Mitochondrial Biology in sporadic Inclusion Body Myositis

Dissertation

Submitted to the Graduate Faculty of Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy
In

Human Nutrition, Foods and Exercise

by

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March 19, 2014
Blacksburg,VA

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Keywords: Amyloid beta; Inclusion Body Myositis; Mitochondria

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ABSTRACT

Sporadic Inclusion Body Myositis (sIBM) is an inflammatory muscle disease that strikes individuals at random and accounts for approximately 1/3 of all idiopathic inflammatory myopathies. It is characterized by progressive weakness of distal and proximal muscles and is the most common muscle disorder in individuals over 50 years of age. Currently, there is no known cause, cure, or enduring treatment for sIBM, although a number of theories as to its cause have been proposed. One theory proposes that activation of the inflammatory/ immune response is the primary trigger resulting in muscle degeneration and protein abnormalities, while an alternative theory suggests that sIBM is a degenerative muscle disease with abnormal pathogenic protein accumulation, in particular Abeta, being a primary cause that triggers an inflammatory/ immune response. Mitochondrial abnormalities have been observed in skeletal muscle from patients diagnosed with the disease, however the role of the mitochondria in disease pathology is still unclear. The aim of this dissertation was to evaluate: 1) the role of the mitochondria in the development of sIBM and 2) the role of amyloid beta on mitochondrial function in skeletal muscle. A better understanding of the role of the mitochondria in the development of sIBM may help to identify novel prevention and/ or treatment strategies.

ACKNOWLEDGMENTS

I could not have completed this dissertation without the priceless support of a several very important individuals. I would like to start off by giving the most heartfelt and meaningful thank you of my entire life to my Ph.D. mentor, Dr. Madlyn Frisard. I am extremely honored and grateful for having had the opportunity to work for her. Her patience, motivation, wisdom, genuine caring and concern, guidance, and faith in me during the dissertation process has been the single most important element in helping me to accomplish earning this Ph.D. Madlyn has selflessly spent her time constantly guiding and educating me without losing sight of the need to mix compassion, fun, and laughter into the learning process. Madlyn you will always remain to be my role model; not just as an accomplished and hardworking scientist, but also as an example as a person that I wish to emulate in life.

Next, I would like to express my gratitude and deep appreciation for Dr. Matt Hulver. He supported me in countless ways throughout my Ph.D. Thank you Matt for providing me with so many opportunities; without your belief in me, and those opportunities, I certainly would not be where I am today.

I would also like to thank Dr. Kevin Davy for his continued encouragement, support and advice during my Ph.D. I am also very grateful to Dr. Richard Helm for his

scientific advice and the many insightful discussions and suggestions he provided for my dissertation.

My success would not have been possible if it were not a result of the support and friendship of each and every member of the Frisard/Hulver lab. In particular, I would like to thank Dr. Ryan McMillian for all his help and contributions to this project.

I would also like to thank my parents, Golnar and Farid, for providing the support and guidance I needed to pursue my dreams. I am also thankful to the rest of my family, Elham and James for their love and constant support throughout this journey. I am also very grateful to Adam for all his support, encouragement, and love during the last stages of my Ph.D., I am glad to have experienced this with you.

I would also like to thank all the members of the HNFE department at Virginia Tech. I have had the best four years of my life here within this department and I am proud to have been a part of it.

I dedicate this dissertation to all of you, without whom I would not have made it this far!

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CHAPTER I

INTRODUCTION

Sporadic Inclusion Body Myositis (sIBM) is an inflammatory muscle disease that strikes individuals at random and accounts for approximately 1/3 of all idiopathic inflammatory myopathies. It is characterized by progressive weakness of distal and proximal muscles and is the most common muscle disorder in individuals over 50 years of age. Because older adults are expected to comprise as much as 20% of the US population by 2030, the number of older adults with sIBM is likely to increase and in turn, sIBM will likely become an even greater public health concern in the future (*1*).

There are two known types of IBM. Sporadic inclusion body myositis is the most common form of IBM that generally occurs in older individuals. The second type is hereditary inclusion body myopathy, also known as inclusion body myopathy 2, which refers to a group of genetic, generally neuromuscular disorders, characterized by muscle weakness, with varying symptoms that develop in young adults (*2*). This review will focus on sIBM.

Currently there is no known cause or cure for sIBM. However, there are two prevailing, but somewhat contrasting theories. One theory proposes that activation of the inflammatory/ immune response is the primary trigger resulting in muscle degeneration and protein abnormalities (*3*). However, the fact that the disease is resistant to immunotherapy is a limitation to this idea. An alternative theory suggests that sIBM is a degenerative muscle disease with abnormal pathogenic protein accumulation, in particular Amyloid beta (Aβ), being a primary cause that triggers an inflammatory/ immune response (*4*). However, this hypothesis is quite controversial and it has been

suggested that methodical issues as well as misinterpretation of data has propagated an incorrect hypothesis (*5*). A third hypothesis, which is more of a combination of the first two, proposes that the autoimmune and degenerative aspects of the disease occur in parallel. It is not clear from the literature which aspects of the disease, inflammation or muscle degeneration occur first, if one causes the other, or if some other factor, such as a viral or bacterial infection, causes both. However, it is likely that sIBM results from the interaction of a number of factors, both genetic and environmental (*6, 7*).

sIBM is characterized by progressive weakness and wasting of the proximal and distal muscles ultimately resulting in restricted movement and mobility. Individuals afflicted with the disorder may not be able to perform activities of daily living and are often confined to the use of a walking aid or wheelchair (*3*). Though not considered a fatal disease, sIBM can have complications, such as falling, that can often be life altering or even fatal. Other characteristics of sIBM include respiratory dysfunction, aspiration, and cachexia. Patients with sIBM may have difficulty swallowing, especially solid food, and may suffer from severe dysphagia, which can introduce food into the lungs and cause death. Average time to wheelchair use for patients is approximately 14-16 years after initial symptom onset. The rate of sIBM progression is not the same in all patients and depends on a number of factors. Disease progression tends to be faster in men than in women and within the same individual proximal and distal limb muscles display faster decline than other muscle groups. Other affected muscles include finger flexors, quadriceps and neck muscles (*8-10*). Preventing falls and tackling the complications of being in a wheelchair are vital for sIBM patients. In some cases of sIBM, weakness of the facial muscles also occurs leading to complications with chewing and talking (*11*).

Currently, there are no long-term treatment therapies (*12*). Patients do not generally respond to anti-inflammatory, immunosuppressant, or immunomodulatory drugs and treatment of the disease usually includes management of symptoms and utilizing therapy to preserve mobilization (*4, 8, 13*).

Muscle disorders can be difficult to diagnose, especially in the initial stages and sIBM is most often misdiagnosed for other diseases with polymyositis being one example. A neurologist or rheumatologist is often needed and generally uses blood creatine kinase, electromyography, or muscle biopsies to confirm diagnoses. Skeletal muscle from patients diagnosed with sIBM is characterized by necrotic, "red, ragged fibers," assessed by Gomori trichrome staining, cytochrome-c oxidase deficient fibers, accumulation of pathogenic protein aggregates including amyloid beta, tau protein, α-syn, parkin, ubiquitin, and the accumulation of immune cells such as macrophages and T cells (*14*).

Skeletal muscle from patients diagnosed with sIBM also displays gross mitochondrial abnormalities including mitochondrial DNA deletions, morphological deformities and fragmentation, and inclusion bodies, although it is not known whether mitochondrial abnormalities are significant to disease progression or just a secondary side effect to some other primary cause. Furthermore, although amyloid beta has been implicated in the development of sIBM, this area is still quite controversial (*12*) and the effects of amyloid beta on mitochondrial function are not yet known.

CHAPTER II

REVIEW OF LITERATURE

2.1. Research/key questions

1) What is the role of mitochondrial biology in the development of sIBM?

2) What is the role of amyloid beta on mitochondrial function in skeletal muscle?

2.2. Search Methods

The databases PUBMED, Web of Science and Health Source (nursing and Academic Edition) were systematically searched. The search terms included 'spontaneous OR sporadic inclusion body myositis' OR 'sIBM AND mitochondria', from which the duplicates from each search engines were excluded. The second key word search included 'mitochondria AND amyloid beta AND skeletal muscle'.

2.3. Inclusion and exclusion criteria

Studies were screened according to defined inclusion and exclusion criteria. Inclusion criteria included published peer-reviewed research articles, English language, human participants or tissue samples of all ages and both genders, and studies conducted only in skeletal muscle. Due to the limited availability of eligible studies, exclusion criteria were kept to a minimum.

2.4. Search Results

PUBMED, Web of Science and Health Source (nursing and Academic Edition) were the databases systematically searched for this review. The search terms 'spontaneous OR sporadic inclusion body myositis and mitochondria ' resulted in a total of 24 articles in PUBMED, 29 articles in Web of Science, and 70 articles in Health Source. A second key word search, 'mitochondria AND amyloid beta AND skeletal muscle' conducted in the same databases resulted in 3 articles. Duplicates were removed and articles were evaluated based on inclusion/ exclusion criteria. A total of 9 studies met inclusion criteria and were included in this review.

2.5. Content results

1) What is the role of the mitochondria in the development of sIBM? Does mitochondrial dysfunction contribute to disease development?

Mitochondria are dynamics organelles that play a significant role in cell survival, not only as a major site of ATP production, but by regulating energy metabolism, protein turnover, cellular proliferation, and apoptosis (*15, 16*). Not surprisingly, defects in this organelle has a profound impact on cellular function and is reported to be a key underlying mechanism in the development of a number of diseases including diabetes, heart disease, Alzheimer's, and Parkinson's as well as contributing to the aging process (*17-19*).

The structure of the mitochondria consists of an outer and inner membrane, both of which are composed of phospholipid bilayers and proteins (*20*) (Figure 1). The

properties of these two membranes are distinct since the outer membrane needs to be freely permeable to small molecules. The inner mitochondrial membrane contains proteins responsible for oxidative phosphorylation and ATP synthesis. Additionally, the inner membrane is compartmentalized into numerous cristae, which expands the surface area of the inner mitochondrial membrane, enhancing its ability to produce ATP. The matrix contains a highly concentrated mixture of enzymes, mitochondrial ribosomes, mRNA, and mitochondrial DNA (*21, 22*).

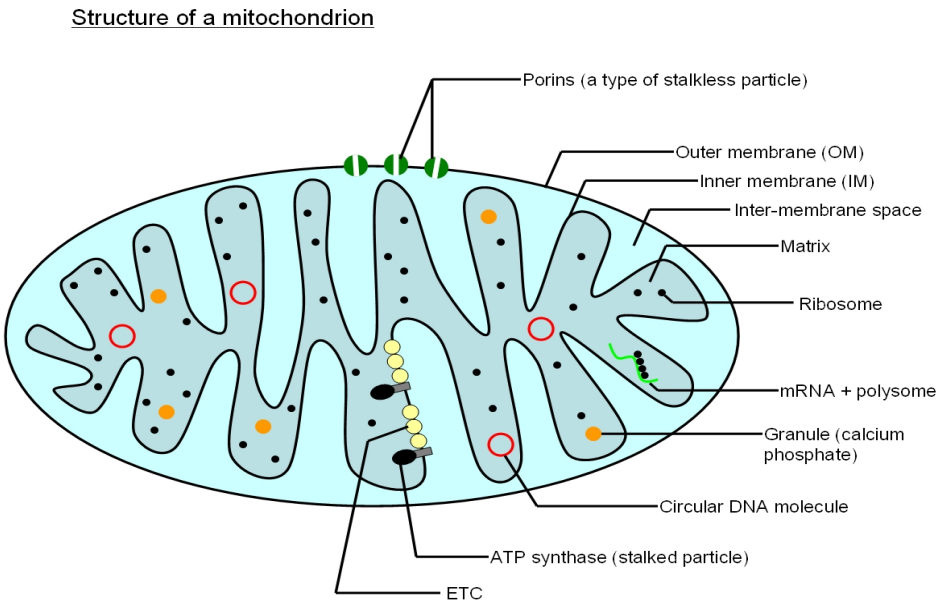

FIGURE ONE: *(a) The structure of the mitochondria consist of an outer and inner membrane. The inner membrane consists of numerous folds called cristae, which are able to increase the surface area of the membrane to increase the capacity for ATP production. Mitochondria also consist of Ribosomes, mitochondrial DNA, and matrix granules.*

The number of mitochondria in a cell depends on the function of the cell and varies widely with organism and tissue type. Mitochondria biogenesis is stimulated by energy demand, and therefore cells with higher energy requirements contain a higher number of mitochondria (*23*). The cells in the brain, skeletal muscle, heart, and the eye contain the highest number of mitochondria (as many as 10,000 per cell) while skin cells contain only a few hundred mitochondria (*24*). One striking physiological characteristic of skeletal muscle is a capacity to rapidly modulate rate of energy production in response to contraction or locomotion (*25*). Therefore, the mitochondria in this tissue must undergo rapid morphological and functional alterations in response to changes in usage and environmental conditions. Not surprisingly, highly endurance athletes have been described as having increased mitochondrial content and cristae (*26*).

Additionally, skeletal muscle can be divided into two primary fiber types categorized in part by the number of mitochondria they contain. Type I fibers, also known as slow, oxidative fibers contain a greater number of mitochondria, while type II fibers, also known as fast, glycolytic fibers have much less mitochondria.

Mitochondrial function is dependent on a number of factors and mitochondrial dysfunction has been linked to the development of a number of diseases. For example, insulin resistance and type 2 diabetes are associated with impairments in mitochondrial function including reduced organelle size and number, disorganized ultra structure, increased reactive oxygen species production, and reduced ATP synthesis (*27-29*). Aging is also characterized by a reduction in mitochondrial content as well as impaired intrinsic activity of mitochondrial machinery (*30*); these impairments are proposed

triggering events for apoptotic signaling in the pathogenesis of myocyte loss and sarcopenia observed with aging (*31*).

Mitochondrial biogenesis is complex and requires the coordinated interaction of membrane synthesis, protein synthesis and import, and replication of mitochondrial DNA (*31*). Mitochondria biogenesis involves the integration of both nuclear and mitochondrial encoded genes. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) co- activates Nuclear Respiratory Factors 1 and 2 (NRF-1 and NRF-2, which in turn activate Transcription factor A mitochondrial (TFAM). TFAM is directly responsible for transcribing nuclear-encoded mitochondrial DNA resulting in the expression of a number of mitochondrial proteins including those involved in mitochondrial structure and the maintenance of the mitochondrial genome. Previous studies have also shown that NRF-1 has transcription factor binding sites in genes that encode proteins of the respiratory chain, F_0F_1 ATP synthase, heme biosynthesis, and mitochondrial protein import machinary (*30*).

AMP-activated protein kinase (*18*) and calcium/calmodulin-activated kinase (*19*) have been known to stimulate PGC-1 expression, just to name a few. In addition, p38 MAP kinase, which is activated by a range of cellular stresses including osmotic shock, inflammatory cytokines, lipopolysaccharides (LPS), ultraviolet light, and growth factors, results in an increase of phosphorylation of PGC-1 (*31*).

Mitophagy was coined by LeMasters to describe the removal of mitochondria by autophagy (*31*). Mitophagy is a form of autophagy in which cells selectively indicate the degradation process of the dysfunctional or damaged mitochondria through vacuolar

engulfment and digestion. Although the mechanisms governing mitophagy are complex and not the focus of this review, mitochondrial depolarization appears to be a requirement for mitophagy activation and it is suggested that fragmented mitochondria, because of their reduced size, are more readily taken up by autophagosomes (*32*). Together, mitophagy and mitochondrial biogenesis maintain mitochondrial quality control by removing damaged, dysfunctional mitochondria from the mitochondrial pool and stimulating the formation of new mitochondria, respectively (*33*).

Mitochondrial dynamics is a concept that includes mitochondrial movement within the cell, alterations in mitochondrial morphology, and mitochondrial interactions controlled by fusion/ fission events (*34*). The importance of these events has recently become evident with the identification of genes directly involved in fusion (MFN1 and 2, OPA1) and fission (DRP1, FIS1) (*35-41*). Pharmacologic or genetic manipulation of either results in mitochondrial abnormalities including increased mitochondrial heterogeneity, reduced oxygen consumption and ATP synthesis, and mtDNA dyshomeostasis (*29, 35*). Furthermore, down regulation and up regulation of DRP1 results in a suppression and augmentation of mitophagy, respectively (*29*). Twig et al, demonstrated that mitochondria that have undergone fusion and fission generally end up in either one of two daughter populations: a depolarized population that will not repeat the fusion/ fission cycle and a population that maintains membrane potential ($\Delta\psi$), and therefore undergoes subsequent cycles of fusion/ fission (*42*). Interestingly, many mitochondria that do not reenter the fusion/ fission cycle end up in autophagosomes for digestion and recycling (*42-44*). It is also suggested that mitochondrial dynamics are involved in the regulation of mitochondrial biogenesis. Inhibition of DRP1 and

mitochondrial division results in reduction in mitochondrial biogenesis and ultimately cell survival (*45*).

There is limited data examining mitochondrial biology in sIBM, but what is available reveals reduced capacity for ATP production, altered morphology and fragmentation, and inclusion bodies (*3, 46-49*). For example, Santorelli et al, (*50*) examined mitochondrial abnormalities in 56 patients diagnosed with sIBM. Their work demonstrated multiple mtDNA deletions in skeletal muscle fibers in 73% of the participants that was associated with ragged-red muscle fibers and cytochrome c oxidase-deficient fibers. Additionally, these effects were observed in conjunction with a reduction in enzyme activity of citrate synthase and complexes I and IV of the electron transport chain. Although aging accounted for a small percentage of mtDNA deletions, the incidence was significantly higher in sIBM patients compared to healthy, elderly individuals. Oldfors et al (*47*) also demonstrated multiple mtDNA deletions among different segments of the same muscle fiber. Their results showed that the majority of muscle fibers in large muscles of most sIBM patients included one or more cytochrome c oxidase (COX)-deficient segments. What's more, these COX deficient fibers were present in the same sections of muscle fibers also containing mtDNA deletions. Schröder et al (*46*) used data retrieval analysis to assess mitochondrial abnormalities in 7,225 muscle cases comprising inflammatory myopathies including polymyositis, systemic scleroderma, vasculitis, and sIBM. Mitochondrial abnormalities were defined as mitochondrial DNA deletions using PCR and Southern blotting techniques. From the 7255 cases electronically analyzed, 8 met all of the inclusion criteria. These 8 included 4 sIBM cases, 2 polymyositis cases, one case of systematic scleoderma, and one case of

vasculitis. In these selected 8 cases, authors noted that severe mtDNA deletions occurred only within the sIBM cases.

Rifai et al (*51*) examined the presence of ragged red fibers, in skeletal muscle samples from 15 young and 13 elderly healthy adults and 27 patients with sIBM, polymyositis, or dermatomyositis. Ragged, red fibers (RRFs) are a pathologic finding in which muscle fibers appear grossly ragged due to peripherally located subsarcolemmal collections of mitochondria that appear red with staining. They are present in a wide variety of muscle diseases and are particularly characteristic of mitochondrial myopathies. The frequency of RFFs in this study was determined using succinate dehydrogenase staining, which is a more sensitive staining technique when compared to the traditional Gomori trichrome stain in detecting accumulation of damaged mitochondria in muscle fibers. Their results demonstrated that there were a significantly higher percentage of RFFs in the sIBM samples compared to aged-matched control samples or young samples. However, the frequency of RRFs in patients with polymyositis or dermatomyositis was comparable to that of age-matched normal control subjects. In addition, the authors also observed reduced COX content in fibers from sIBM subjects. The high percentage of RRFs in sIBM samples suggest that mitochondrial function may be impaired in this disease, however Rifai et al, comment that although RRFs are an important characteristic of sIBM disease, mitochondrial dysfunction still may only be a secondary side effect of the disease.

Temiz et al (*3*) compared the clinical topographies and muscle biopsy features of patients with polymyositis (steroid responsive inflammatory myopathy; n=12),

polymyositis with mitochondrial pathology (PM-Mito; n=23), and sIBM (n=26). Their results revealed skeletal muscle weakness in the quadriceps and finger flexors shared in both PM-Mito and sIBM. In addition, the autophagy marker, LC3 was increased in aggregates in both PM-Mito and sIBM, but not in polymyositis. Mitochondrial staining revealed that while the rate of progression of the disease determined the quantity of cytochrome c oxidase deficient fibers in the PM-Mito patients, there was no difference in the frequency of cytochrome oxidase negative muscle fibers comparing fast and slow progressing sIBM patients. Both PM-Mito and sIBM biopsies muscle samples had mildly elevated CK blood levels and increased staining intensity for SDH, also suggesting impaired oxidative capacity. Since PM-Mito and sIBM samples share many features, these finding suggest that both PM-Mito and sIBM may be part of the same disease spectrum and thus may have the same underlying pathogenic mechanisms including mitochondrial dysfunction.

Argov et al (*52*) investigated mitochondrial oxidative capacity using phosphorus magnetic resonance spectroscopy (31PMRS) in the calf muscles of 7 participants diagnosed with sIBM compared to 8 healthy, elderly controls. Phosphorus magnetic resonance spectroscopy is used to monitor muscle energy metabolism by measuring the ratio of phosphocreatine (PCr) to inorganic phosphate (Pi) at rest, during muscle contraction, and recovery (*53*). Results of the study showed that 6/7 patients had significantly elevated Pi signal at rest, while one patient had a borderline high Pi compared to controls. Abnormally low PCr at rest also was reported in 3/7 sIBM patients, and the calculated ADP concentration was significantly higher in 4/7 patients compared to controls. However, the half recovery time (t1⁄2) of ADP following exercise

was normal in all but 1 of the sIBM patients. Also, other indicators of oxidative metabolism such as PCr recovery and Qmax were reported normal in sIBM patients in comparison to the control group. Qmax is considered an estimate of oxidative capacity and is calculated from PCr kinetics during recovery from 30s muscle contraction (*54, 55*). The lack of impairment during recovery suggests that the abnormalities observed with histologic and molecular genetic techniques in sIBM are not related to defective oxidative metabolism. Additionally, elevated Pi concentration at rest may be caused by nuclear breakdown, a slower Pi efflux, or derived from cells other than myocytes, such as inflammatory cells.

In a similar study, Lodi et al (*56*), used 31P magnetic resonance spectroscopy to measure in vivo skeletal muscle mitochondrial function during rest and following muscle contraction in calf muscles of 12 sIBM patients. Their results showed that 11 patients contained multiple mitochondrial DNA deletions in skeletal muscle and 8 patients displayed ragged, red fibers and/or cytochrome c oxidase deficient fibers. Their results also demonstrated abnormalities in metabolite ratios in all of the sIBM participants at rest. Nevertheless, maximum rates of mitochondrial ATP production and post-exercise ADP recovery rates were both normal in sIBM patients and therefore the authors concluded that the mitochondrial abnormalities observed in skeletal muscle from sIBM patients are a secondary characteristic and not substantial in the pathogenesis of sIBM.

Although information is limited, it appears that mitochondria from sIBM patients display mtDNA deletions, red ragged fibers, inclusion bodies, cytochrome c oxidase deficient fibers, increased LC3 content, decreased activity of citrate synthase and

complexes I and IV of the electron transport chain. However, these defects do not appear to result in decreased mitochondrial function as measured by ATP flux.

2) What is the role of amyloid beta on mitochondrial function in skeletal muscle?

In 1853, Rudolf Virchow first used histochemical stains to characterize amyloid deposits in pathologic brain samples and named these cerebral deposits "amyloid". Since the pattern of staining that was used was also used to stain cellulose, Virchow concluded that the amyloid deposits were composed of cellulose or starch structures thus resulting in the term "amyloid." However, it is now known that these amyloid deposits are comprised mainly of protein and not cellulose or starch (*57*). Amyloid protein aggregates can be formed from a number of protein precursors and are associated with diseases such as Alzheimer's, Parkinson's, spongiform encephalopathies, sIBM, and others, however the exact role of amyloid in each disease state is not completely understood. Amyloid Beta (Aβ) is a peptide of 36–43 amino acids and is processed from the transmembrane glycoprotein amyloid precursor protein (APP) (*58*). APP is approximately 695 amino acids in length and is expressed in many tissues and concentrated in the synapses of neurons. The primary role of APP is not known, however it has been suggested that it has roles in regulation of synapse formation (*59*), neural plasticity, iron export (*60*), kinase activation, protection against reactive oxygen species and oxidative stress, regulation of cholesterol transport, transcriptional regulation, and anti-microbial activity (*61, 62*). Aβ is the result of serial cleavage of amyloid precursor protein. The normal

function of Aβ is also not well known. Intracellular Aβ accumulation is mostly linked to cellular toxicity and damage, however it has been proposed that Aβ accumulation may also occur in response to cellular inflammation (*63, 64*).

APP undergoes consecutive enzymatic cleavages by two membrane-bound endoproteases, β- and γ-secretase to form Aβ (Figure 2). Cleavage by β secretase results in the formation of secreted amyloid precursor β (sAPPβ). The resulting fragment is then cleaved by Gamma secretase (γ-secretase) to produce Amyloid Beta (Aβ). The γ secretase cleaves within the transmembrane region of APP and can generate a number of isoforms of 36-43 amino acid residues in length. The most common isoforms are $A\beta_{40}$ and $A\beta_{42}$; the longer form is typically produced by cleavage occurring in the endoplasmic reticulum, while the shorter form is produced by cleavage occuring in the trans-Golgi network. APP can also go through post-translational modification such as glycosylation, sulfation, and phosphorylation (*65*). The molecular mechanisms involved in APP cleavage and Aβ assembly have yet to be fully understood. The $A\beta_{40}$ form is the more common of the two, but $A\beta_{42}$, which includes ~5-10% of the amyloid beta fragments is the more hydrophobic and fibrillogenic and therefore more cytotoxic and associated with the development of disease states (*8, 66*). Mutations in APP associated with early-onset Alzheimer's have been noted to increase the relative production of $A\beta_{42}$, and thus one suggested avenue of Alzheimer's therapy involves modulating the activity of β and γ secretases to produce mainly $A\beta_{40}$ (*67*). Since β- and γ-secretase have essential roles amyloid beta production, they have been targets for pharmaceutical therapies with mixed results.

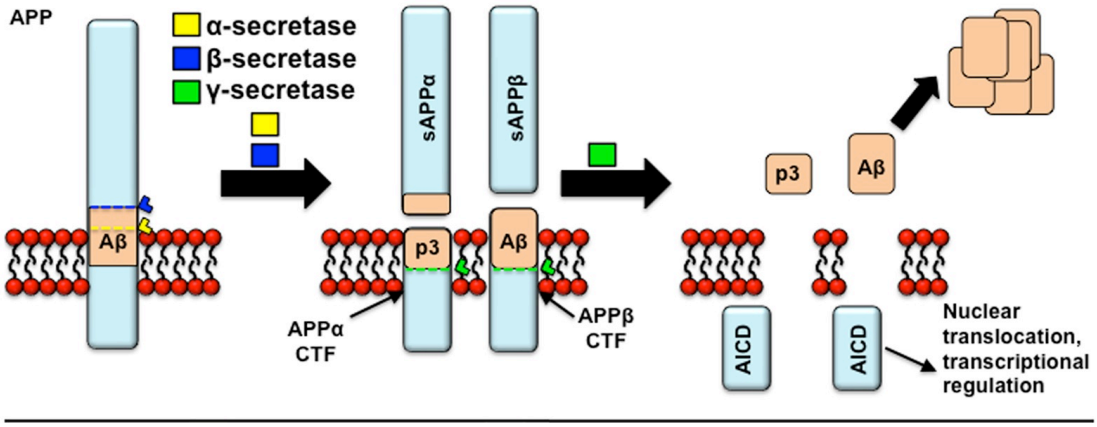

FIGURE TWO: Aβ formation. APP undergos proteolytic cleavage by α-, β- and γ-secretases. Cleavage by α-secretase or β-secretase within the luminal or the extracellular domain results in two large soluble APP derivatives, APPsα and APPsβ, and also the generation of membrane-tethered α- or β-carboxyl-terminal fragments (APP-CTFα and APP-CTFβ). The APP-CTα fragment is then cleaved by γ-secretase to generate a benign 3-kDa product, p3. Aβ is formed from the APP-CTFβ segment. In both fates the generation of APP intracellular domain (AICD) also occurs.

The role of Aβ in the development of sIBM is quite controversial. Askanas et al, and others have demonstrated a link between overexpression of APP and abnormal accumulation of Aβ with the development of sIBM like symptoms. However, Greenberg has suggested that methodical issues as well as citation bias have propagated an incorrect hypothesis (5). What's more, he states that Aβ accumulation is not specific to sIBM and occurs in many muscle disorders suggesting that it is not specific to sIBM pathology or is the underlying cause. Nonetheless, the most widely used models for study of sIBM utilize skeletal muscle cell culture and rodent models overexpressing amyloid precursor protein, which results in the accumulation of Aβ in skeletal muscle and sIBM like

symptoms (*68, 69*). However, much less research is available on the effect of Aβ on mitochondrial function in skeletal muscle.

Askanas et al (*70*), transfected normal human muscle cells with constructed adenovirus vector and demonstrated that APP overexpression can lead to structural mitochondrial abnormalities and reduced cytochrome C oxidase (COX) activity. Authors suggest that excessive production of APP may be responsible for the mitochondrial alternations in observed in sIBM muscle and Alzheimer's disease brain. Askanas and her colleagues performed COX histochemical staining to detect COX reactivity in the muscle fibers. They reported more than a 50% decrease in COX activity in transfected fibers 5-7 days after transfection. After 14 days, COX staining had decreased further and was visually undetectable in 80% of the muscle fibers. They also demonstrated mitochondrial abnormalities including enlarged and inflated mitochondria, paracrystalline inclusions, and disorganized, scarcity, or complete lack of cristae. These mitochondria abnormalities resemble those also observed in sIBM biopsied muscle. Unfortunately, this is the only study conducted to examine the accumulation of Aβ on mitochondrial function in human skeletal muscle.

Boncompagni et al (*71*) investigated the affects of amyloid beta aggregations on mitochondrial structure and function using muscle specific transgenic mice (MCK-APP) that overexpress APP in skeletal muscle only. As a result of overexpression, mice accumulate intramyofiber Aβ, display characteristics of, and are often used as a model of sIBM. Electron microscopic analysis demonstrated that myofibers from 2–3-month-old MCK-APP mice showed significant mitochondrial abnormalities. Mitochondria from

MCK-APP, but not wild type (WT) muscle fibers appear swollen, highly variable in size and shape, and the mitochondrial matrix appeared translucent and devoid of electron density. In addition, the cristae were fragmented or sometimes entirely absent and in some of the more severely affected mitochondria, the outer membrane was disrupted. They also observed reduced cytoplasmic pH, increased ROS production, and partially depolarized mitochondrial membrane in isolated muscle fibers. Finally, they reported a reduction in TCA cycle activity and a shift from aerobic to anaerobic glucose metabolism as measured by 13C NMR spectroscopy. It is important to point out that these effects are observed at 2-3 months of age approximately 8 months prior to the onset of initial symptoms and Aβ accumulation.

In summary, the accumulation of Aβ appears to be a key feature of sIBM, however its role in disease pathology as well as mitochondrial function is not yet confirmed. Both Askanas et al (*70*), and Boncompagni et al (*71*) demonstrated that APP overexpression can result in gross mitochondrial abnormalities, though the mitochondrial defects observed in MCK-APP mice occurred approximately 8 months prior to the significant accumulation of Aβ. These findings would suggest that the observed effects were not directly due to Aβ accumulation. Yet Glabe et al (*72*), has argued that amyloid oligomers rather than the mature amyloid plaques may be responsible for the pathologic effects. They contend that the conflicting lines of evidence regarding the pathogenic role of Aβ is due to the fact that it is the oligomer rather than the plaques that result in calcium dyshomeostasis, production of reactive oxygen species, altered signaling pathways, and mitochondrial dysfunction. This may be why the observed mitochondrial defects in Boncompagni et al's work appears to occur prior to the onset of Aβ accumulation.

2.6. Discussion

Based on the results of the systematic literature review it appears that skeletal muscle from patients diagnosed with sIBM contain mtDNA deletions, inclusion bodies, red ragged fibers, cytochrome c oxidase deficient fibers, and decreased enzyme activity of citrate synthase and complexes I, IV of the electron transport chain (*49, 73-76*). These results alone would imply that mitochondrial function is impaired in sIBM patients. Although, MRS studies conducted in two small samples of diagnosed patients (n=19, total) compared to elderly controls demonstrated no impairment in exercise capacity or recovery suggesting no defects in mitochondrial function in these individuals. Limitations of these studies include the applicability of the measurement in elderly individuals with impaired mobility and varying levels of fitness. Furthermore, mitochondrial ROS production and the resulting shift to a more oxidized redox environment in the absence of overt mitochondrial dysfunction as measured by ATP flux could still have detrimental effects on skeletal muscle function. Importantly, these aspects of mitochondrial function have not been adequately examined.

In summary, the lack of consistent data at this time makes it impossible to conclude whether the structural and morphological mitochondrial abnormalities observed in sIBM patients contribute to disease pathology or to the functional limitations observed with disease progression. Unfortunately, the very low prevalence rate (0.002% in individuals over 50 years of age in the US) makes acquiring skeletal muscle samples and studying the disease quite difficult (*77*). Furthermore, the pathogenesis of sporadic inclusion-body myositis is complex involving multidimensional pathways with some of

the most critical issues still unresolved. Despite an increase in the understanding of sIBM pathology, it remains controversial if the inflammatory mechanisms are a cause or consequence of the degeneration or if both cascades occur independently. However, the consistent yet unsuccessful results yielded by immunosuppressant or anti-inflammatory treatment in sIBM patients has continually suggested this may be a secondary effect to abnormal protein homeostasis (*78*). On the other hand, while it is unlikely that Aβ or any one protein is the sole underlying cause of the disease, there is considerable evidence supporting the role of global protein dyshomeostasis in the development of sIBM. The accumulation of multiple toxic protein aggregates including Aβ, phosphorylated tau, and alpha Syn as well as the fact that specific aggregate accumulation is not consistent across all patients provide support for this idea. In addition, evidence of endoplasmic or sarcoplasmic reticulum stress as well as impairments in both the 26S proteasome and lysosomal function may also provide potential mechanisms (*78*). Delaunay et al (*79*), in their study showed that the expression of ER-bound RING finger protein 5 RNF5 (RMA1) which is responsible for the identification and handling of misfolded proteins is elevated in muscle biopsies in sIBM patients. The presence of abnormally functioning mitochondria could also be the result of abnormal protein degradation since mitochondria are removed via mitophagy. Studies are currently ongoing to examine whether treatment with lithium, an inhibitor of the tau-phosphorylating enzyme glycogen synthase kinase-3β, or arimoclomol, a drug that reduces the heat shock response has any effect on disease progression.

As a result of the complexity of disease pathology, research models with which to study the role of mitochondrial function in the development of the disease are lacking.

Askanas et al (*78*), has taken the approach of modifying the microenvironment in cell culture models to mimic various pathologic aspects of sIBM (*70, 80, 81*). However, limitations to these models as with any cell culture model include the isolated conditions and the inability to account for input from other organs or systems along with the fact that their models isolate specific characteristics of the disease, none of which identified as the primary, underlying cause. Additionally, the only available animal model for the disease are transgenic mice that over express APP and demonstrate accumulation of Aβ and sIBM like pathology in their skeletal muscle along with motor deficits (*82-84*). Although the role of Aβ as the primary cause of the disease has been questioned, these models have consistently been used to study disease progression and test hypotheses regarding possible underlying mechanism(s).

While there is insufficient evidence to prove that mitochondrial dysfunction is integral in the development of sIBM, there is evidence for mitochondrial impairment in the development of a number of conditions with similar pathology to sIBM.

Alzheimer's disease is the most common type of dementia. According to the World Health Organization (WHO), approximately 18 million people worldwide live with AD and by 2025, this number is predicted to grow to 34 million. A neuropathological hallmark of AD is the accumulation of Aβ, which occurs in the presence of a heightened inflammatory state characterized by influx of inflammatory cytokines. In fact, the similar presence of Aβ in both AD and sIBM had researchers suggesting a common pathology. Interestingly, another characteristic common to both diseases is the observed mitochondrial alterations including mtDNA deletions,

cytochrome c oxidase deficiency (*85*). Moreover, increased ROS production, decreased oxidative phosphorylation, reduced membrane potential, and calcium dysregulation have all been observed in mitochondria in AD and have been implicated in disease development. Both excessive ROS production and calcium dysregulation can result in activation of the mitochondrial permeability transition pore ultimately leading to neuronal death (*86-88*). In PC12 cells, a model system for neuronal cell differentiation, Aβ exposure results in depolarization of the mitochondrial membrane, decreased oxygen consumption, and inhibition of complexes I, III and IV of the mitochondrial respiratory chain. Several antioxidants prevented this inhibitory response, suggesting that oxidative stress may involved in Aβ-induced cytotoxicity in neuronal cells. These data also suggest that mitochondrial dysfunction may contribute to Aβ-protein cytotoxicity and may play a major role in the abnormalities of energy metabolism observed in Alzheimer's disease. Furthermore, cytoplasmic hybrid (cybrid) studies conducted in NT2 neuronal cells demonstrated that the addition of mtDNA from AD patients led to reduced cytochrome oxidase activity, elevated ROS concentrations, and reduced ATP levels. Furthermore, when these cell lines were exposed to Abeta 1-40, excessive mitochondrial membrane potential depolarization, increased cytoplasmic cytochrome c, and elevated caspase-3 activity was observed when compared to cells receiving mtDNA from control subjects. Finally, events associated with programmed cell death were activated to a greater extent, suggesting an additive effect of mitochondrial dysfunction and Aβ-protein cytotoxicity in AD degeneration (*89*).

Parkinson's disease is a degenerative disease of the central nervous system resulting from depletion of dopamine-producing cells in substantia nigra region of the

brain (*90*). Approximately one million Americans live with Parkinson's disease (*91*). While medical research has failed to find a cure, a growing body of research has implicated oxidative stress, inflammation, and mitochondrial dysfunction in the disease process. For example, most of the gene mutations related to familial Parkinson's are closely linked to mitochondrial dysfunction and increased free radical production and oxidative stress (*92*). Additionally, age-related mutations in mtDNA seem to play a crucial role in the pathogenesis of this disease (*93*). Finally, as observed in sIBM, abnormalities in protein metabolism including mis-folding and aggregation of toxic fragments such as Aβ are also implicated in the pathology of Parkinson's disease (*94*).

sIBM is often misdiagnosed for polymyositis, an inflammatory myopathy also of unknown cause with symptoms including chronic muscle weakness and tenderness, difficulty speaking and swallowing, fatigue, and skin complications such as thickening of the skin (sclerodactyly) (*95*). Analogous with sIBM, mitochondrial abnormalities (cytochrome oxidase negative muscle fibers, multiple mtDNA, abnormal protein metabolism and elevated serum creatine kinase levels) are also reported in polymyositis, just to a lesser degree (*96, 97*). However, as also with sIBM, the role of the mitochondria in the pathology of polymyositis is not currently known.

In summary, although there is limited evidence for a role of mitochondrial function in the development of sIBM, additional support is provided by work conducted in other disease states with similar symptoms and/or pathology. Furthermore, this work identifies potential mechanisms linking mitochondrial function or dysfunction to disease progression. For example, in both Alzheimer's and Parkinson's disease intracellular

ROS production and oxidative stress have been identified as potential mechanism(s) in disease development. The mitochondria are the primary site of ROS production and the mitochondrial abnormalities observed in sIBM patients could easily result in increased ROS production and altered redox state in skeletal muscle in absence of changes in oxygen consumption or ATP synthesis or kinetics. Future studies are necessary to better understand mitochondrial biology in the progression of the disease. Finally, future work should also focus on protein dyshomeostasis as well as whether amyloid oligomers rather than the mature amyloid plaques are more significant to disease pathology.

As a final note, we conclude that sIBM is a complex disorder and potentially multiple factors contribute to the pathophysiology of the disease. Therefore, the identification of a single cause of the disease is not likely. In this report, the role of mitochondrial function in sIBM pathology was reviewed and it was determined that while a number of studies have examined structural and morphological aspects of the mitochondria in sIBM, very limited data exists on the role of mitochondrial function in disease development. What's more, a more comprehensive understanding of mitochondrial biology in the development of sIBM is needed. Even if overt mitochondrial dysfunction is not the primary cause of sIBM, any alteration in mitochondrial function could adversely affect cellular homeostasis thus altering disease outcomes. A better understanding of the role of the mitochondria in disease progression could still be used to impact disease development by discovering new mechanism(s) in sIBM pathology and identifying novel therapeutic treatment strategies.

CHAPTER III

Mitochondrial Dysregulation in Skeletal Muscle from Patients Diagnosed with Alzheimer's Disease and sporadic Inclusion Body Myositis

3.1 Abstract

Mitochondrial dysfunction is implicated in Alzheimer's disease (AD) and disruption of mitochondrial dynamic pathways has been documented in brains from patients diagnosed with AD; although it is unclear whether other tissues are also affected. Much less is known about the mitochondria in patients diagnosed with sporadic Inclusion Body Myositis (sIBM). The current study examined mitochondrial biology in skeletal muscle from AD and sIBM patients compared to healthy, elderly individuals. Skeletal muscle samples were obtained from the National Disease Research Interchange and mRNA, protein content, and enzyme activity was used to assess mitochondrial parameters. Patients diagnosed with AD or sIBM demonstrated reduced mitofusin 2 and optic atrophy protein 1 protein. AD patients also displayed increased mRNA of superoxide dismutase 2, catalase, and uncoupling protein 3. Amyloid β precursor protein mRNA was higher in sIBM patients only compared to both AD patients and elderly individuals. Both total and phosphorylated AMPK protein content, an upstream regulator of mitochondrial dynamics and biogenesis, was also reduced in sIBM patients. The current study demonstrates a disruption in signaling pathways regulating mitochondrial dynamics in both AD and sIBM patients, although the underlying causes may differ.

3.2 Introduction

Mitochondria are dynamic organelles that play a pivotal role in cellular function, not only as a major site of ATP production, but also as an organelle regulating energy metabolism, protein turnover, cellular proliferation, and apoptosis (*98*). Not surprisingly, defects in this organelle can have a profound impact on cellular function and are implicated in the development of metabolic and neurological disease (*99, 100*). Mitochondrial function is dependent on a number of factors including mitochondrial biogenesis, mitophagy, and dynamics (*101, 102*). Mitochondrial dynamics is a concept that includes mitochondrial movement within the cell and mitochondrial interactions controlled by fusion/ fission events (*103*). The importance of these events has recently become evident with the identification of genes responsible for fusion (mitofusin 1 and 2, optic atrophy protein 1) and fission (dynamin Related Protein 1 and fission 1) (*36, 104-106*). Pharmacologic and/ or genetic manipulation of these genes can result in gross mitochondrial abnormalities including, altered substrate metabolism, reduced oxygen consumption, a decline in ATP synthesis, and mtDNA nucleotide dyshomeostasis (*103*). Furthermore, impairment in mitochondrial function is detrimental to myofiber health and can result in fiber death and muscle atrophy (*107*).

Disruption of mitochondrial dynamic pathways has been documented in the brains of Alzheimer's patients and occurs in conjunction with mitochondrial network fragmentation, increased reactive oxygen species (ROS) production, and accumulation of pathological protein fragments including amyloid β and tau (*108-112*), although it is not known if other tissues are affected. Much less is known about the role of the mitochondria in the development of sporadic Inclusion Body Myositis (sIBM), however,

mtDNA dyshomeostasis along with structural and functional abnormalities have been observed in patients diagnosed with the disease (*46, 49, 112*). Furthermore, Boncompagni, et al (*71*) observed structural and functional alterations in mitochondria from MCK-βAPP mice, which over express amyloid β precursor protein (AβPP) in skeletal muscle and display characteristics of the disease. Importantly, these alterations are observed preceding the reported appearance of histopathological and clinical features and may represent a key early event in disease pathology (*47, 71, 112*).

Both AD and sIBM are characterized by the accumulation of protein fragments such as amyloid β (Aβ), a peptide fragment processed from the AβPP, in the brain and skeletal muscle, respectively (*113, 114*). In fact, the presence of Aβ in both diseases originally suggested a common pathology. However, while the role of Aβ in the development of AD has been established, whether Aβ accumulation in skeletal muscle from sIBM patients is a cause or secondary side effect is not known (*115, 116*).

Recent work has demonstrated that AβPP can interact with both the outer and inner mitochondrial membrane import channels and prevent import of de novo synthesized nuclear-encoded mitochondrial proteins (*117-119*). Furthermore, intramitochondrial amyloid β may directly impair mitochondrial function by disrupting mitochondrial dynamic pathways (*120, 121*). This provides a potential mechanism for the observed impairments in mitochondrial function and suggests a role for mitochondrial dynamic pathways in the development of sIBM. The purpose of the current paper was to examine pathways regulating mitochondrial fusion and fission as well as regulators of

mitochondrial biogenesis and autophagy in skeletal muscle from patients diagnosed with AD and sIBM compared with healthy, elderly individuals.

3.3 Methods

Human samples. Human skeletal muscle samples were obtained from the National Register Disease Interchange (New York, New York). Samples were obtained from patients diagnosed with AD (n=5; age=79+3; 2 males and 3 females), sIBM (n=2; age=69y+4; all males), and elderly controls with no evidence of muscle disease (n=6; age=66+4; all males). Samples were collected from the vastus lateralis muscle within 12 hours post mortem. Samples were shipped overnight on dry ice and processed for assessment of mRNA, protein, and enzyme activity.

Gene expression. RNA was extracted using an RNeasy Mini Kit (Qiagen) and DNase I treatment (Qiagen, Valencia, CA), according to the manufacturer's instructions. qRT-PCR was performed using an ABI 7900 Sequence Detection System instrument and TaqMan Universal PCR Master Mix according to the manufacturer's specifications (Applied Biosystems, Foster City, CA) and as previously described (*122*). mRNA of uncoupling protein three (UCP3), manganese superoxide dismutase (SOD2), catalase, beclin, peroxisome proliferator-activated receptor gamma coactivator 1-alpha, myosin heavy chain 7B, myosin heavy chain 2, and myosin heavy chain 4 were assessed. Primers and 5# FAM-labeled TaqMan probes were purchased as prevalidated assays (Applied Biosystems, Foster City, CA). Relative quantification of target genes was calculated

using the 2−ΔCT method. Derivation of the 2−ΔCT equation has been described in Applied Biosystems User Bulletin no. 2 (P/N 4303859). Target gene expression was normalized against GAPDH.

Protein content. Western analysis was performed as previously described using cell lysates harvested in Mammalian Cell Lysis Buffer (Sigma Aldrich) (31). Proteins (30 μg) were separated using a 10% Criterion-Tris·HCl gel (Bio-Rad, Hercules, CA) and subsequently transferred to a polyvinylidene difluoride membrane (Bio-Rad). Blots were probed with primary antibodies against GAPDH (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), peroxisome proliferator-activated receptor (PPARα) (1:1,000; Abcam, Cambridge, MA), mitofusin 2 (1:1,000; Cell Signaling, Danvers, MA), optic atrophy protein 1 (1:1,000; Abcam, Cambridge, MA), dynamin related protein 1 (1:1,000; Abcam, Cambridge, MA) and phosphorylated 5' AMP-activated protein kinase (AMPK) and total AMPK (1:1,000; both Abcam, Cambridge, MA) followed by anti-rabbit, mouse, or goat secondary antibodies (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA). Proteins were visualized using Super-Signal Chemiluminescent Substrate (Pierce, Rockville, IL) and a ChemiDoc XRS Imaging System (Bio-Rad). Protein content was normalized to GAPDH and phosphorylated AMPK was adjusted for total AMPK and normalized to GAPDH.

Enzyme activity. Enzyme activities were assessed in muscle homogenates (20-fold dilution). Sample buffer consisted of 0.1 mol/l KH2PO4/Na2PHO4 and 2 mmol/l EDTA, pH 7.2. Phosphofructokinase (PFK), citrate synthase (CS), malate dehydrogenase

(MDH) and beta hydroxyacyl-CoA dehydrogenase (β-HAD) activities were determined spectrophotometrically as previously described (*122, 123*).

Citrate synthase catalyzes the formation of citrate and coenzyme A (CoASH) from acetyl-CoA and oxaloactetate. CoASH reduces DTNB and CS activity was determined from the reduction of DTMB over time. Ten microliters of a 1:5 diluted muscle homogenate was added, in triplicate, to 170µl of a solution containing Tris buffer (0.1M, pH 8.3), DNTB (1mM, in 0.1M in Tris buffer) and oxaloacetate (0.01M, in 0.1M Tris buffer). Following a 2-minute background reading, the spectrophotometer (SPECTRAmax ME, Molecular Devices Corporation, Sunnyvale California) was calibrated and 30µl of 3 mM acetyl CoA was added to initiate the reaction. Absorbance was measured at 405nm at 37C every 12 seconds for 7 minutes. Maximum CS activity was calculated and reported as µmol/min/mg.

For the determination of β-hydroxyacyl-CoA dehydrogenase, oxidation of NADH to NAD was measured. In triplicate, 35 µl of whole muscle homogenate was added to 190µl of a buffer containing 0.1M liquid triethanolamine, 5mM EDTA tetrasodium salt dihydrate, and 0.45mM NADH. The spectrophotometer (SPECTRAmax PLUS 384, Molecular Devices Corporation, Sunnyvale California) was calibrated and 15µl of 2mM acetoacetyl CoA was added to initiate the reaction. Absorbance was measured at 340 nm every 12 seconds for 6 minutes at 37C. Maximum β-HAD activity was calculated and reported as µmol/min/mg.

Malate dehydrogenase reversibly catalyzes the oxidation of malate to oxaloacetate using the reduction of NAD+ to NADH. The rate of the disappearance of NADH was

measured spectrophotometrically at 340nm at 37°C. Briefly, 10ul of sample were pipetted in triplicate in wells. Then, 290ul of reaction media (0.1 M potassium phosphate buffer, pH 7.4 plus 0.006 M oxaloacetic acid, prepared in potassium phosphate buffer plus 0.00375 M NADH, prepared in potassium phosphate buffer) was added to the wells and samples were read for 5 minutes at 340nm. The rate of disappearance of NADH was analyzed and expressed relative to protein content. Data is expressed as means ± SEM.

Phosphofructokinase phosphorylates fructose-6-phosphate to fructose-1,6-bisphosphate, and is a key regulatory step in glycolysis. Phosphofructokinase activity was measured spectrophotometrically at 340nm at 37°C., Briefly, 30ul of sample homogenate were pipetted in triplicate. Assay buffer (12 mM MgCl2 , 400 mM KCL, 2 mM AMP, 1 mM ATP, 0.17 mM NADH, 0.0025 mg/mL, Antimycin 0.05 mg/mL Aldolase 0.05 mg/mL GAPDH, in100mM Tris buffer, pH=8.2) was then added to each well. After a 2-minute background reading, 3 mM fructose-6-phosphate was added to each sample well and followed by a 7-minute kinetic reading. Changes in absorbance across time were recorded and expressed relative to protein content. Data is expressed as means ± SEM.

Statistical analysis. Results were analyzed with non-parametric Kruskal–Wallis one-way analysis of variance analysis. Comparisons between groups were assessed using a Mann–Whitney–Wilcoxon (MWW) test. The level of significance was set at $P<0.05$.

3.3. Results

Enzyme activity in skeletal muscle from patients diagnosed with sIBM and AD. Due to lack of sample, we were only able to measure enzyme activity in one patient diagnosed with sIBM, although we were able to measure enzyme activity in all of the AD and control samples. While there were no significant differences in enzyme activity of phosphofructokinase, malate dehydrogenase, or citrate synthase (Figure 3 A-B and D), there was a trend for reduced activity of beta hydroxyacyl CoA dehydrogenase activity, the primary regulator of beta-oxidation in AD patients compared to elderly controls (Figure 3 C).

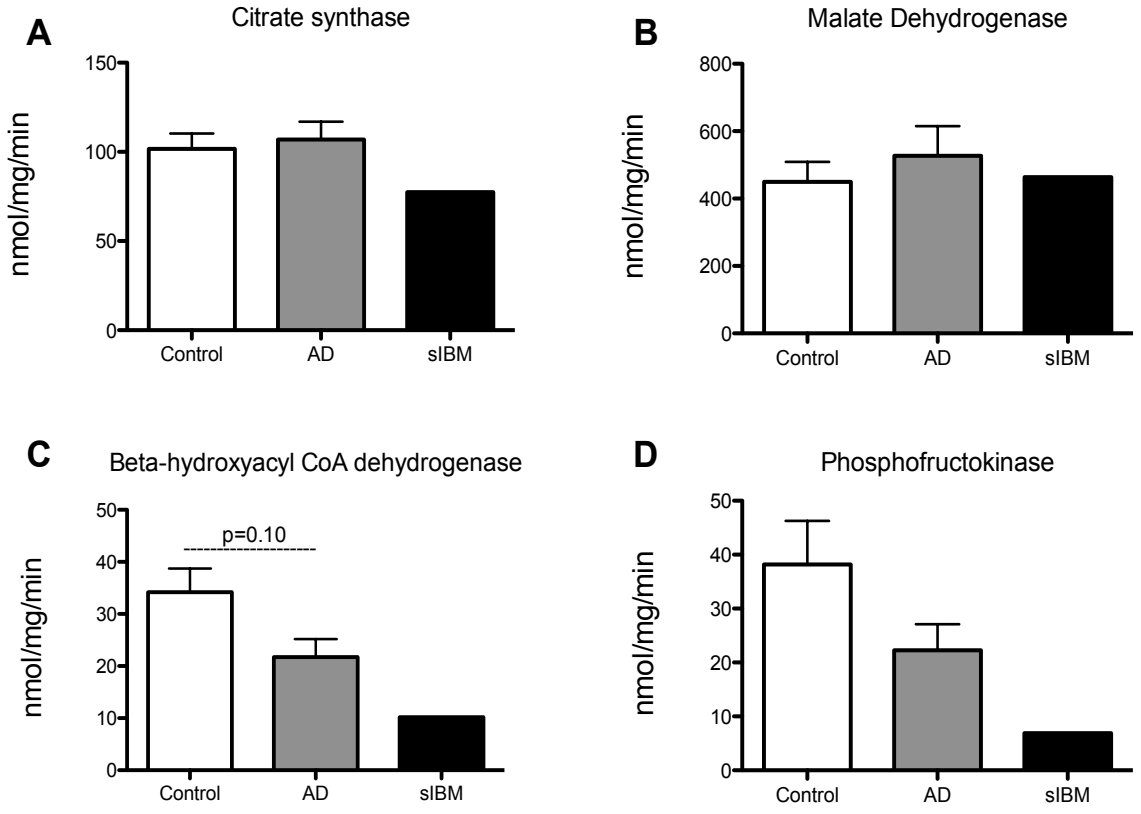

FIGURE THREE: *Metabolic enzyme activity was measured in skeletal muscle from patients diagnosed with sIBM, AD and healthy controls*. Maximal enzymatic activities of citrate synthase (A), malate dehydrogenase (B), beta hydroxyacyl-CoA dehydrogenase (C), and phosphofructokinase-1 (D) in skeletal muscle from patients diagnosed with AD, sIBM, and elderly individuals. Data presented at means ± SEM and presented as nmol of mg protein per minute. *P<0.05, compared to controls.

Transcriptional regulation in skeletal muscle from patients diagnosed with sIBM and Alzheimer's disease. mRNA analysis revealed that AβPP was significantly higher in patients diagnosed with sIBM compared to AD patients and controls (Figure 4 A). Despite this, mRNA of UCP3 and the antioxidants catalase and SOD2 were all significantly higher in AD patients compared to elderly controls (Figure 4 B-D). While there were no significant differences in UCP3 concentrations between sIBM patients and controls, there were trends for higher and lower catalase and SOD2 mRNA, respectively. Beclin, a regulator of autophagy, was significantly higher in the AD patients with a trend for higher mRNA observed in sIBM patients compared to controls (Figure 4 E). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) expression, a regulator of mitochondrial biogenesis, was significantly higher in both sIBM and AD compared to controls (Figure 5 A). To determine whether changes in expression patterns were associated with differences in skeletal muscle fiber type, mRNA of myosin heavy chain 7b, 2, and 4, markers of type 1, type 2A, and type 2B, respectively, were assessed (Figure 5 B-D). There were no differences in expression of genes responsible for fiber type suggesting that observed differences may not be due to differences in fiber type. We were however only able to measure MHC4 in one sIBM sample, and therefore only limited conclusions can be made.

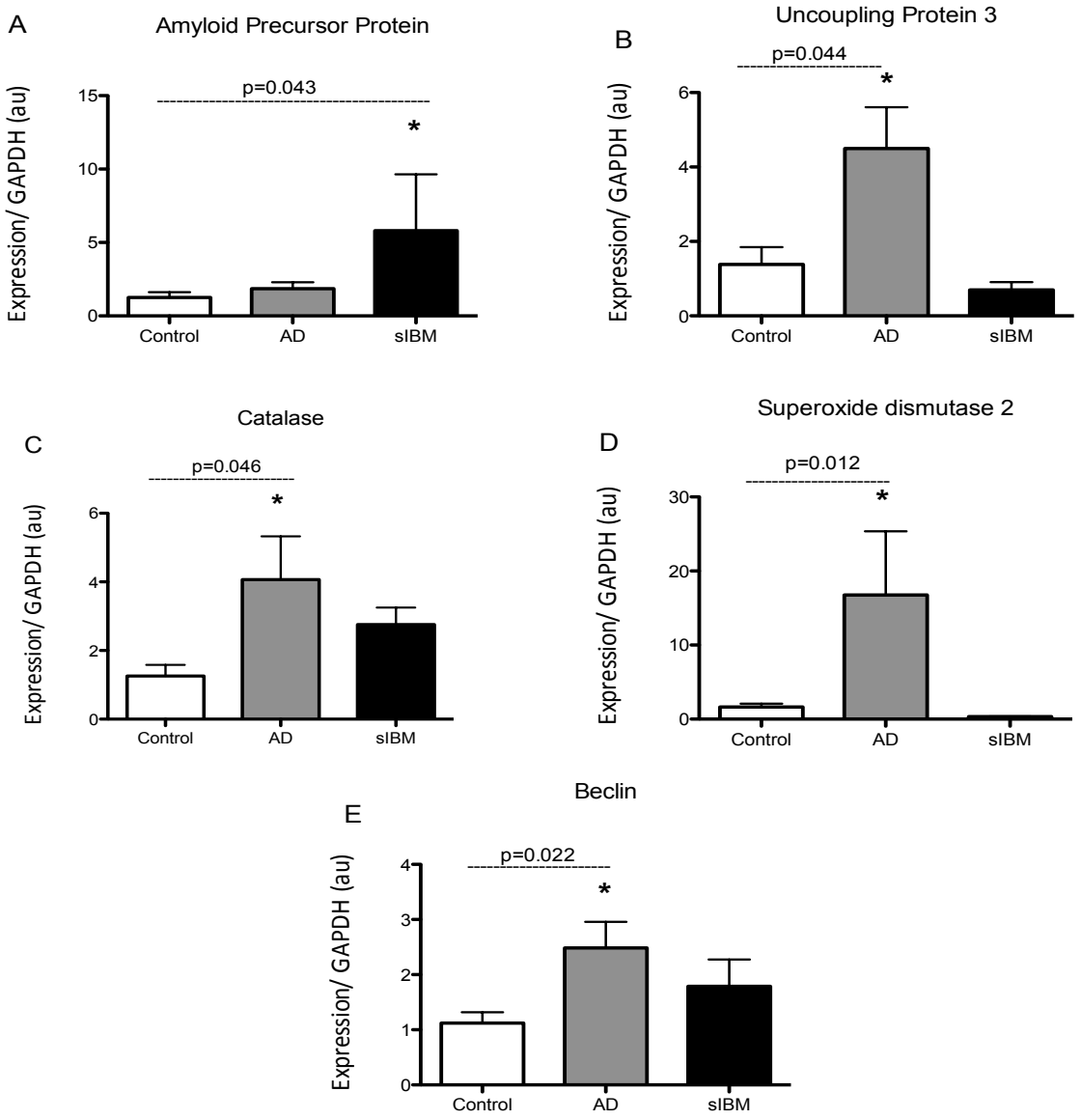

FIGURE FOUR: *Transcriptional regulation in skeletal muscle from patients diagnosed with AD and sIBM.* Relative mRNA content of amyloid precursor protein (A), uncoupling protein 3 (B), catalase (C), superoxide dismutase 2 (D), and beclin (E) in skeletal muscle from patients diagnosed with AD, sIBM, and elderly individuals. Data are presented as means ± SEM and normalized to GAPDH mRNA *P<0.05.

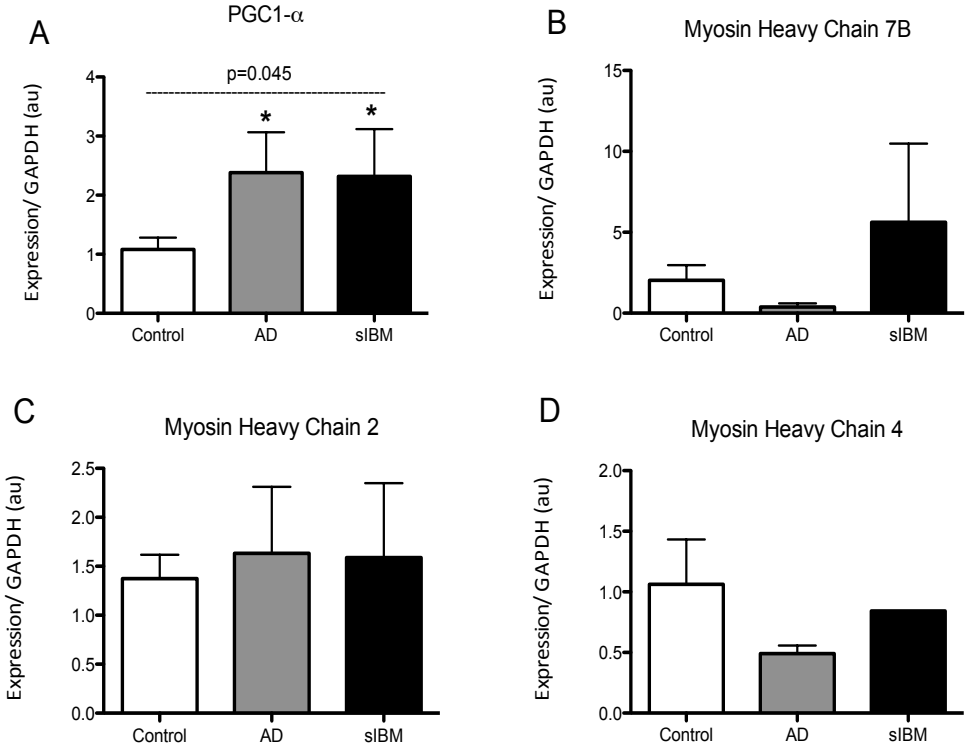

FIGURE FIVE: *Transcriptional regulation in skeletal muscle from patients diagnosed with AD and sIBM.* Relative mRNA content of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (A), myosin heavy chain 7B (B), myosin heavy chain 2 (C), and myosin heavy chain 4 (D) in skeletal muscle from patients diagnosed with AD, sIBM, and elderly individuals. Data are presented as means ± SEM and normalized to GAPDH mRNA *P<0.05.

Protein content in skeletal muscle from patients diagnosed with sIBM and Alzheimer's disease. While there were no significant differences in PGC1α protein content, MFN2, OPA1, and DRP1 were all significantly lower in both patient populations compared to elderly controls (Figure 6 B-D). Additionally, Both phosphorylated and total 5' AMP-activated protein kinase (AMPK) protein content was significantly lower in sIBM patients compared to both AD and controls, respectively (Figure 6 E).

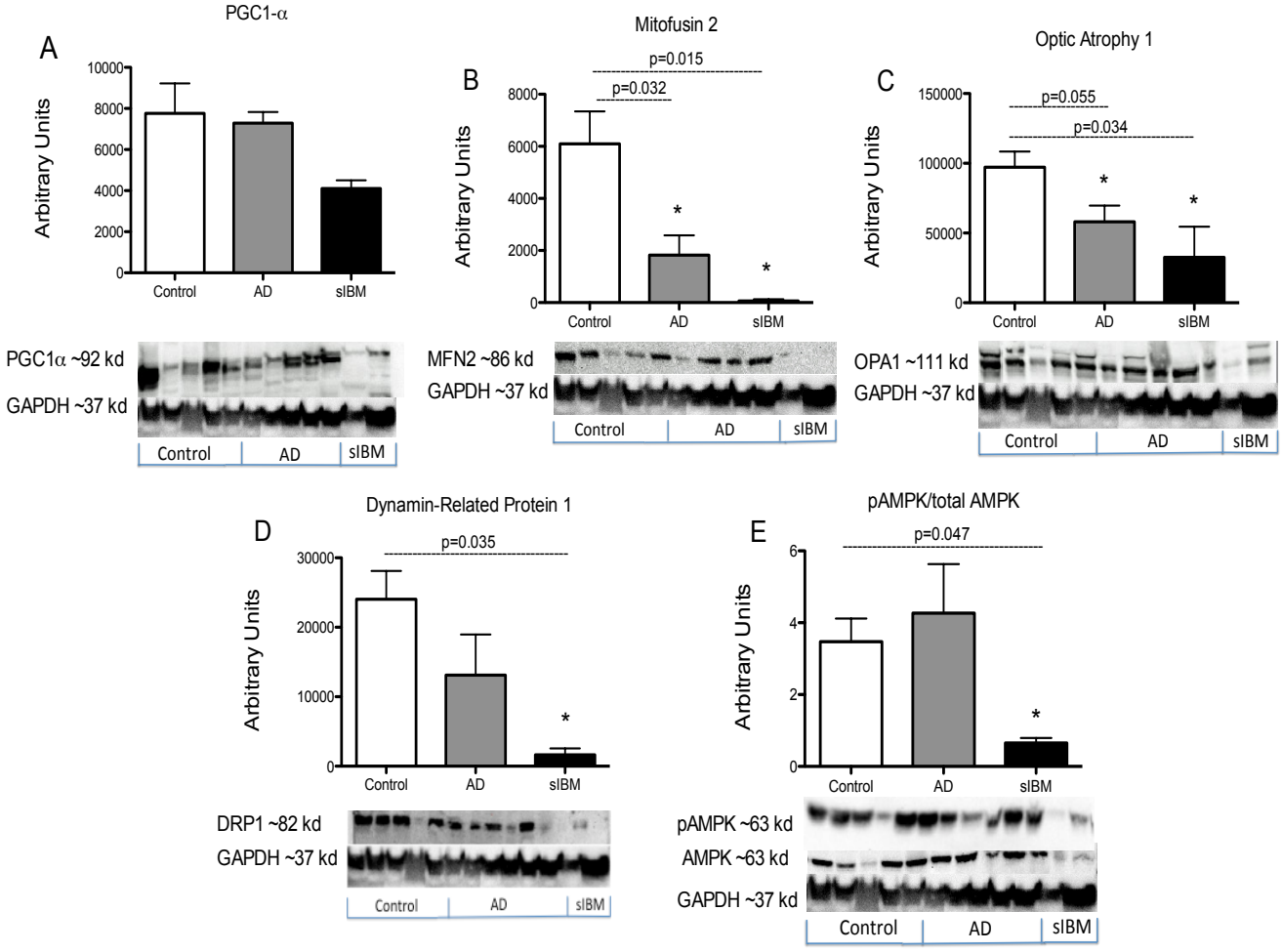

FIGURE SIX. *Protein content in skeletal muscle from patients diagnosed with AD and sIBM.* Protein content of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (A), mitofusin 2 (B), optic atrophy protein 1 (C), dynamin related protein 1 (D), and phosphorylated 5' AMP-activated protein kinase (AMPK) and total AMPK (E). Data are presented as means +SEM and normalized to GAPDH protein content. *P<0.05, compared to controls.

3.5. Discussion

The current study supports previous findings and provides a potential mechanism of mitochondrial dysfunction by demonstrating reduced protein content of MFN2 and OPA1 in patients diagnosed with AD and sIBM (*121, 124*). Additionally, we observed a similar decline in DRP1 protein in sIBM patients, although it did not reach statistical significance in AD patients. Mitochondrial dynamics, the repetitive cycles of fusion and fission, have

been recognized as a critical process in the maintenance of mitochondrial homeostasis. These opposing processes determine the architecture of the entire mitochondrial population of the cell with fission events segregating dysfunctional mitochondria from the network and fusion events allowing for equilibration of matrix metabolites and membrane components (*42, 43, 102, 125, 126*). A down regulation of the primary regulators of these pathways could account for the structural and functional alterations previously observed in sIBM as well as demonstrating for the first time, alterations in mitochondrial signaling pathways in skeletal muscle from patients diagnosed with AD.

The above effects are observed in conjunction with a significant increase in PGC1α mRNA in both patient populations, although there were no significant differences in PGC1 protein content. PGC1α is regulated at a number of levels and it has been previously demonstrated that PGC1α mRNA does not reflect protein content (*127, 128*). Furthermore, PGC1α protein has been shown to track with glycogen content and therefore current results may indicate a reduction in glycogen content in sIBM patients compared to elderly controls (*129*). The observed increase in PGC1α mRNA along with increased expression of regulators of mitochondrial autophagy, may indicate a compensatory effect to remove and replace damaged, dysfunctional mitochondria in the face of dysregulated mitochondrial dynamic pathways (*98*).

While these findings indicate a potential disruption of mitochondrial dynamics and function, the mechanisms underlying the observed similarities between the phenotypes may differ. While AβPP mRNA was higher in sIBM patients, which supports previous findings (*130*); there were no differences between AD patients and

elderly controls. The lack of an increase in APP mRNA in AD patients in the current study does not support a link between mitochondrial defects and increased AβPP expression in skeletal muscle in AD patients. Additionally, there was reduced content of both phosphorylated and total AMPK in sIBM patients compared to elderly controls. It has been demonstrated that increased AMPK activity results in an up regulation of MFN2, OPA1, and DRP1 in murine skeletal muscle (*131*). Therefore, a down regulation of AMPK may explain the down regulation of MFN2, OPA1, and DRP1 observed in the patients with sIBM. The fact that there was reduced AMPK protein content may also suggest a potential therapeutic strategy for treatment of individuals afflicted with this disease.

While there was reduced phosphorylated and total AMPK content in sIBM patients, there were no significant differences between AD patients and controls. This indicates that something other than AMPK may be responsible for the decline in MFN2 and OPA1 protein content in AD patients. Interestingly, there was an increase in UCP3, SOD2, and Catalase mRNA. This transcriptional up regulation of antioxidant genes may be a protective mechanism in response to an increase in intracellular reactive oxygen species concentrations in skeletal muscle from AD patients (*132*). ROS has been demonstrated to regulate expression of both fusion and fission proteins and may therefore also be responsible for the alterations observed in the current work (*133, 134*). On the other hand, genetic or pharmaceutical alterations of mitochondrial fusion or fission pathways has been shown to result in increased production of reactive oxygen species (*103*). Unfortunately, it is not possible to determine cause and effect from the current study.

There are limitations to the current study. The very low N of our sIBM patients is a concern. However sIBM, although considered the most common myopathy in older individuals, still has a low prevalence rate with only 0.002% in the US in individuals over 50 diagnosed with the disease (*77*). Additionally, the AD patients were significantly older than the controls and sIBM patients, making it difficult to exclude an age effect. However, all of the individuals studied were over the age of 60 and the observed similarities along with differences between the two patients populations would suggest that the observations in the current work are not simply due to age.

In conclusion, we are reporting for the first time that regulators of mitochondrial fusion and fission are down regulated in skeletal muscle from patients diagnosed with AD and sIBM. These findings suggest a potential for mitochondrial dysfunction in skeletal muscle in both disease states although the underlying causes may differ. This work highlights the need for future study in an effort to identify new opportunities for prevention or treatment of these life-altering diseases.

CHAPTER IV

Mitochondrial Function and Substrate Metabolism in a Mouse Model of Sporadic Inclusion Body Myositis

4.1. Abstract

Sporadic Inclusion body myositis (sIBM) is an idiopathic inflammatory myopathy that involves inflammation and damage to skeletal muscle tissue. Studies in humans demonstrate altered mitochondrial morphology in skeletal muscle from patients diagnosed with sIBM suggesting mitochondrial defects may be a contributor to disease progression. MCK-APP mice, overexpress amyloid precursor protein specifically in skeletal muscle, display characteristics of sIBM, and are an accepted model of the disease. The present study examined mitochondrial function, substrate metabolism, and production of reactive oxygen species in skeletal muscle from 3, 6, and 9-month old MCK-APP mice compared to wild type littermates to determine whether mitochondrial dysfunction occurs prior to the onset of disease symptoms. While there were no significant differences in mitochondrial bioenergetics, fat metabolism, or production of reactive oxygen species in red gastrocnemius muscle, there was a significant reduction in fat oxidation and oxidative efficiency in white gastrocnemius muscle in 9 month-old MCK-APP mice compared to wild type animals. Additionally, protein content of light chain 3 (LC3) and Beclin, was significantly higher in red gastrocnemius muscle from 3 month-old MCK-APP mice. These results suggest that functional alterations in mitochondria are not yet pronounced in 3,6 and 9-month MCK-APP mice prior to symptom development, however alterations in fat metabolism in white skeletal muscle may be present. In conclusion, this work suggests that mitochondrial abnormalities may

not represent crucial initial incidents in the development of sIBM-like symptoms in MCK-APP mice. Nonetheless, there is a significant reduction in fat metabolism as well as an up regulation of autophagic pathways suggesting that certain alterations in skeletal muscle from MCK-APP mice occur prior to the onset of disease symptoms.

4.2. Introduction

Sporadic Inclusion Body Myositis (sIBM) is an inflammatory muscle disease that strikes individuals at random and accounts for approximately 1/3 of all idiopathic inflammatory myopathies (*8*). The disease is characterized by progressive weakness and wasting of the proximal and distal muscles ultimately resulting in restricted movement and mobility (*135*). Individuals afflicted with the disorder may be restricted from performing activities of daily living and are often confined to the use of a walking aid or wheelchair (*135*). Currently, there is no known cause or cure for sIBM, nor is there any long-term treatment options (*7*). Patients do not generally respond to anti-inflammatory, immunosuppressant, or immunomodulatory drugs and treatment of the disease usually includes symptom management and utilizing therapy to maintain mobilization (*8, 136, 137*). The identification of novel mechanism(s) contributing to disease progression and/ or muscle defects can provide new opportunities for prevention and/ or treatment of the disorder.

Two processes, one autoimmune and the other degenerative, appear to occur in parallel in sIBM and as a result two prevailing theories have emerged,(*6, 138*). However, disease progression most likely results from the interaction of a number of factors, both

genetic and environmental (*8*). Patients diagnosed with sIBM display gross skeletal muscle abnormalities including accumulation of pathogenic protein aggregates including Abeta and phosphorylated tau, inflammation, and necrosis (*6, 8, 139*). Abeta has been extensively studied in sIBM pathology, however its significance to disease progression is still debated (*140*). Skeletal muscle from sIBM patients also display gross mitochondrial abnormalities including mtDNA deletions, fragmentation, lack of cristae, and increased ROS production, however the role of mitochondrial biology in disease progression has not been widely studied (*3, 46-49, 112, 141, 142*).

Earlier work demonstrated no differences in oxidative capacity in sIBM patients, however sample and methodological limitations of these earlier studies makes conclusions difficult (*56, 143, 144*). Not surprisingly, the rarity and often misdiagnosis of the disease makes access to sIBM patients a challenge. MCK-APP mice, overexpress amyloid precursor protein specifically in skeletal muscle and display characteristics of the disease starting at approximately 10 months of age. MCK-APP mice are considered an accepted model of sIBM and have been used to identify potential mechanism(s) contributing to disease pathology (*68*). Recently, mitochondrial impairments such as mitochondrial membrane depolarization, and calcium dysregulation have been observed in 3-month-old MCK-APP mice, which (*145, 146*). Furthermore, glutathione administration to reduce intracellular ROS concentrations also reduced calcium leak and restored membrane potential in cells cultured from this model implicating the role of intracellular ROS concentrations in mitochondrial dysfunction and disease progression. However, whether mitochondrial dysfunction (reduced oxygen consumption, dysregulated substrate metabolism, increased mitochondrial reactive oxygen species

production) occurs in these mice is not yet known (*71, 147*). Therefore, the purpose of this study was to examine mitochondrial bioenergetics, substrate metabolism, and production of reactive oxygen species in skeletal muscle from 3, 6, and 9-month old MCK-APP mice.

4.3 Methods

Animal model: MCK-APP mice with muscle-specific over-expression of the APP gene were obtained from Dr. Alex Shtifman and used for the experiments proposed in this study (*148*). The transgenic mice (MCK-APP) mice selectively overexpress human APP and accumulate Aβ42 in affected muscle fibers, both of which are important features observed in sIBM patients (*149*). This animal model also shows motor impairments, which is usually the partial or total loss of function of limbs as a result of muscle weakness, which become exacerbated in an age-dependent manner. Based on previous studies this animal model is an accepted model of sIBM (*82-84*). Three, six, and nine month old MCK-APP mice and their wild type littermates were used for the current studies.

Immediately following a 12-hour fast, the animals were sacrificed using carbon dioxide asphyxiation. Skeletal muscle was harvested (gastrocnemius and quadriceps) for functional measures and mRNA and protein. Red and white skeletal muscle was manually separated based on visual detection. Mitochondria were isolated from red muscle for measures of mitochondrial respiration, fatty acid oxidation, and reactive oxygen species production.

Mitochondrial isolation. Mitochondria were isolated from red gastrocnemius muscle. Briefly, tissue samples were collected in buffer containing 67mM sucrose, 50mM Tris/HCl, 50mM KCl, 10mM EDTA/ Tris, and 10% bovine serum albumin (all from Sigma-Aldrich, St. Louis, MO). Samples were minced and digested in 0.05% trypsin (Invitrogen, Carlsbad, CA) for 30 minutes. Samples were homogenized and mitochondria were isolated by differential centrifugation.

Respiration in isolated mitochondria. Respirometry measures of isolated mitochondria were performed using an XF24 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA). Immediately following mitochondrial isolation, protein was quantified using a Pierce bicinchoninic acid assay (Thermo Scientific, Rockford, IL) and mitochondria were plated on Seahorse cell culture plates at a concentration of 5 ug/ well in the presence of 10 mM pyruvate (P5280; Sigma-Aldrich, St. Louis, MO) and 5 mM malate (P5280; Sigma-Aldrich, St. Louis, MO). Experiments consisted of 25 second mixing and 4-7 minute measurement cycles. Oxygen consumption was measured under basal conditions, ADP (5 mM, Sigma-Aldrich, St. Louis, MO) state 3 stimulated respiration (State 3), oligomycin (2μM) insensitive state 4 respiration State 4_O), and uncoupled, maximal respiration in the presence of FCCP (0.3 μM) to assess respiratory capacity (State 3_u). Respiratory control ratio (RCR) was calculated as the ratio of ADP stimulated state 3 and oligomycin induced state 4 respiration. Oligomycin induced state 4 respiration was used to account for any contaminating ATPase activity that may prevent the restoration of a low respiration. Data are expressed as pmol/min. All experiments were performed at 37 °C.

Fatty acid oxidation. Total palmitate and oxidation were assessed from red and white gastrocnemius homogenates by measuring and summing 14-CO_2 production and 14C-labeled acid-soluble metabolites from the oxidation of [1-14C]-palmitic acid (Perkin Elmer, Waltham, MA), respectively (*123*). The measurement of 14-C-water-soluble metabolites accounted for any14C label that did not result in14-CO_2 due to isotopic exchange in the tricarboxylic acid cycle.

Gaseous 14-CO_2 produced from the oxidation of [1-14C] palmitate during the incubation was measured by transferring 1.0 ml of the incubation medium to a 20-ml glass scintillation vial containing 1.0 ml of 1 M H_2SO_4 and a 0.5-ml Fisher microcentrifuge tube containing 400 µl of benzethonium hydroxide. Liberated 14-CO_2 was trapped in the benzethonium hydroxide for 60 min, and the microcentrifuge tube containing trapped 14-CO_2 was placed in a scintillation vial and counted. 14-C-water-soluble metabolites were measured by sampling 0.5 ml of aqueous phase of the lipid extraction, which was placed in a scintillation vial and counted (*150*).

Pyruvate dehydrogenase activity (PDH), metabolic flexibility and oxidative efficiency. Pyruvate oxidation was assessed by measuring 14-CO_2 production from the oxidation of [1-14C] pyruvate (Perkin-Elmer, Waltham, MA) as previously described (*151, 152*). Metabolic flexibility was assessed by measuring [1-14C] pyruvate oxidation ± non-labeled BSA (0.5%) bound-palmitic acid. Flexibility is denoted by the percentage decrease in pyruvate oxidation in the presence of free fatty acid (e.g. a higher percentage is indicative of greater metabolic flexibility). Oxidative efficiency was calculated by dividing CO_2 production by acid soluble metabolites (ASMs) and expressed as a ratio.

Enzyme activity. Maximal enzyme activities were assessed in muscle homogenates prepared in a sample buffer containing modified sucrose EDTA medium (SET) on ice containing 250 mM sucrose, 1 mM EDTA, 10 mM tris-HCl, and 1mM ATP, pH 7.4. Citrate synthase (CS), malate dehydrogenase (MDH) and beta hydroxyacyl-CoA dehydrogenase (β-HAD) activities were determined spectrophotometrically as previously described (*122, 123*).

Citrate synthase catalyzes the formation of citrate and coenzyme A (CoASH) from acetyl-CoA and oxaloactetate. CoASH reduces DTNB and CS activity was determined from the reduction of DTMB over time. Ten microliters of muscle homogenate was added, in triplicate, to 170µl of a solution containing Tris buffer (0.1M, pH 8.3), DNTB (1mM, in 0.1M in Tris buffer) and oxaloacetate (0.01M, in 0.1M Tris buffer). Following a 2-minute background reading, 30µl of 3 mM acetyl CoA was added to initiate the reaction. Absorbance was measured at 405nm at 37C every 12 seconds for 5 minutes. Maximum CS activity was calculated and reported as µmol/mg/min. Data is expressed as means ± SEM.

For the determination of β-hydroxyacyl-CoA dehydrogenase activity, the oxidation of NADH to NAD was measured. In triplicate, 20 µl of muscle homogenate was added to 190µl of a buffer containing 0.1M liquid triethanolamine, 5mM EDTA tetrasodium salt dihydrate, and 0.45mM NADH. Following a 2 min background reading, 15µl of 2mM acetoacetyl CoA was added to initiate the reaction. Absorbance was measured at 340 nm every 12 seconds for 5 minutes at 37C. Maximum β-HAD activity was calculated and reported as µmol/mg/min. Data is expressed as means ± SEM.

Malate dehydrogenase reversibly catalyzes the oxidation of malate to oxaloacetate and the rate of the disappearance of NADH was measured spectrophotometrically at 340nm at 37°C. Briefly, 10ul of sample were pipetted in triplicate in wells. Then, 290ul of reaction media (0.1 M potassium phosphate buffer, pH 7.4 plus 0.006 M oxaloacetic acid, prepared in potassium phosphate buffer plus 0.00375 M NADH, prepared in potassium phosphate buffer) was added to the wells and samples were read for 5 minutes at 340nm. The rate of disappearance of NADH was analyzed and expressed relative to protein content. Data is expressed as means ± SEM.

Reactive oxygen species measures in isolated mitochondrial. Amplex Red Hydrogen Peroxide/Peroxidase assay Kit was used for measures of ROS production. To measure ROS production from complex 1, complex 3, and reverse electron transfer (REV), isolated mitochondria were plated on a 96-well black plate at a concentration of 5ug/well under three different conditions, respectively. The three conditions were pyruvate (20mM)/malate (10mM)/oligomycin (2μM)/rotenone (200nM) for complex 1, pyruvate (20mM)/malate (10mM)/oligomycin (2μM)/SOD (400U/ml)/antimycin A (2μM) for complex 3, and succinate (20mM)/oligomycin (2μM) for reverse electron flow to complex 1 (REV). Experiments were conducted in sucrose/mannitol solution in order to maintain mitochondrial integrity. Experiments consisted of 1-minute delay and 1 minute reading cycles, followed by a 5 second mixing cycle performed every third reading. All experiments were performed at 37°C. Measures for ROS levels were conducted on a microplate reader (Biotek synergy 2, Winooski, VT). Fluorescence of Amplex Red was measured using a 530nm excitation filter and a 560nm emission filter.

Real-time quantitative PCR (RTQ-PCR). Tissues were harvested in 1.0 ml Trizol reagent for RNA extraction. Tissues were then collected in Trizol, homogenized and RNA was extracted. Total mRNA were prepared using Trizol reagent according to the manufacturer's protocol (Life Technology), treated with DNase I (Ambion, Austin, TX), and quantified using an Agilent Bioanalyzer. Real-time quantitative PCR (RTQ-PCR) was performed using an ABI 7900 Fast HT RTQ-PCR Instrument and software (PE Applied Biosystems, Foster City, CA). ABI gene expressions assays for light chain 3 and beclin, superoxide dismutase 2, catalase, and uncoupling protein 3 were assessed and results were normalized to beta actin RNA levels by RTQ-PCR. Primers and 5# FAM-labeled TaqMan probes were purchased as prevalidated assays (Applied Biosystems, Foster City, CA). Relative quantification of target genes was calculated using the $2^{-\Delta CT}$ method. Derivation of the $2^{-\Delta CT}$ equation has been described in Applied Biosystems User Bulletin no. 2 (P/N 4303859).

Western blot analysis. Western analysis was performed as previously described using cell lysates harvested in Mammalian Cell Lysis Buffer (Sigma Aldrich) (*122*). Proteins (30 μg) were separated using a 10% Criterion-Tris·HCl gel (Bio-Rad, Hercules, CA) and subsequently transferred to a polyvinylidene difluoride membrane (Bio-Rad). Blots were probed with primary antibodies against GAPDH (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), light chin 3 (1:1,000; Cell Signaling, Danvers, MA), and beclin (1:1,000; Cell Signaling, Danvers, MA), followed by anti-rabbit, mouse, or goat secondary antibodies (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA). Proteins were visualized using Super-Signal Chemiluminescent Substrate

(Pierce, Rockville, IL) and a ChemiDoc XRS Imaging System (Bio-Rad). Protein content was normalized to GAPDH.

Blood inflammatory marker Analysis. Interleukin 6 (IL6) and C-reactive protein inflammatory markers were measured via enzyme-linked immunosorbent assay (ELISA) from Alpco (Salem, NH) and R&D Systems (Minneapolis, MN) respectively, according to manufacture's instructions.

Statistical analysis. Results were analyzed with two-way ANOVA and multiple comparions were assessed Bonferroni post tests. Results were expressed as means ± SEM. The level of significance was set at $P < 0.05$.

4.4. Results

Respiration in isolated mitochondria. Respiration was examined in mitochondria isolated from red gastrocnemius muscle from 3, 6, and 9-month MCK-APP mice and their wild-type littermates. There were no significant differences in any of the oxygen consumption measures between MCK-APP and wild-type mice indicating no evidence of reduced oxidative capacity (Figure 7 A-D). There was however, a significant reduction in RCR, basal, state III, and FCCP stimulated respiration in both the MCK-APP and wild type 9 month old compared to 3 and 6 month old mice.

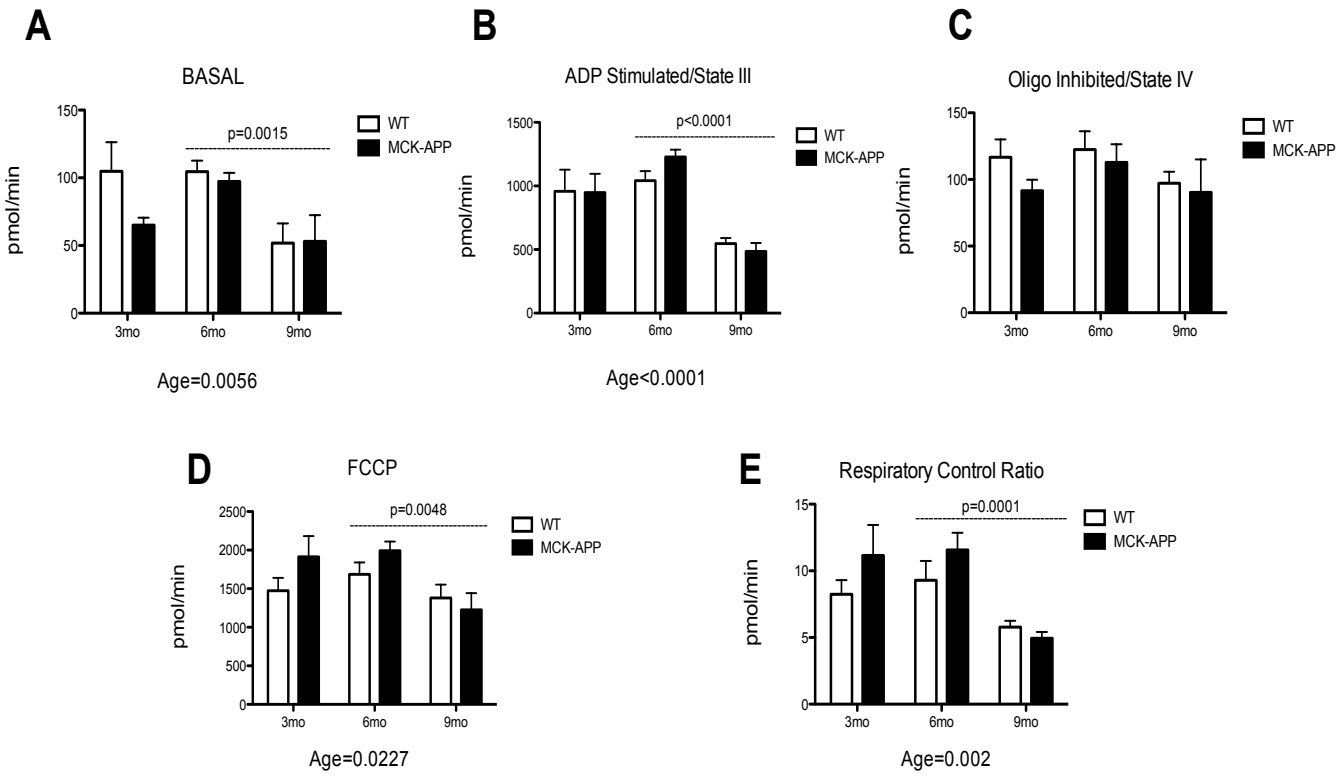

FIGURE SEVEN: Mitochondrial respiration parameters in MCK-APP mice versus wild-type littermates. Isolated mitochondria was measured under basal (A), State III respiration (B), State III/State IV respiration (C), in the presence of oligomycin (inhibitor of ATP synthesis), and FCCP-stimulated (mitochondrial uncouplet) maximal respiration (D) and respiratory control ratio (E).

Fatty acid oxidation. Fatty acid oxidation measures are displayed in Figure 8. No significant interactions were observed in red muscle from 3, 6, or 9-month transgenic or wild type mice (Figure 8 A-C). However, in white muscle, there was a significant interaction between genotype ($p=0.0149$) and age with CO_2 production ($p=0.0012$) and total oxidation ($p<0.0001$) and a trend for an interaction with ASM production ($p<0.0001$) (Figure 8 D-F). In 9-month-old mice, CO_2 production ($p=0.0149$) and total oxidation ($p=0.0281$) was significantly lower in transgenic mice compared to wild type

mice (Figure D and F). This same effect trended for ASM production (p=0.0865) (Figure 8 E).

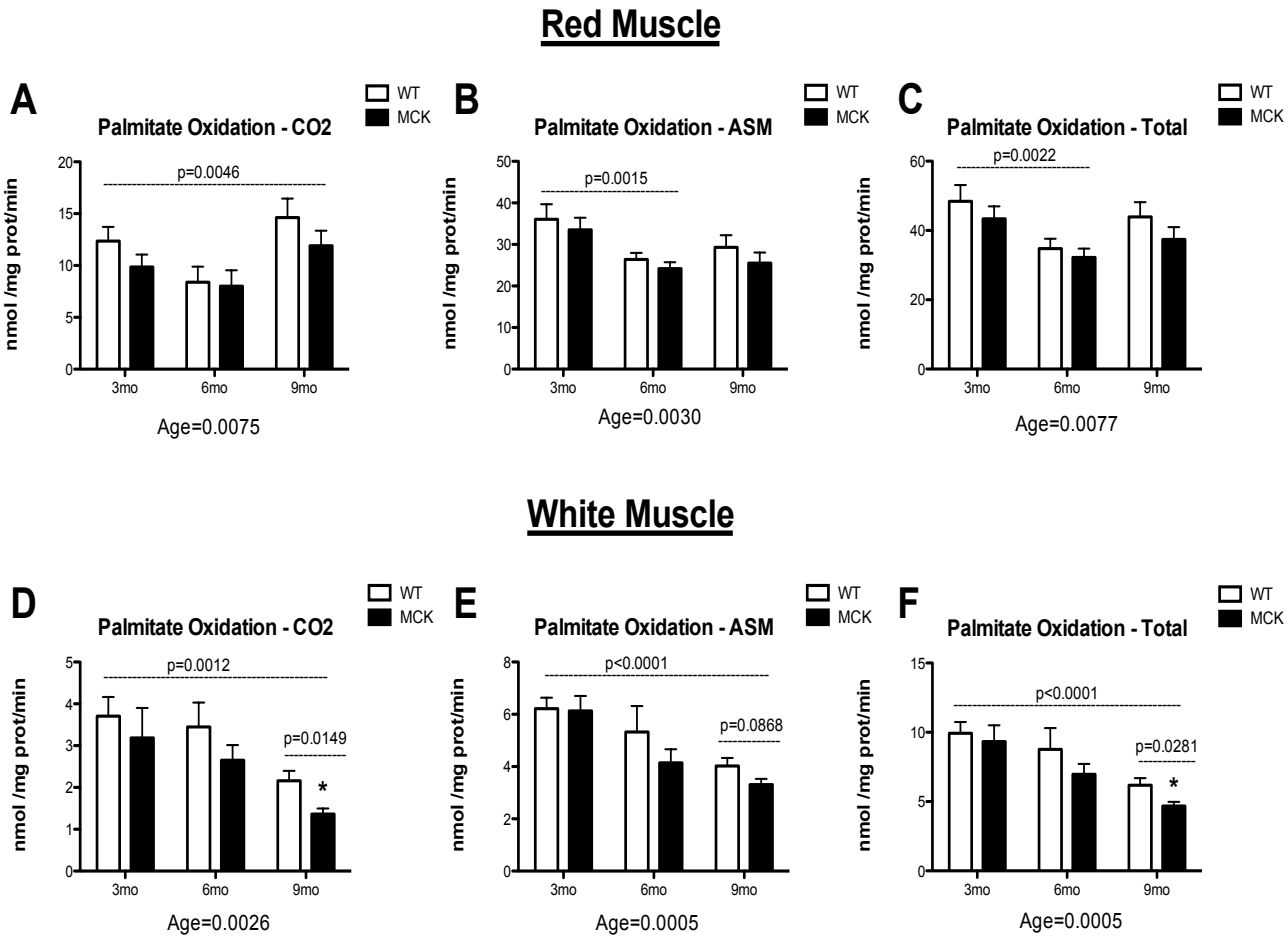

FIGURE EIGHT: *Fatty Acid Oxidation in 3,6 and 9-month MCK-APP versus wild-type littermates in red and white muscles. Fatty acid oxidation was measured under palmitate oxidation-CO2 in red muscle (A), palmitate oxidation-ASM in red muscle (B), total palmitate oxidation in red muscle (C), palmitate oxidation-CO2 in white muscle (D), palmitate oxidation-ASM in white muscle (E), and total palmitate oxidation in white muscle (F). Data are presented as means +SEM and *P<0.05.*

Pyruvate dehydrogenase activity (PDH), metabolic flexibility, and oxidative efficiency. Pyruvate dehydrogenase activity, metabolic flexibility, and oxidative efficiency are displayed in Figure 9. There were significant interactions in pyruvate dehydrogenase activity ($p=0.014$), metabolic flexibility ($p=0.003$), and oxidation efficiency ($p=0.007$) in red muscle (Figure 9 A-C). Transgenic animals were significantly less efficient compared to wild type mice ($p=0.05$) (Figure 9 F). There were also significant interactions with pyruvate dehydrogenase activity and oxidation efficiency in white muscle ($p=0.011$). PDH activity was significantly lower in 6-month-old transgenic animals while 6 and 9-month-old transgenic mice were significantly less efficient compared to wild type animals.

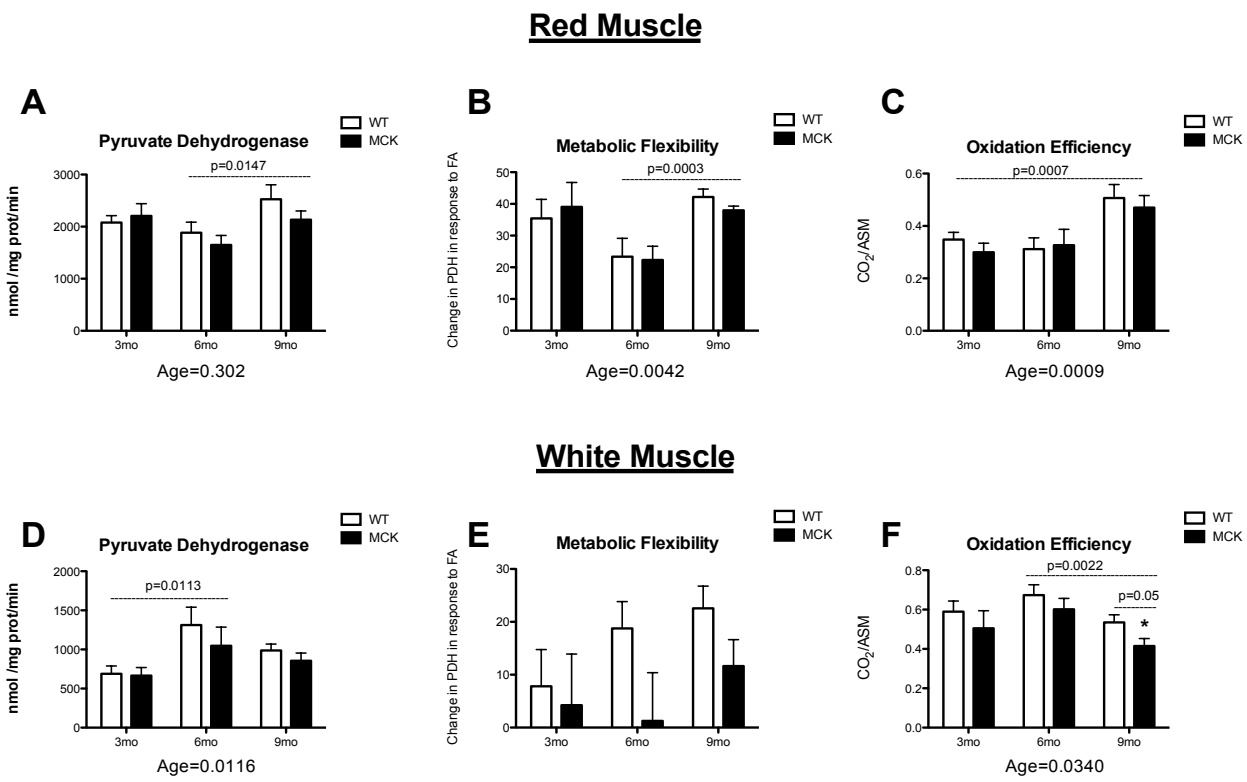

FIGURE NINE: *Pyruvate Dehydrogenase Activity (PDH) and Metabolic flexibility in 3,6 and 9-month MCK-APP versus wild-type littermates in red and white muscles. PDH activity in red muscle (A), metabolic flexibility in red muscle (B), oxidation efficiency in red muscle (C), PDH activity in white muscle (D), metabolic flexibility in white muscle (E), oxidation efficiency in white muscle (F), Data are presented as means +SEM and *P<0.05.*

Metabolic enzyme activity. Metabolic enzymatic activity was assessed in red and white muscle homogenates at all three age groups and displayed in figure 10. There were no significant interactions with regards to CS, β-HAD and MDH activity in red or white muscle. Citrate synthase was significantly lower in red muscle in both transgenic and wild type animals 6 month old animals compared to both 3 and 9 months (Figure 10 A), while β-HAD was significantly higher in 9 month old mice (Figure 10 B) and MDH was significantly lower in both 6 and 9 month old mice (Figure 10 C).

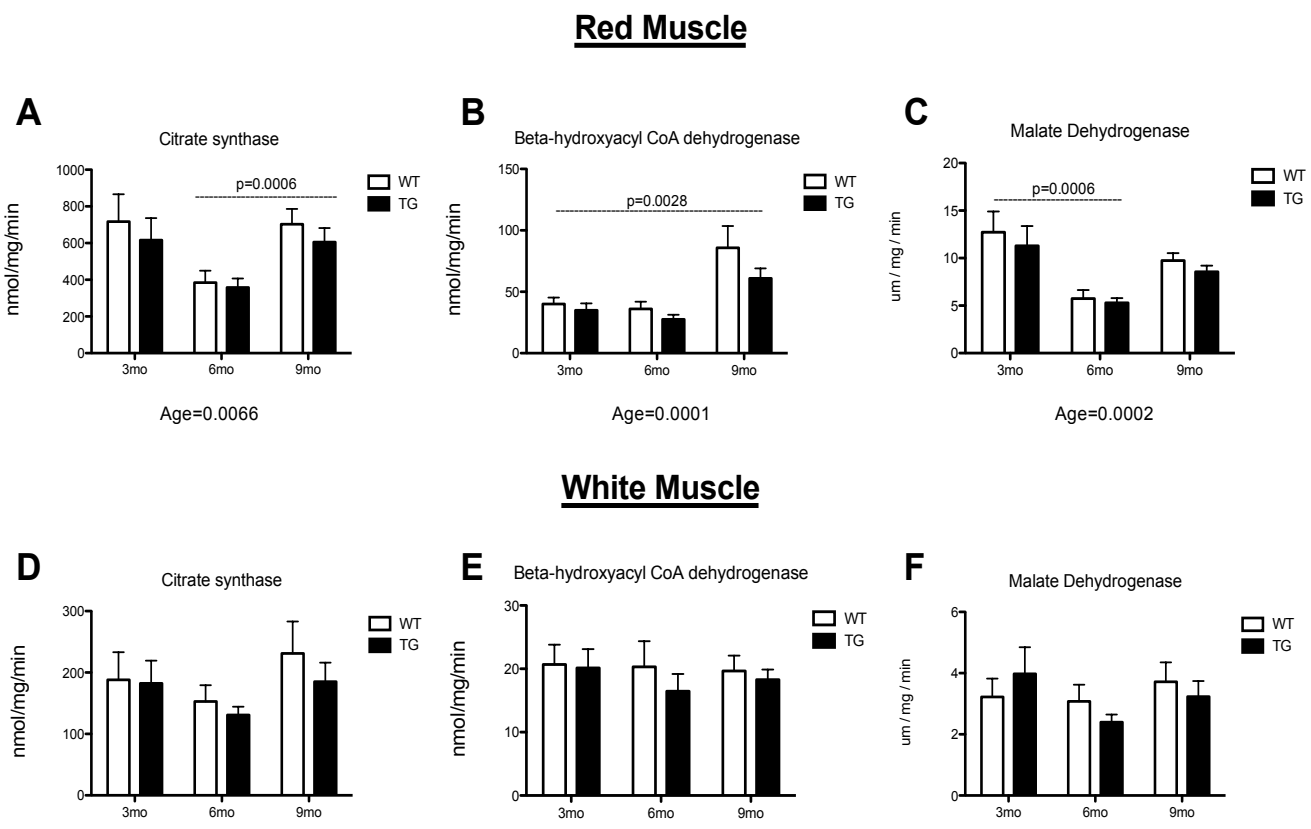

FIGURE TEN: *CS, β-HAD and MDH was measured in red and white muscle of 3,6 and 9-month mice CS in red muscle (A), β-HAD in red muscle (B), MDH in red muscle (C), CS in white muscle (D), β-HAD in white muscle (E), MDH in white muscle (F) Data are presented as means +SEM and *P<0.05.*

Reactive oxygen species generation. There were no significant differences in ROS generation between transgenic and wild type animals at any of the time points measured. There was, however, a significant decrease in ROS production from complex I (Figure 11 A) and complex III (Figure 11 B), but not REV complex (Figure 11 C) between 6 and 9-month mice.

Red Muscle

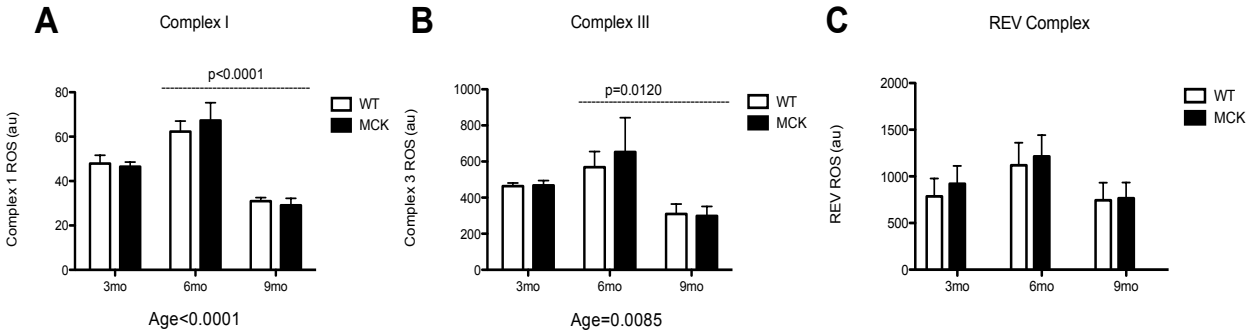

FIGURE ELEVEN: *Reactive Oxygen Species (ROS) Generation in 3,6 and 9-mo mice. ROS generation in complex I (A), ROS generation in complex II (B), ROS generation in complex III (C). Data are presented as means +SEM and *P<0.05.*

Transcriptional regulation in red and white skeletal muscle from MCK-APP mice and their littermate controls. Markers of autophagy were assessed in red and white skeletal muscle along with antioxidant genes in white muscle (since ROS production was not assessed). Beclin mRNA was significantly higher in the red muscle of 3-month (p=0.0256) and 9-month-old MCK mice (p=0.0244) compared to wild type controls (Figure 12 A and B). Also, LC3 mRNA was significant higher in red muscle of 9-month-old mice (p=0.0256). mRNA analysis of white muscle in MCK-APP mice revealed no significant differences in the antioxidants catalase, manganese superoxide dismutase (SOD2), or LC3 (Figure 13 A, B and D). While UCP3 mRNA was significantly higher in 9-month-old mice compared to younger mice (p=0.025), it was lower in MCK-APP mice when compared to wild type mice (p=0.0413) (Figure 13 C).

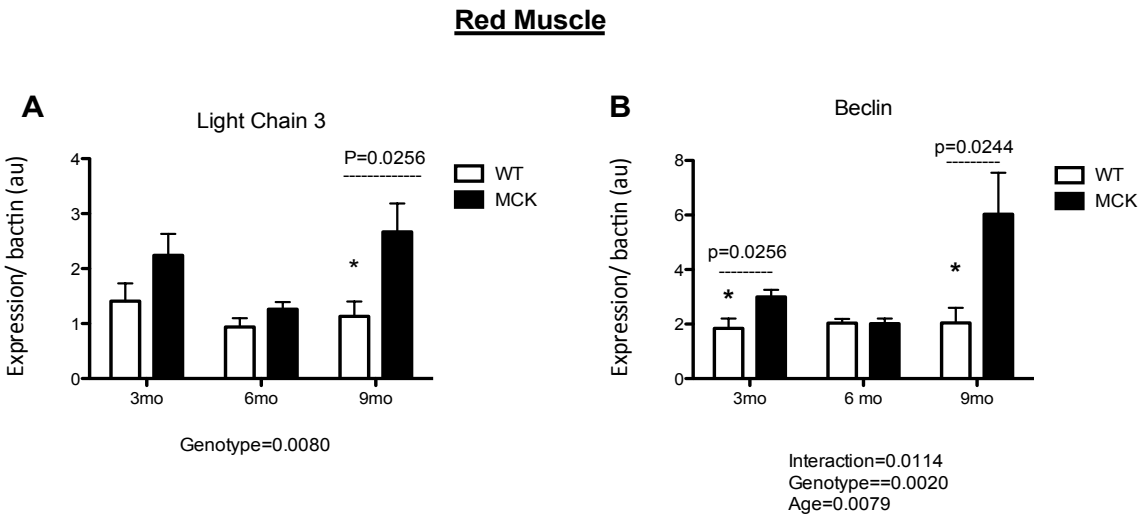

FIGURE TWELVE: *Transcriptional regulation in red skeletal muscle from 3,6 and 9 mo mice.. Relative mRNA content of Light Chain 3 (A) and Beclin (B). Data are presented as means ± SEM and normalized to GAPDH mRNA *P<0.05.*

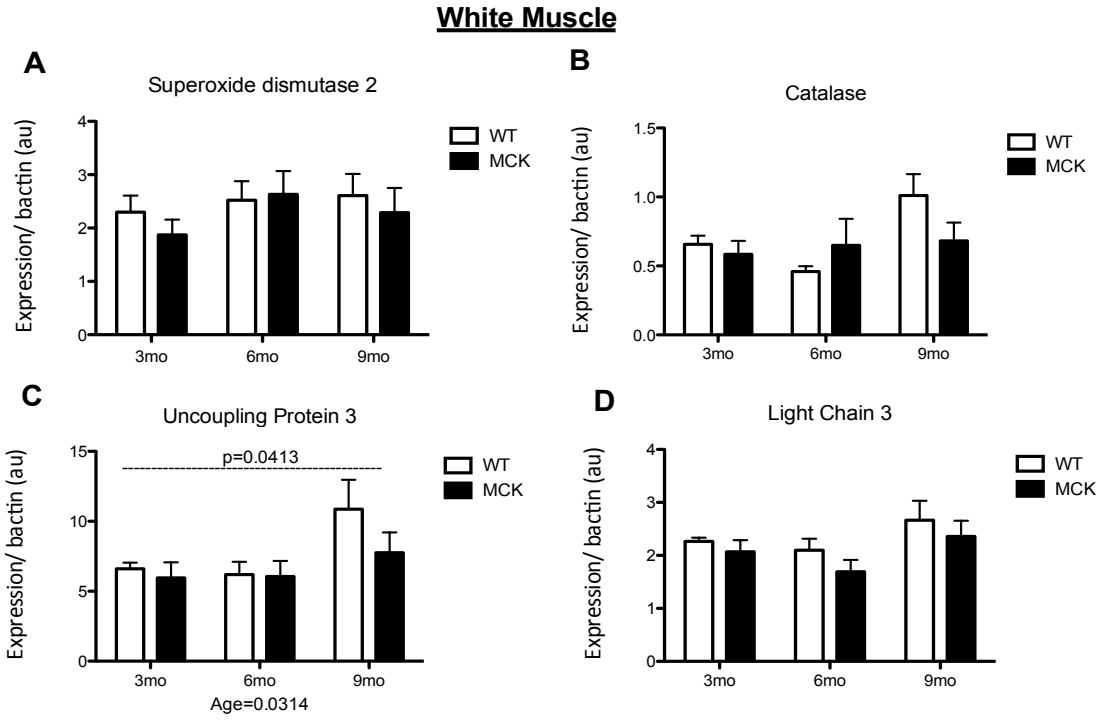

FIGURE THIRTHEEN*: Transcriptional regulation in white skeletal muscle from 3,6 and 9 mo MCK-APP mice and controsl. Relative mRNA content of superoxide dismutase 2 (A), catalase (B), uncoupling protien 3 (C), and light chain 3 (D) from white muscles of MCK-APP and contol mice. Data are presented as means +SEM and normalized to GAPDH mRNA. *P<0.05,*

Markers of autophagy in MCK-APP mice and wildtype littermates. Protein content for markers of autophagy was assessed in red gastrocnemius and quadriceps muscle from MCK-APP mice and their wild-type littermates. LC3 was significantly higher in 3-month-old MCK-APP mice compared to wild type mice (p=0.03) (Figure 14 A). Although there was no interaction, there was a significant effect of age on Beclin content in both transgenic and wild type mice (Figure 14 B).

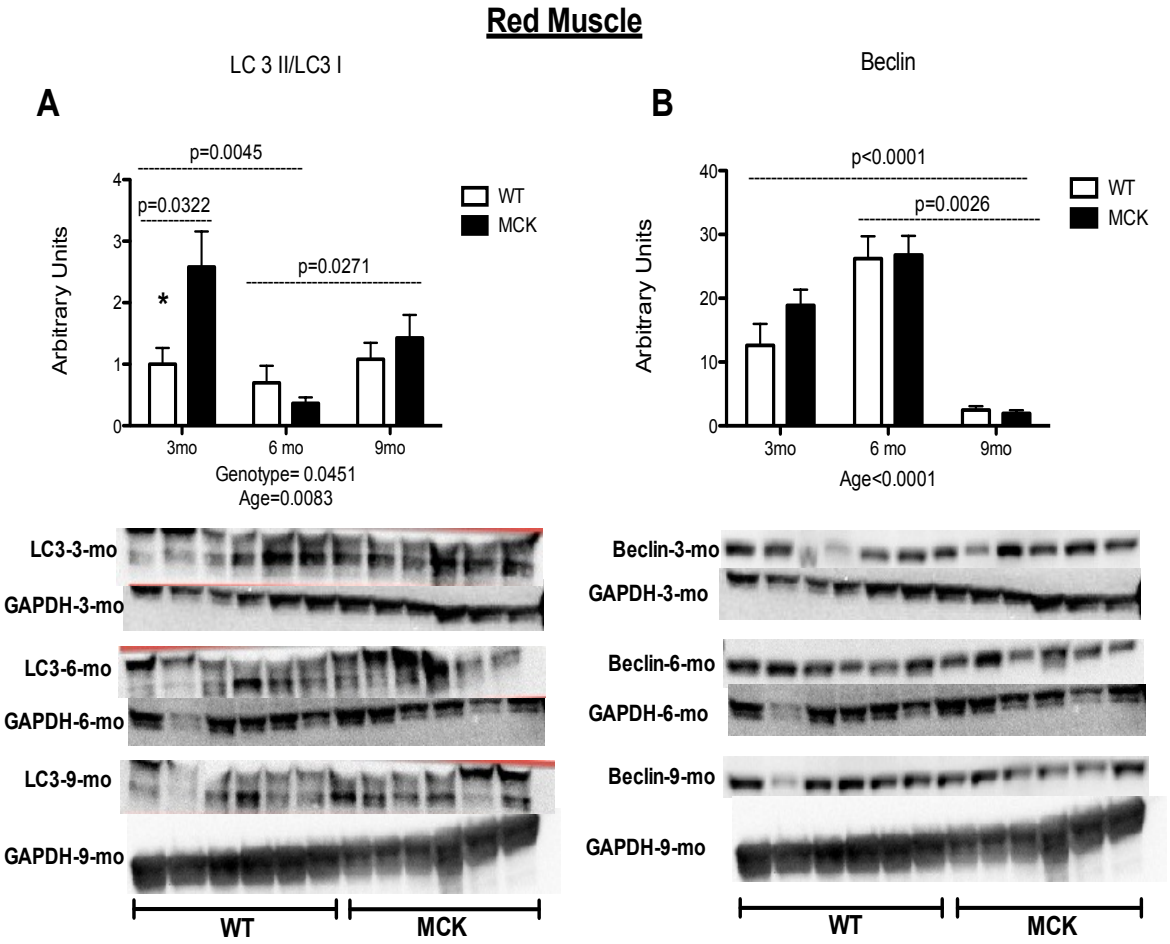

FIGURE FOURTEEN. Protein content in red skeletal muscle from 3,6 and 9-month MCK-APP and wild-type mice. Light chain 3 (A), and Beclin (B). Data is presented as mean + SEM and normalized to GAPDH protien content. *P<0.05 compared to control.

Markers of systemic inflammation in MCK-APP mice and wild-type littermates. To assess systemic inflammation, fasting measures of C-reactive protein (CRP) and interlukin-6 (IL- 6) were measured and shown in figure 15. Although at 3-months CRP concentrations were slightly lower in MCK-APP mice compared to wild type animas, there were no significant differences between genotype observed at 6 or 9-months (p= 0.0511) (Figure 15 A). Serum IL-6 measurements showed no significant differences between MCK-APP and wild-type mice, however there was a significant difference in IL-6 concentrations between the 6-month and 9-month animals (p=0.05) (Figure 15 B).

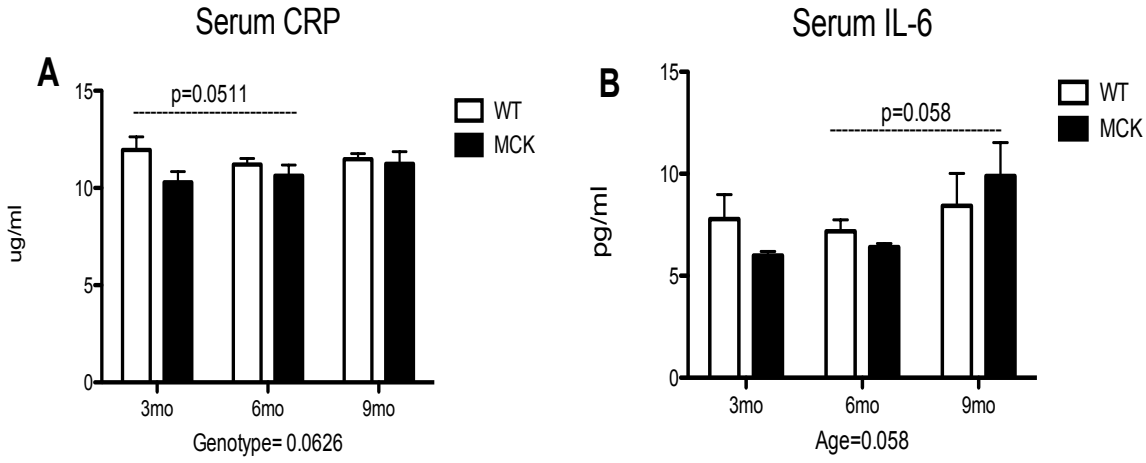

FIGURE FIFTEEN: Systemic Inflammation in 3,6 and 9-month MCK-APP and WT mice of fasting measures of C reactive protein (A) and IL- 6 (B). Data are presented as means ± SEM and *P<0.05.

4.5 Discussion

sIBM is the most prevalent muscle disease among the elderly and risk increases with age (*8*). Furthermore, there is no known cause or successful treatment for the disorder leaving patients with limited options following diagnosis (*153*). The current study tested whether mitochondrial dysfunction contributes to the development of sIBM like symptoms in a mouse model of sIBM. The results from the current study demonstrate that mitochondrial function from skeletal muscle from MCK-APP mice is not disrupted in sIBM. Additionally, there were no differences in substrate metabolism or reactive oxygen species generation in red muscles of MCK-APP mice compare to the wild types. Conversely, decreased fat metabolism and decreased oxidative efficiency in white muscles from MCK-APP mice compared to wild type mice was observed in the current study. Increased protein content of LC3 a marker of autophagy was also reported in MCK-APP mice compared to wild type controls.

These results are in contrast to data reported by Boncompagni et al (*154*), which demonstrated structural and functional alterations in mitochondria of 2-3 month old MCK-APP mice. Their study reported disruption of TCA cycle activity, i.e., reductions in radiolabeled glutamate, and succinate, in MCK-APP mice compared to wild type controls. Increased ROS production in MCK-APP mice was also reported in this study. Differences in findings between the current study and Boncompagni (*71*) may be due to the differences in methodologies used to assess mitochondrial function. While the current study assessed mitochondrial function by measuring mitochondrial oxygen consumption, fatty acid oxidation, and oxidative enzyme activity, Boncompagni's study (*71*) assessed structural and morphological differences within the muscle along with TCA cycle activity,

rate of appearance and disappearance of radiolabeled glutamate and succinate. Furthermore the two studies used different techniques to assess reactive oxygen species production. The precise measurement of ROS in cells and tissues is a challenge because of extremely low concentrations and short lifespan. The current study employed Amplex Red. Amplex red is highly specific and sensitive, with a limit of detection of ≈5 pmol of H_2O_2. Also, the stoichiometry of Amplex Red and H_2O_2 is 1:1; thus, the assay results are linear over the range of values encountered in tissues and cells (*155*). On the other hand, Boncompagni assessed ROS production by measuring intracellular ROS concentrations with Cellular Reactive Oxygen Species Detection Assay (DCF-DA). The DCFH-DA technique is often criticized since photoreduction of DCF results in artificial production of a semiquinone radical that in turn can reduce oxygen to free radicals, and the oxidation of DCFH to the DCF can be self-catalyzed by peroxidases (*156*). Therefore, conditions that alter cellular peroxidase levels could affect DCF fluorescence independent of actual cellular ROS levels (*156, 157*).

There were also differences between the two studies with regards to the fiber types assessed. While Boncompagni et al (*71*) examined mitochondrial parameters in extensor digitorum longus (EDL) and flexor digitorum brevis (FDB), both predominately white muscle types, the current study assessed quadriceps and gastrocnemius muscles, both considered mixed muscle groups. We chose these muscle groups because they are more physiologically relevant to the muscles affected in patients diagnosed with sIBM (vastus laterali)s (*158*). It is important to note that red and white muscle was separated for the current studies and while we did not measure mitochondria bioenergetics from white skeletal muscle we did note a decrease in fat oxidation (total oxidation and CO_2

production) and oxidative efficiency in white gastrocnemius and quadriceps femoris muscle. These results also coincide with the previous human proteomic studies that found reductions in proteins exclusively in the white muscles (*159*). Finally, Boncompagni et al (*71*) identified and separated "amyloid beta affected" muscle fibers for their study. These fibers were characterized by amyloid beta accumulation, amorphous material, the presence of vacuoles, and mitochondrial structural alterations, Not all muscle fibers within a muscle are affected by sIBM and in fact affected fibers are interspersed between many healthy fibers (*71*). It was not possible in the current study to only select affected fibers. As a result, the lack of differences observed in the current study could be due to dilution of affected fibers interspersed within many healthy fibers. Nonetheless, these data along with the work from Boncompagni et al (*71*) suggest that other factors such as amyloid beta accumulation are required for the initiation of mitochondrial abnormalities in skeletal muscle and are therefore more likely the cause (or causes) of sIBM.

While data from the current study do not indicate mitochondria as a primary factor in the initial development of sIBM, it is possible that mitochondria could still be a contributor to disease progression. The current study specifically chose to employ 3,6 and 9-month old MCK-APP mice to explore the effects of mitochondrial dysfunction prior to the occurrence of sIBM symptoms including inflammation, amyloid beta accumulation, and motor defects (*139, 160*). It is possible that mitochondrial dysfunction occurs following disease onset and may contribute to the decreased functionality occurring with disease progression. This idea is supported by previous work conducted in humans in which mitochondrial abnormalities are observed following diagnosis (*81, 161, 162*).

Data from the current study demonstrate increase in LC3 mRNA and protein in MCK-APP mice, which is an indication of increased autophagosome formation. During autophagy, LC3 is lipidated, and the LC3-phospholipid conjugate (LC3-II) is localized to the autophagosome (*163*). The autophagosome travels through the cytoplasm of the cell to a lysosome, which then fuses with the autophagosome resulting in the formation of the autolysosome. As such, the LC3-phospholipid conjugate system is important for the development and transport of the autophagosome (*164*). Additionally, any disruption in any of the steps of autophagy, i.e., disruption in lysosome formation or fusion of the autophagosome with the lysosome, could result in the accumulation of damaged cellular debris including degraded cellular protein. While the current study suggests an increase in autophagosome formation, it is not evident whether this results in the formation of the autolysosome or if there are defects in the formation of the lysosome or autolysosome.

There are some limitations to the current work. For example, while the MCK-APP animal model is an accepted animal model for the study of sIBM, the role of amyloid beta precursor protein and subsequent accumulation of amyloid beta protein in the development of the disease is still up for debate. In addition, in order to understand the effects of age on the role of the mitochondria in the development of sIBM, additional time points with older animals (12, 15, 18 months) should be investigated. sIBM is an age-related disease and the effect of age is an important factor to be investigated in MCK-APP animals. However, it was the focus of the current study to determine the effects of the mitochondria on sIBM prior to the initiation of symptoms, which is why we focused efforts on animals younger than 10 months of age.

In summary, the present work demonstrates that mitochondrial abnormalities and ROS production are not observed in red gastrocnemius and quadriceps femoris muscle and therefore do not appear to be a primary cause of sIBM like symptoms in MCK-APP mice. Nonetheless, there is a significant reduction in fat metabolism as well as an up regulation of autophagic pathways suggesting that certain alterations in skeletal muscle from MCK-APP mice occur prior to the onset of disease symptoms.

CHAPTER V
IMPLICATIONS/ FUTURE DIRECTIONS

Sporadic Inclusion body myositis is the most common muscle disease in elderly individuals. However, to date, the pathogeneses of sIBM is still unknown (*165*). A fundamental feature of sIBM disease is the presence of abnormal expression of the amyloid precursor protein, as well as the buildup of its proteolytic products including amyloid beta (*139*). In fact, it has been suggested that amyloid beta may play a central role in the early events that lead to sIBM (*166, 167*). However, while mitochondrial abnormalities and decreased cytochrome c oxidase have been reported to be mediated by amyloid beta aggregation in cell culture studies (*70, 81, 168, 169*), the role of mitochondrial function in pathology of sIBM has not been investigated.

The central objective of this project was to elucidate the role of the mitochondria in the development of sporadic inclusion body myositis using skeletal muscle samples from patients diagnosed with the disease and a mouse model of sIBM. Overall, results of this project demonstrate 1) disruption of mitochondrial pathways in patients diagnosed with sIBM, 2) reduced fatty acid metabolism and oxidative efficiency in white gastrocnemius muscle, and 3) increased expression of autophagy markers, LC3 and Beclin in red gastrocnemius muscle in MCK-APP mice compared to wild type littermates.

The current study demonstrates that mitochondrial dysfunction does not occur prior to symptom development in a mouse model of sIBM. Nonetheless, the presence of mitochondrial abnormalities in patients diagnosed with sIBM suggests a role in disease progression as well as the functional impairment observed with the disease (*75, 170*).

There are a number of future directions for the current project. A better understanding of the role of autophagy, or disrupted autophagy in disease progression is warranted. For example, determining whether there is a disruption in lysosome and autolysosome formation in this mouse model would highlight novel potential mechanisms for disease pathology. Additionally, future studies are planned to determine the mechanisms whereby mitochondrial lipid composition may play a role in disease progression. In particular, Cardiolipin is an inner mitochondrial membrane phospholipid, which accounts for ~ 17% of mitochondrial lipid membrane. Additionally, LC3 recognition of cardiolipin-binding sites is a requirement for the engulfment of mitochondria by autophagosomes (*171*). Any disruption in this glycerophospholipid can lead to interruption in the delivery of mitochondria to autophagosomes (*172*). The maintenance of the lipid component of the mitochondrial membranes is also crucial for mitochondrial function, integrity and membrane homeostasis.

Additional work should focus on mitochondrial bioenergetics and reactive oxygen species production in white muscles under same conditions as well as studying mice at 12, 15, and 18 months of age following the onset of disease symptoms.

Finally, future studies should be also planned to investigate whether increased oxidative capacity may improve outcomes in sIBM. Data from our laboratory (first manuscript) demonstrate that phosphorylated and total AMPK content is down regulated in skeletal muscle from sIBM patients. AMPK is a master regulator of cellular energy homeostasis and responsible for regulating glucose uptake, fatty acid oxidation, and mitochondrial biogenesis in skeletal muscle (*173-175*). AMPK activation following exercise and treatment with AICAR, an AMPK activator results in improvements in

mitochondrial biogenesis and function that coincides with enhanced running performance (*176-179*). Activation of AMPK activity also leads to reductions in intracellular ROS concentrations. Future work examining whether activation of AMPK via AICAR for example, results in improvement in oxidative capacity and disease symptoms are warranted.

In conclusion, while this work suggests that mitochondrial function is not a primary contributor the development of sIBM, future work in needed to better understand the mechanisms of its contribution to pathology and/ or functional impairment in sIBM.

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