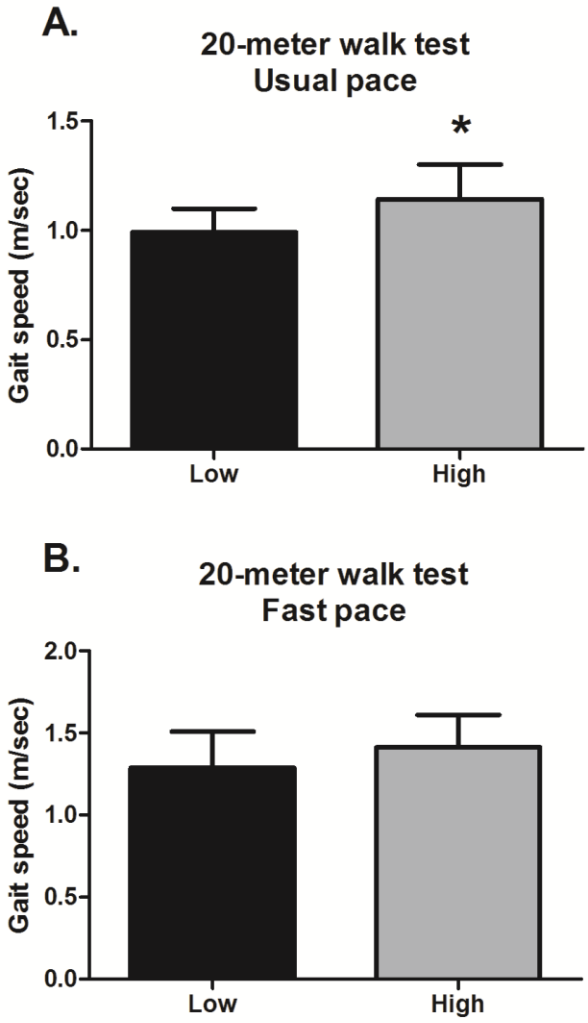

Figure 17. Gait speed on the 20-meter walk test in Low and High mitochondrial respiration groups.

(A) Gait speed on the 20-meter walk test, usual pace. **(B)** Gait speed on the 20-meter walk test, fast pace. Data presented as Mean and SD; *$p<0.05$, n=11 per group.

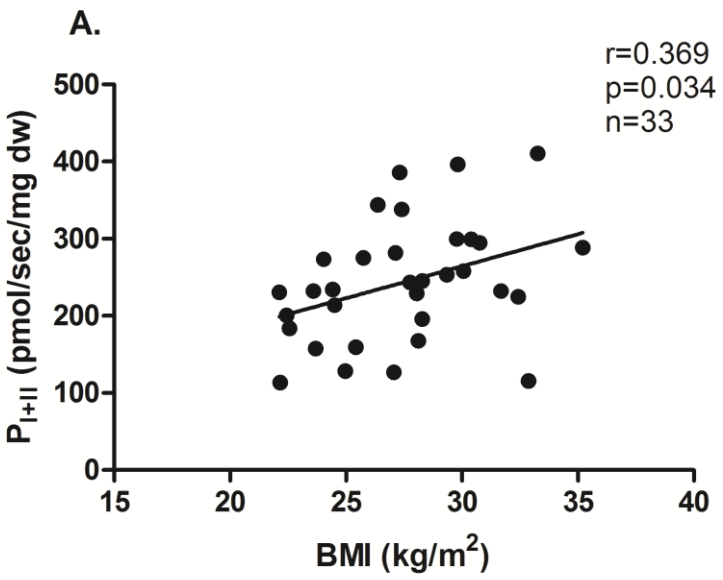

Figure 18. Bivariate correlation between mitochondrial respiration and BMI.

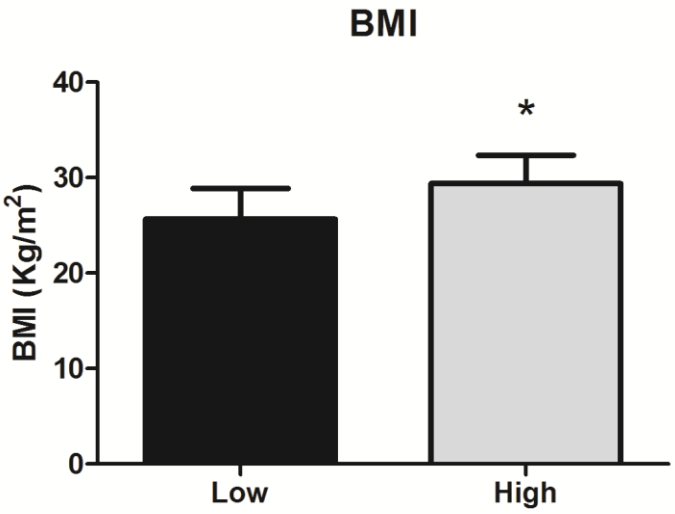

Figure 19. Body mass index in Low and High mitochondrial respiration groups.

Data presented as Mean and SD; *p<0.05, n=11 per group.

3.5 DISCUSSION

Sarcopenia is a public health concern, and may lead to loss of independence, frailty, disability, institutionalization and mortality. While several factors have been associated with its onset and progression, little is known about the contribution of mitochondrial capacity to the age-related loss of muscle mass and strength. In the present study we investigated the influence of mitochondrial respiration on measures of sarcopenia in a group of 41 very old individuals (85-95years). While mitochondrial respiration did not explain a significant amount of variation in myofiber cross-sectional area and intramyocellular lipid content, it demonstrated to be associated with grip strength, knee extension peak torque, and preferred gait speed.

Recent data from animal studies have strongly suggested that there is a mechanistic link between mitochondria and sarcopenia. During muscle wasting, apoptotic and necrotic muscle fibers have shown to exhibit electron transport chain abnormalities, and it is suggested that mitochondrial dysfunction is a major contributor to the activation of cell death pathways in aged muscle fibers (144). Skeletal muscle of old mice have reduced association between calcium release units and mitochondria when compared to young mice, which contributes to impaired cross-talk between the two organelles and result in reduced efficiency in ATP production and muscle performance (36). Additionally, muscle-specific Atg 7 knockout mice, a model in which skeletal muscle lacks a crucial autophagy gene, have shown to present not only abnormal mitochondria, but also profound muscle atrophy and decreased strength (19). While these studies have provided evidence that muscle mass and function are compromised with mitochondria dysfunction, others have demonstrated that improvements in mitochondrial capacity results in diminished loss of muscle mass and function. Mice overexpressing PGC-1α, a major regulator of mitochondrial biogenesis, have preserved mitochondrial function and reduced muscle wasting with aging (20).

Loss of muscle mass in these animals are prevented by reduced apoptosis, autophagy, and proteasome degradation (20). Additionally, old animals treated with a mitochondria-targeted ROS and electron scavenger demonstrate improved activity of respiratory complexes, reduced protein oxidation and higher muscle contractility (37).

Despite the large number of animal studies supporting the potential link between mitochondria and sarcopenia, few human studies have been performed to test this association and to investigate the mechanisms involved on these processes (14, 18, 22, 62). Mitochondrial-mediated apoptotic signaling is increased in muscle from older individuals, with an accumulation of dysfunctional mitochondria due to impaired mitophagy (62). Additionally, skeletal muscle of older subjects present increased mitochondrial protein carbonyl abundance, which have demonstrated to be negatively correlated with muscle strength (149). Other recent cross-sectional human studies have suggested that decreased mitochondrial function is associated with lower muscle mass, strength and/or poor physical performance. When compared to a group of active older adults, it was found that sedentary older adults have a reduced mitochondrial capacity, as well as a decreased fat-free mass, declined maximal isometric torque, poorer performance on the walk test and stair-climb test (18). Likewise, mitochondrial respiration is reduced in low-functioning elderly, determined by the short physical performance battery (SPPB) score, when compared to high-functioning elderly (14). Additionally, an association between PGC-1α and gait speed was observed in these elderly subjects. Similarly, previous study conducted by our research group demonstrated an association between mitochondrial efficiency and preferred walking speed in a group of older adults (22). However, no studies to date have investigated these associations in a group of very older adults, and utilized a comprehensive approach to evaluate physical function.

In the present study we evaluated the association of mitochondrial function with muscle size at the cellular level. The association of mitochondrial respiration with type I, IIa, and IIx myofiber cross-sectional area was tested after controlling for age, sex, race, and BMI. We found that after controlling for these covariates mitochondrial respiration do not explain a significant amount of variation in myofiber area. Considering that muscle size is not the only factor that influences muscle strength and function, we also investigated the quality of these myofibers. No association between mitochondrial respiration with intramyocellular lipid content in type I, IIa and IIx myofibers was found.

While no associations were observed between mitochondrial respiration and lipid content at the cellular level, we found that BMI was positively associated with maximal OXPHOS. This finding is in contrast with other studies that have reported a negative correlation between mitochondrial respiration and BMI (17), and with the results of chapter 2 of this dissertation. This result may suggest that while having a higher BMI may be detrimental for young and older adults and increase the likelihood of several chronic diseases, at very old age it may be beneficial or protective. Future analysis will be performed to investigate the contribution of fat free mass and fat mass to the BMI of these very older adults, and will be essential to further understand the association between BMI with mitochondrial respiration in different age populations.

In addition to examining the influence of mitochondrial respiration on myofiber cross-sectional area and intramyocellular lipid content, we also investigated whether mitochondrial respiration translates to physical function, including muscle strength, balance and physical performance. We found that mitochondrial respiration explained a significant additional amount of variation in grip strength, and knee extension torque, after controlled for covariates. Additionally, we found that subjects with higher preferred walking speed have higher level of

mitochondrial respiration. Considering that these measurements are strong predictors of overall health and mortality (150), these findings may also support an essential role of mitochondria for overall health status. The results of this study are in agreement with other cross-sectional studies that tested the same associations (14, 18, 22). However, these previous studies were conducted in younger older adults, and had an age average of 70 to 78yrs. The present study was conducted in slightly older subjects (88.5±2.4), and included a total of 10 individuals that were older than 90yrs. This may suggest that even after very old age, when several neurological changes occur, mitochondrial function may still have an essential role on physical function.

We acknowledge that multiple comparisons were performed in this study in order to investigate the association between mitochondrial respiration (5 respiratory states) and myofiber cross-sectional area (4 outcome measures), intramyocellular lipid content (4 outcome measures), and physical function (total of 10 outcome measures). However, we opted to not statistically control for multiple comparisons due to the exploratory nature of the study.

3.5.1 Limitations

This is the first study to investigate mitochondrial respiration of permeabilized myofibers in a group of very old individuals. Despite recruiting 41 subjects to be part of this study, some of the physical function outcomes were only collected in a subset of the study population. Therefore, the sample size was relatively low for the number of associations tested. We addressed this issue by further investigating the association of mitochondrial respiration and measures of sarcopenia with group comparisons for the subjects in the higher and lower tertiles for mitochondrial respiration. However, future studies that include a larger sample are desired.

Another limitation of the study was that the population included was very homogeneous in terms of general health. We hypothesized that there would be more heterogeneity in mitochondrial capacity and physical function in these very old subjects. Future research that include a more heterogeneous population with pronounced physical function limitations would provide essential information on the association between mitochondrial function and sarcopenia. Additionally, in this study we were limited to investigate mitochondrial function by analysis of mitochondrial respiration. Futures studies that include a more comprehensive analysis of mitochondrial capacity, such as information on mitochondrial content, quality control, and production of reactive oxygen species are desired.

Moreover, we only investigated the influence of mitochondrial respiration on muscle mass and quality at the cellular level. Future studies that investigate the association of mitochondrial capacity and the whole muscle cross-sectional area are essential. Additionally, analysis of muscle quality including measures of intramuscular fat are required. Finally, the development of longitudinal studies that examine whether changes in muscle mass, and physical function are linked with changes in mitochondrial function is an essential step to establish the influence of mitochondria on sarcopenia.

3.6 CONCLUSION

Skeletal muscle mitochondrial respiration is associated with physical function at very old age. Future studies that incorporate additional measures of mitochondrial capacity and utilizes a longitudinal design should be performed, and will provide essential information on the role of mitochondrial function in sarcopenia.

4.0 SIGNIFICANCE AND DIRECTION OF FUTURE RESEARCH

Sarcopenia is a major public health concern as life expectancy continues to increase. The condition is a well-establish risk factor for several negative health-related conditions such as disability, frailty, institutionalization, and mortality (23). While numerous factors have been associated with the onset and development of sarcopenia, the primary mechanisms contributing to this process are still uncertain. Among the intramuscular factors hypothesized to play a determinant role on skeletal muscle aging is mitochondria. Elucidating the specific role of mitochondria on the age-related loss of muscle mass and function may assist in the development of preventive approaches and therapeutic interventions for sarcopenia, and improve quality of life in the older population.

In study 1 of this dissertation we investigated whether mitochondrial respiratory capacity and quality control are affected with chronological aging. Considering that aging is usually associated with decreased physical activity and higher adiposity, we also examined the influence of BMI and cardiorespiratory fitness on these associations. While age-related mitochondrial dysfunction has been a topic of investigation for several decades, results are contradictory and several questions remain to be elucidated. Our study was unique as included a group of 68 subjects with a wide range of age, BMI and cardiorespiratory fitness. Additionally, it examined changes in mitochondrial quality control with chronological aging, a topic investigated only in a limited number of human studies.

Our findings showed that cardiorespiratory fitness and BMI are more influential to mitochondrial capacity than age *per se*. While we observed no decreases in maximal mitochondrial respiration with chronological age, we found that both BMI and cardiorespiratory fitness play a significant role on this association. The findings of this study support the idea that improvements

in lifestyle, such as increased physical activity and better nutrition could benefit individuals by preventing decreases in mitochondrial respiration and alterations in mitochondrial quality control. Future longitudinal studies focusing on the lifelong effect of physical activity and body composition on mitochondrial function would be crucial to further investigate our findings. Additionally, future investigations on the topic should include additional measures of mitochondrial function, such as reactive oxygen species generation, ATP production, capacity for calcium uptake and others, to provide a more comprehensive understanding of mitochondrial capacity in aging.

In study 2 of this dissertation we investigated the association of mitochondrial function with sarcopenia. While several animal studies have provided strong evidence that there is a mechanistic link between mitochondrial function and loss of mass and strength (19-21), few studies have examined these associations in humans (14, 22). Study 2 was conducted to investigate the association between mitochondrial respiration and myofiber cross-sectional area, intramyocellular lipid content, and physical function in very old individuals. A cohort of very old subjects was selected because at this age decreases in muscle mass and physical function are pronounced and associations with mitochondria more likely to be established.

While we found no association between mitochondrial respiration and myofiber cross-sectional area and intramyocellular lipid content, mitochondrial respiration was associated with grip strength, knee extension torque and preferred gait speed, predictors of overall health and mortality. This is the first study, to our knowledge, that investigates mitochondrial respiration of permeabilized myofibers in a group of very old individuals. We were limited to evaluate the influence of mitochondrial respiration on muscle size and lipid content at the cellular level only. Future studies investigating these associations in the whole muscle cross-sectional area and quality

will complement the findings of this study, and provide a more comprehensive understanding of the influence of mitochondrial function to muscle mass. Additionally, studies with a longitudinal design should be performed to determine whether changes in muscle mass, strength and physical performance are linked with changes in mitochondrial function.

The results of this dissertation study provide a better understanding of the association between mitochondrial capacity and chronological aging, the age-related changes in mitochondrial quality control, the influence of cardiorespiratory fitness and BMI on mitochondrial capacity, and the association between mitochondrial function with muscle size, muscle function and physical performance.

We acknowledge that this dissertation study has limitations, such as the research design utilized (cross-sectional versus longitudinal) and different inclusion and exclusion criteria for each parent study. It would be extremely relevant to be able to compare mitochondrial capacity between the old group (Study#1 of this dissertation) and very old group (Study #2 of this dissertation). However, we were limited since we didn't have many common variables between them, such as cardiorespiratory fitness and measures of physical function.

The influence of mitochondrial function on measures of sarcopenia will be further investigated using additional data collected for the Health ABC study (study 2 of this dissertation). Analysis of whole muscle cross-sectional area as well as muscle quality acquired with computed tomography (CT) scan will be used to classify subjects with and without sarcopenia, and further investigate the association of mitochondrial function with the age-related loss of muscle mass and function. Moreover, additional measures of mitochondrial function, including mitochondrial content, ROS production, and expression of proteins that mediate mitochondrial fusion, fission

and autophagy will be evaluated to provide a more comprehensive analysis of mitochondrial capacity.

The translational initiative utilized in this dissertation study is an essential step to establish the possible role of mitochondria on age-related decreases in muscle mass and function, and it is the basis for the development of preventive approaches and effective rehabilitation for sarcopenia. The findings of this study contributes to the clinical field by providing evidence that physical activity and dietary habits play an essential role on mitochondrial capacity and physical function. Understanding the specific role of mitochondria on skeletal muscle aging will allow the development of rehabilitation that target the mitochondria.

BIBLIOGRAPHY

1. Clark BC, Manini TM. Sarcopenia =/= dynapenia. The journals of gerontology Series A, Biological sciences and medical sciences. 2008;**63**:829-834.

2. Clark BC, Manini TM. Functional consequences of sarcopenia and dynapenia in the elderly. Curr Opin Clin Nutr Metab Care. 2010;**13**:271-276.

3. Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, *et al.* Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People. Age and ageing. 2010;**39**:412-423.

4. Baumgartner RN, Koehler KM, Gallagher D, Romero L, Heymsfield SB, Ross RR, *et al.* Epidemiology of sarcopenia among the elderly in New Mexico. American journal of epidemiology. 1998;**147**:755-763.

5. Janssen I, Heymsfield SB, Ross R. Low relative skeletal muscle mass (sarcopenia) in older persons is associated with functional impairment and physical disability. Journal of the American Geriatrics Society. 2002;**50**:889-896.

6. Zoico E, Di Francesco V, Guralnik JM, Mazzali G, Bortolani A, Guariento S, *et al.* Physical disability and muscular strength in relation to obesity and different body composition indexes in a sample of healthy elderly women. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 2004;**28**:234-241.

7. Newman AB, Kupelian V, Visser M, Simonsick E, Goodpaster B, Nevitt M, *et al.* Sarcopenia: alternative definitions and associations with lower extremity function. Journal of the American Geriatrics Society. 2003;**51**:1602-1609.

8. Visser M, Kritchevsky SB, Goodpaster BH, Newman AB, Nevitt M, Stamm E, *et al.* Leg muscle mass and composition in relation to lower extremity performance in men and women aged 70 to 79: the health, aging and body composition study. Journal of the American Geriatrics Society. 2002;**50**:897-904.

9. Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol. 1956;**11**:298-300.

10. Harman D. The biologic clock: the mitochondria? Journal of the American Geriatrics Society. 1972;**20**:145-147.

11. Trounce I, Byrne E, Marzuki S. Decline in skeletal muscle mitochondrial respiratory chain function: possible factor in ageing. Lancet. 1989;**1**:637-639.

12. Cooper JM, Mann VM, Schapira AH. Analyses of mitochondrial respiratory chain function and mitochondrial DNA deletion in human skeletal muscle: effect of ageing. J Neurol Sci. 1992;**113**:91-98.

13. Boffoli D, Scacco SC, Vergari R, Solarino G, Santacroce G, Papa S. Decline with age of the respiratory chain activity in human skeletal muscle. Biochimica et biophysica acta. 1994;**1226**:73-82.

14. Joseph AM, Adhihetty PJ, Buford TW, Wohlgemuth SE, Lees HA, Nguyen LM, *et al.* The impact of aging on mitochondrial function and biogenesis pathways in skeletal muscle of sedentary high- and low-functioning elderly individuals. Aging cell. 2012;**11**:801-809.

15. Brierley EJ, Johnson MA, James OF, Turnbull DM. Effects of physical activity and age on mitochondrial function. QJM. 1996;**89**:251-258.

16. Barrientos A, Casademont J, Rotig A, Miro O, Urbano-Marquez A, Rustin P, *et al.* Absence of relationship between the level of electron transport chain activities and aging in human skeletal muscle. Biochemical and biophysical research communications. 1996;**229**:536-539.

17. Hutter E, Skovbro M, Lener B, Prats C, Rabol R, Dela F, *et al.* Oxidative stress and mitochondrial impairment can be separated from lipofuscin accumulation in aged human skeletal muscle. Aging cell. 2007;**6**:245-256.

18. Safdar A, Hamadeh MJ, Kaczor JJ, Raha S, Debeer J, Tarnopolsky MA. Aberrant mitochondrial homeostasis in the skeletal muscle of sedentary older adults. PLoS One. 2010;**5**:e10778.

19. Masiero E, Agatea L, Mammucari C, Blaauw B, Loro E, Komatsu M, *et al.* Autophagy is required to maintain muscle mass. Cell Metab. 2009;**10**:507-515.

20. Wenz T, Rossi SG, Rotundo RL, Spiegelman BM, Moraes CT. Increased muscle PGC-1alpha expression protects from sarcopenia and metabolic disease during aging. Proc Natl Acad Sci U S A. 2009;**106**:20405-20410.

21. Romanello V, Guadagnin E, Gomes L, Roder I, Sandri C, Petersen Y, *et al.* Mitochondrial fission and remodelling contributes to muscle atrophy. EMBO J. 2010;**29**:1774-1785.

22. Coen PM, Jubrias SA, Distefano G, Amati F, Mackey DC, Glynn NW, *et al.* Skeletal muscle mitochondrial energetics are associated with maximal aerobic capacity and walking speed in older adults. The journals of gerontology Series A, Biological sciences and medical sciences. 2013;**68**:447-455.

23. Visser M, Schaap LA. Consequences of sarcopenia. Clinics in geriatric medicine. 2011;**27**:387-399.

24. Marzetti E, Calvani R, Cesari M, Buford TW, Lorenzi M, Behnke BJ, *et al.* Mitochondrial dysfunction and sarcopenia of aging: from signaling pathways to clinical trials. Int J Biochem Cell Biol. 2013;**45**:2288-2301.

25. Kuznetsov AV, Veksler V, Gellerich FN, Saks V, Margreiter R, Kunz WS. Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. Nature protocols. 2008;**3**:965-976.

26. Aon MA. From isolated to networked: a paradigmatic shift in mitochondrial physiology. Frontiers in physiology. 2010;**1**:20.

27. Youle RJ, van der Bliek AM. Mitochondrial fission, fusion, and stress. Science. 2012;**337**:1062-1065.

28. McBride HM, Neuspiel M, Wasiak S. Mitochondria: more than just a powerhouse. Curr Biol. 2006;**16**:R551-560.

29. Jeong SY, Seol DW. The role of mitochondria in apoptosis. BMB Rep. 2008;**41**:11-22.

30. Sanz A, Pamplona R, Barja G. Is the mitochondrial free radical theory of aging intact? Antioxid Redox Signal. 2006;**8**:582-599.

31. Hroudova J, Singh N, Fisar Z. Mitochondrial dysfunctions in neurodegenerative diseases: relevance to Alzheimer's disease. Biomed Res Int. 2014;**2014**:175062.

32. Wohlgemuth SE, Calvani R, Marzetti E. The interplay between autophagy and mitochondrial dysfunction in oxidative stress-induced cardiac aging and pathology. J Mol Cell Cardiol. 2014;**71**:62-70.

33. Nassir F, Ibdah JA. Role of mitochondria in nonalcoholic fatty liver disease. Int J Mol Sci. 2014;**15**:8713-8742.

34. Martin SD, McGee SL. The role of mitochondria in the aetiology of insulin resistance and type 2 diabetes. Biochimica et biophysica acta. 2014;**1840**:1303-1312.

35. Gaude E, Frezza C. Defects in mitochondrial metabolism and cancer. Cancer Metab. 2014;**2**:10.

36. Pietrangelo L, D'Incecco A, Ainbinder A, Michelucci A, Kern H, Dirksen RT, *et al.* Age-dependent uncoupling of mitochondria from Ca2+ release units in skeletal muscle. Oncotarget. 2015.

37. Javadov S, Jang S, Rodriguez-Reyes N, Rodriguez-Zayas AE, Soto Hernandez J, Krainz T, *et al.* Mitochondria-targeted antioxidant preserves contractile properties and mitochondrial function of skeletal muscle in aged rats. Oncotarget. 2015.

38. Hiona A, Leeuwenburgh C. The role of mitochondrial DNA mutations in aging and sarcopenia: implications for the mitochondrial vicious cycle theory of aging. Experimental gerontology. 2008;**43**:24-33.

39. Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. Cell. 2005;**120**:483-495.

40. Poljsak B, Suput D, Milisav I. Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. Oxid Med Cell Longev. 2013;**2013**:956792.

41. Short KR, Bigelow ML, Kahl J, Singh R, Coenen-Schimke J, Raghavakaimal S, *et al.* Decline in skeletal muscle mitochondrial function with aging in humans. Proc Natl Acad Sci U S A. 2005;**102**:5618-5623.

42. Menshikova EV, Ritov VB, Fairfull L, Ferrell RE, Kelley DE, Goodpaster BH. Effects of exercise on mitochondrial content and function in aging human skeletal muscle. The journals of gerontology Series A, Biological sciences and medical sciences. 2006;**61**:534-540.

43. Drew B, Phaneuf S, Dirks A, Selman C, Gredilla R, Lezza A, *et al.* Effects of aging and caloric restriction on mitochondrial energy production in gastrocnemius muscle and heart. American journal of physiology Regulatory, integrative and comparative physiology. 2003;**284**:R474-480.

44. Mansouri A, Muller FL, Liu Y, Ng R, Faulkner J, Hamilton M, *et al.* Alterations in mitochondrial function, hydrogen peroxide release and oxidative damage in mouse hind-limb skeletal muscle during aging. Mechanisms of ageing and development. 2006;**127**:298-306.

45. Gouspillou G, Bourdel-Marchasson I, Rouland R, Calmettes G, Biran M, Deschodt-Arsac V, *et al.* Mitochondrial energetics is impaired in vivo in aged skeletal muscle. Aging cell. 2014;**13**:39-48.

46. Kerner J, Turkaly PJ, Minkler PE, Hoppel CL. Aging skeletal muscle mitochondria in the rat: decreased uncoupling protein-3 content. American journal of physiology Endocrinology and metabolism. 2001;**281**:E1054-1062.

47. Kumaran S, Panneerselvam KS, Shila S, Sivarajan K, Panneerselvam C. Age-associated deficit of mitochondrial oxidative phosphorylation in skeletal muscle: role of carnitine and lipoic acid. Mol Cell Biochem. 2005;**280**:83-89.

48. Chabi B, Ljubicic V, Menzies KJ, Huang JH, Saleem A, Hood DA. Mitochondrial function and apoptotic susceptibility in aging skeletal muscle. Aging cell. 2008;**7**:2-12.

49. Crane JD, Devries MC, Safdar A, Hamadeh MJ, Tarnopolsky MA. The effect of aging on human skeletal muscle mitochondrial and intramyocellular lipid ultrastructure. The journals of gerontology Series A, Biological sciences and medical sciences. 2010;**65**:119-128.

50. Conley KE, Jubrias SA, Esselman PC. Oxidative capacity and ageing in human muscle. J Physiol. 2000;**526 Pt 1**:203-210.

51. Orlander J, Kiessling KH, Larsson L, Karlsson J, Aniansson A. Skeletal muscle metabolism and ultrastructure in relation to age in sedentary men. Acta Physiol Scand. 1978;**104**:249-261.

52. Lanza IR, Short DK, Short KR, Raghavakaimal S, Basu R, Joyner MJ, *et al.* Endurance exercise as a countermeasure for aging. Diabetes. 2008;**57**:2933-2942.

53. Rooyackers OE, Adey DB, Ades PA, Nair KS. Effect of age on in vivo rates of mitochondrial protein synthesis in human skeletal muscle. Proc Natl Acad Sci U S A. 1996;**93**:15364-15369.

54. Proctor DN, Sinning WE, Walro JM, Sieck GC, Lemon PW. Oxidative capacity of human muscle fiber types: effects of age and training status. Journal of applied physiology. 1995;**78**:2033-2038.

55. Tonkonogi M, Fernstrom M, Walsh B, Ji LL, Rooyackers O, Hammarqvist F, *et al.* Reduced oxidative power but unchanged antioxidative capacity in skeletal muscle from aged humans. Pflugers Archiv : European journal of physiology. 2003;**446**:261-269.

56. Houmard JA, Weidner ML, Gavigan KE, Tyndall GL, Hickey MS, Alshami A. Fiber type and citrate synthase activity in the human gastrocnemius and vastus lateralis with aging. Journal of applied physiology. 1998;**85**:1337-1341.

57. McCully KK, Fielding RA, Evans WJ, Leigh JS, Jr., Posner JD. Relationships between in vivo and in vitro measurements of metabolism in young and old human calf muscles. Journal of applied physiology. 1993;**75**:813-819.

58. Larsen RG, Callahan DM, Foulis SA, Kent-Braun JA. Age-related changes in oxidative capacity differ between locomotory muscles and are associated with physical activity behavior. Appl Physiol Nutr Metab. 2012;**37**:88-99.

59. Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, *et al.* Mitochondrial dysfunction in the elderly: possible role in insulin resistance. Science. 2003;**300**:1140-1142.

60. Rasmussen UF, Krustrup P, Kjaer M, Rasmussen HN. Experimental evidence against the mitochondrial theory of aging. A study of isolated human skeletal muscle mitochondria. Experimental gerontology. 2003;**38**:877-886.

61. Rasmussen UF, Krustrup P, Kjaer M, Rasmussen HN. Human skeletal muscle mitochondrial metabolism in youth and senescence: no signs of functional changes in ATP formation and mitochondrial oxidative capacity. Pflugers Archiv : European journal of physiology. 2003;**446**:270-278.

62. Gouspillou G, Sgarioto N, Kapchinsky S, Purves-Smith F, Norris B, Pion CH, *et al.* Increased sensitivity to mitochondrial permeability transition and myonuclear translocation of endonuclease G in atrophied muscle of physically active older humans. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2014;**28**:1621-1633.

63. Chretien D, Gallego J, Barrientos A, Casademont J, Cardellach F, Munnich A, *et al.* Biochemical parameters for the diagnosis of mitochondrial respiratory chain deficiency in humans, and their lack of age-related changes. Biochem J. 1998;**329 (Pt 2)**:249-254.

64. Kent-Braun JA, Ng AV. Skeletal muscle oxidative capacity in young and older women and men. Journal of applied physiology. 2000;**89**:1072-1078.

65. Lanza IR, Befroy DE, Kent-Braun JA. Age-related changes in ATP-producing pathways in human skeletal muscle in vivo. Journal of applied physiology. 2005;**99**:1736-1744.

66. Lanza IR, Larsen RG, Kent-Braun JA. Effects of old age on human skeletal muscle energetics during fatiguing contractions with and without blood flow. J Physiol. 2007;**583**:1093-1105.

67. DeLany JP, Dube JJ, Standley RA, Distefano G, Goodpaster BH, Stefanovic-Racic M, *et al.* Racial Differences In Peripheral Insulin Sensitivity And Mitochondrial Capacity In The Absence Of Obesity. The Journal of clinical endocrinology and metabolism. 2014:jc20142512.

68. Kim JY, Hickner RC, Cortright RL, Dohm GL, Houmard JA. Lipid oxidation is reduced in obese human skeletal muscle. American journal of physiology Endocrinology and metabolism. 2000;**279**:E1039-1044.

69. Thyfault JP, Kraus RM, Hickner RC, Howell AW, Wolfe RR, Dohm GL. Impaired plasma fatty acid oxidation in extremely obese women. American journal of physiology Endocrinology and metabolism. 2004;**287**:E1076-1081.

70. Aon MA, Bhatt N, Cortassa SC. Mitochondrial and cellular mechanisms for managing lipid excess. Frontiers in physiology. 2014;**5**:282.

71. Wende AR, Symons JD, Abel ED. Mechanisms of lipotoxicity in the cardiovascular system. Current hypertension reports. 2012;**14**:517-531.

72. Walther TC, Farese RV, Jr. Lipid droplets and cellular lipid metabolism. Annual review of biochemistry. 2012;**81**:687-714.

73. Greenberg AS, Coleman RA. Expanding roles for lipid droplets. Trends in endocrinology and metabolism: TEM. 2011;**22**:195-196.

74. Greenberg AS, Coleman RA, Kraemer FB, McManaman JL, Obin MS, Puri V, *et al.* The role of lipid droplets in metabolic disease in rodents and humans. The Journal of clinical investigation. 2011;**121**:2102-2110.

75. Wojtczak L, Schonfeld P. Effect of fatty acids on energy coupling processes in mitochondria. Biochimica et biophysica acta. 1993;**1183**:41-57.

76. Itani SI, Ruderman NB, Schmieder F, Boden G. Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha. Diabetes. 2002;**51**:2005-2011.

77. Bruce CR, Thrush AB, Mertz VA, Bezaire V, Chabowski A, Heigenhauser GJ, *et al.* Endurance training in obese humans improves glucose tolerance and mitochondrial fatty acid oxidation and alters muscle lipid content. American journal of physiology Endocrinology and metabolism. 2006;**291**:E99-E107.

78. Bergman BC, Perreault L, Hunerdosse DM, Koehler MC, Samek AM, Eckel RH. Increased intramuscular lipid synthesis and low saturation relate to insulin sensitivity in endurance-trained athletes. Journal of applied physiology. 2010;**108**:1134-1141.

79. Lowell BB, Shulman GI. Mitochondrial dysfunction and type 2 diabetes. Science. 2005;**307**:384-387.

80. Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. The New England journal of medicine. 2004;**350**:664-671.

81. Dohm GL, Huston RL, Askew EW, Fleshood HL. Effects of exercise, training, and diet on muscle citric acid cycle enzyme activity. Canadian journal of biochemistry. 1973;**51**:849-854.

82. Holloszy JO, Oscai LB, Don IJ, Mole PA. Mitochondrial citric acid cycle and related enzymes: adaptive response to exercise. Biochemical and biophysical research communications. 1970;**40**:1368-1373.

83. Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, *et al.* Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2002;**16**:1879-1886.

84. Geng T, Li P, Okutsu M, Yin X, Kwek J, Zhang M, *et al.* PGC-1alpha plays a functional role in exercise-induced mitochondrial biogenesis and angiogenesis but not fiber-type transformation in mouse skeletal muscle. American journal of physiology Cell physiology. 2010;**298**:C572-579.

85. Ding H, Jiang N, Liu H, Liu X, Liu D, Zhao F, *et al.* Response of mitochondrial fusion and fission protein gene expression to exercise in rat skeletal muscle. Biochimica et biophysica acta. 2010;**1800**:250-256.

86. Cartoni R, Leger B, Hock MB, Praz M, Crettenand A, Pich S, *et al.* Mitofusins 1/2 and ERRalpha expression are increased in human skeletal muscle after physical exercise. The Journal of physiology. 2005;**567**:349-358.

87. Perry CG, Lally J, Holloway GP, Heigenhauser GJ, Bonen A, Spriet LL. Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. The Journal of physiology. 2010;**588**:4795-4810.

88. Smuder AJ, Kavazis AN, Min K, Powers SK. Exercise protects against doxorubicin-induced markers of autophagy signaling in skeletal muscle. Journal of applied physiology. 2011;**111**:1190-1198.

89. Ringholm S, Bienso RS, Kiilerich K, Guadalupe-Grau A, Aachmann-Andersen NJ, Saltin B, *et al.* Bed rest reduces metabolic protein content and abolishes exercise-induced mRNA responses in human skeletal muscle. American journal of physiology Endocrinology and metabolism. 2011;**301**:E649-658.

90. Zampieri S, Pietrangelo L, Loefler S, Fruhmann H, Vogelauer M, Burggraf S, *et al.* Lifelong Physical Exercise Delays Age-Associated Skeletal Muscle Decline. The journals of gerontology Series A, Biological sciences and medical sciences. 2014.

91. Toledo FG, Menshikova EV, Azuma K, Radikova Z, Kelley CA, Ritov VB, *et al.* Mitochondrial capacity in skeletal muscle is not stimulated by weight loss despite increases in insulin action and decreases in intramyocellular lipid content. Diabetes. 2008;**57**:987-994.

92. Toledo FG, Watkins S, Kelley DE. Changes induced by physical activity and weight loss in the morphology of intermyofibrillar mitochondria in obese men and women. The Journal of clinical endocrinology and metabolism. 2006;**91**:3224-3227.

93. Coen PM, Menshikova EV, Distefano G, Zheng D, Tanner CJ, Standley RA, *et al.* Exercise and Weight Loss Improve Muscle Mitochondrial Respiration, Lipid Partitioning and Insulin Sensitivity Following Gastric Bypass Surgery. Diabetes. 2015.

94. Lanza IR, Nair KS. Mitochondrial function as a determinant of life span. Pflugers Archiv : European journal of physiology. 2010;**459**:277-289.

95. Gnaiger E. Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply. Respiration physiology. 2001;**128**:277-297.

96. Hutter E, Unterluggauer H, Garedew A, Jansen-Durr P, Gnaiger E. High-resolution respirometry--a modern tool in aging research. Experimental gerontology. 2006;**41**:103-109.

97. Picard M, Ritchie D, Wright KJ, Romestaing C, Thomas MM, Rowan SL, *et al.* Mitochondrial functional impairment with aging is exaggerated in isolated mitochondria compared to permeabilized myofibers. Aging cell. 2010;**9**:1032-1046.

98. Twig G, Hyde B, Shirihai OS. Mitochondrial fusion, fission and autophagy as a quality control axis: the bioenergetic view. Biochimica et biophysica acta. 2008;**1777**:1092-1097.

99. Luce K, Weil AC, Osiewacz HD. Mitochondrial protein quality control systems in aging and disease. Advances in experimental medicine and biology. 2010;**694**:108-125.

100. Ono T, Isobe K, Nakada K, Hayashi JI. Human cells are protected from mitochondrial dysfunction by complementation of DNA products in fused mitochondria. Nature genetics. 2001;**28**:272-275.

101. Eura Y, Ishihara N, Yokota S, Mihara K. Two mitofusin proteins, mammalian homologues of FZO, with distinct functions are both required for mitochondrial fusion. Journal of biochemistry. 2003;**134**:333-344.

102. Misaka T, Miyashita T, Kubo Y. Primary structure of a dynamin-related mouse mitochondrial GTPase and its distribution in brain, subcellular localization, and effect on mitochondrial morphology. The Journal of biological chemistry. 2002;**277**:15834-15842.

103. Twig G, Elorza A, Molina AJ, Mohamed H, Wikstrom JD, Walzer G, *et al.* Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. The EMBO journal. 2008;**27**:433-446.

104. Smirnova E, Griparic L, Shurland DL, van der Bliek AM. Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. Molecular biology of the cell. 2001;**12**:2245-2256.

105. Mozdy AD, McCaffery JM, Shaw JM. Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. The Journal of cell biology. 2000;**151**:367-380.

106. Kroemer G, Marino G, Levine B. Autophagy and the integrated stress response. Molecular cell. 2010;**40**:280-293.

107. Ding WX, Yin XM. Mitophagy: mechanisms, pathophysiological roles, and analysis. Biological chemistry. 2012;**393**:547-564.

108. Iqbal S, Ostojic O, Singh K, Joseph AM, Hood DA. Expression of mitochondrial fission and fusion regulatory proteins in skeletal muscle during chronic use and disuse. Muscle & nerve. 2013;**48**:963-970.

109. Konopka AR, Suer MK, Wolff CA, Harber MP. Markers of human skeletal muscle mitochondrial biogenesis and quality control: effects of age and aerobic exercise training. The journals of gerontology Series A, Biological sciences and medical sciences. 2014;**69**:371-378.

110. Rajawat YS, Hilioti Z, Bossis I. Aging: central role for autophagy and the lysosomal degradative system. Ageing research reviews. 2009;**8**:199-213.

111. Wohlgemuth SE, Seo AY, Marzetti E, Lees HA, Leeuwenburgh C. Skeletal muscle autophagy and apoptosis during aging: effects of calorie restriction and life-long exercise. Experimental gerontology. 2010;**45**:138-148.

112. Russ DW, Krause J, Wills A, Arreguin R. "SR stress" in mixed hindlimb muscles of aging male rats. Biogerontology. 2012;**13**:547-555.

113. Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, *et al.* Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell. 2004;**117**:399-412.

114. Tong JF, Yan X, Zhu MJ, Du M. AMP-activated protein kinase enhances the expression of muscle-specific ubiquitin ligases despite its activation of IGF-1/Akt signaling in C2C12 myotubes. Journal of cellular biochemistry. 2009;**108**:458-468.

115. Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del Piccolo P, *et al.* FoxO3 controls autophagy in skeletal muscle in vivo. Cell metabolism. 2007;**6**:458-471.

116. Bureau USC. Projections of the total resident population by 5-year age groups, and sex with special age categories: middle series, 1999 to 2000.; 2000.

117. Bureau USC. Projections of the total resident population by 5-year age groups, and sex with special age categories: middle series, 2025 to 2045. Washington D.C.; 2000.

118. Calvani R, Joseph AM, Adhihetty PJ, Miccheli A, Bossola M, Leeuwenburgh C, *et al.* Mitochondrial pathways in sarcopenia of aging and disuse muscle atrophy. Biol Chem. 2013;**394**:393-414.

119. Goodpaster BH, Park SW, Harris TB, Kritchevsky SB, Nevitt M, Schwartz AV, *et al.* The loss of skeletal muscle strength, mass, and quality in older adults: the health, aging and body composition study. The journals of gerontology Series A, Biological sciences and medical sciences. 2006;**61**:1059-1064.

120. Metter EJ, Lynch N, Conwit R, Lindle R, Tobin J, Hurley B. Muscle quality and age: cross-sectional and longitudinal comparisons. The journals of gerontology Series A, Biological sciences and medical sciences. 1999;**54**:B207-218.

121. Roth SM, Ferrell RF, Hurley BF. Strength training for the prevention and treatment of sarcopenia. J Nutr Health Aging. 2000;**4**:143-155.

122. Santanasto AJ, Glynn NW, Jubrias SA, Conley KE, Boudreau RM, Amati F, *et al.* Skeletal Muscle Mitochondrial Function and Fatigability in Older Adults. The journals of gerontology Series A, Biological sciences and medical sciences. 2014.

123. Porter C, Hurren NM, Cotter MV, Bhattarai N, Reidy PT, Dillon EL, *et al.* Mitochondrial respiratory capacity and coupling control decline with age in human skeletal muscle. American journal of physiology Endocrinology and metabolism. 2015:ajpendo 00125 02015.

124. Larsen S, Hey-Mogensen M, Rabol R, Stride N, Helge JW, Dela F. The influence of age and aerobic fitness: effects on mitochondrial respiration in skeletal muscle. Acta Physiol (Oxf). 2012;**205**:423-432.

125. Gram M, Vigelso A, Yokota T, Hansen CN, Helge JW, Hey-Mogensen M, *et al.* Two weeks of one-leg immobilization decreases skeletal muscle respiratory capacity equally in young and elderly men. Experimental gerontology. 2014;**58**:269-278.

126. Campello S, Scorrano L. Mitochondrial shape changes: orchestrating cell pathophysiology. EMBO reports. 2010;**11**:678-684.

127. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. The Journal of pathology. 2010;**221**:3-12.

128. Peterson CM, Johannsen DL, Ravussin E. Skeletal muscle mitochondria and aging: a review. J Aging Res. 2012;**2012**:194821.

129. Seo AY, Joseph AM, Dutta D, Hwang JC, Aris JP, Leeuwenburgh C. New insights into the role of mitochondria in aging: mitochondrial dynamics and more. Journal of cell science. 2010;**123**:2533-2542.

130. Dube JJ, Coen PM, DiStefano G, Chacon AC, Helbling NL, Desimone ME, *et al.* Effects of acute lipid overload on skeletal muscle insulin resistance, metabolic flexibility, and mitochondrial performance. American journal of physiology Endocrinology and metabolism. 2014;**307**:E1117-1124.

131. Thompson WR GN, Pescatello LS. ACSM's Guidlines for Exercise Testing and Prescription. 8 ed. Philadelphia, PA: Wolters Kluwer/Lippincott Williams & Wilkins; 2010.

132. Pruchnic R, Katsiaras A, He J, Kelley DE, Winters C, Goodpaster BH. Exercise training increases intramyocellular lipid and oxidative capacity in older adults. American journal of physiology Endocrinology and metabolism. 2004;**287**:E857-862.

133. Coen PM, Hames KC, Leachman EM, DeLany JP, Ritov VB, Menshikova EV, *et al.* Reduced skeletal muscle oxidative capacity and elevated ceramide but not diacylglycerol content in severe obesity. Obesity. 2013;**21**:2362-2371.

134. Lanza IR, Sreekumaran Nair K. Regulation of skeletal muscle mitochondrial function: genes to proteins. Acta physiologica (Oxford, England). 2010;**199**:529-547.

135. Gnaiger E. Mitochondrial pathways and respiratory control. An introduction to OXPHOS analysis. In: Network MP, ed. Innsbruck: OROBOROS MiPNet Publications; 2012:64.

136. Gnaiger E. Capacity of oxidative phosphorylation in human skeletal muscle: new perspectives of mitochondrial physiology. The international journal of biochemistry & cell biology. 2009;**41**:1837-1845.

137. Bori Z, Zhao Z, Koltai E, Fatouros IG, Jamurtas AZ, Douroudos, II, *et al.* The effects of aging, physical training, and a single bout of exercise on mitochondrial protein expression in human skeletal muscle. Experimental gerontology. 2012;**47**:417-424.

138. Konopka AR, Suer MK, Wolff CA, Harber MP. Markers of Human Skeletal Muscle Mitochondrial Biogenesis and Quality Control: Effects of Age and Aerobic Exercise Training. The journals of gerontology Series A, Biological sciences and medical sciences. 2013.

139. Fry CS, Drummond MJ, Glynn EL, Dickinson JM, Gundermann DM, Timmerman KL, *et al.* Skeletal muscle autophagy and protein breakdown following resistance exercise are similar in younger and older adults. The journals of gerontology Series A, Biological sciences and medical sciences. 2013;**68**:599-607.

140. Caffin F, Prola A, Piquereau J, Novotova M, David DJ, Garnier A, *et al.* Altered skeletal muscle mitochondrial biogenesis but improved endurance capacity in trained OPA1-deficient mice. The Journal of physiology. 2013;**591**:6017-6037.

141. Lira VA, Okutsu M, Zhang M, Greene NP, Laker RC, Breen DS, *et al.* Autophagy is required for exercise training-induced skeletal muscle adaptation and improvement of physical performance. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2013;**27**:4184-4193.

142. Gallagher D, Visser M, Sepulveda D, Pierson RN, Harris T, Heymsfield SB. How useful is body mass index for comparison of body fatness across age, sex, and ethnic groups? American journal of epidemiology. 1996;**143**:228-239.

143. Hughes VA, Frontera WR, Wood M, Evans WJ, Dallal GE, Roubenoff R, *et al.* Longitudinal muscle strength changes in older adults: influence of muscle mass, physical activity, and health. The journals of gerontology Series A, Biological sciences and medical sciences. 2001;**56**:B209-217.

144. Cheema N, Herbst A, McKenzie D, Aiken JM. Apoptosis and necrosis mediate skeletal muscle fiber loss in age-induced mitochondrial enzymatic abnormalities. Aging cell. 2015.

145. Coldham F, Lewis J, Lee H. The reliability of one vs. three grip trials in symptomatic and asymptomatic subjects. Journal of hand therapy : official journal of the American Society of Hand Therapists. 2006;**19**:318-326; quiz 327.

146. Hogrel JY. Grip strength measured by high precision dynamometry in healthy subjects from 5 to 80 years. BMC musculoskeletal disorders. 2015;**16**:139.

147. Goldberg A, Chavis M, Watkins J, Wilson T. The five-times-sit-to-stand test: validity, reliability and detectable change in older females. Aging clinical and experimental research. 2012;**24**:339-344.

148. Goodpaster BH, Theriault R, Watkins SC, Kelley DE. Intramuscular lipid content is increased in obesity and decreased by weight loss. Metabolism: clinical and experimental. 2000;**49**:467-472.

149. Beltran Valls MR, Wilkinson DJ, Narici MV, Smith K, Phillips BE, Caporossi D, *et al.* Protein carbonylation and heat shock proteins in human skeletal muscle: relationships to age and sarcopenia. The journals of gerontology Series A, Biological sciences and medical sciences. 2015;**70**:174-181.

150. Bohannon RW. Hand-grip dynamometry predicts future outcomes in aging adults. Journal of geriatric physical therapy. 2008;**31**:3-10.