Effects of Creatine Supplementation on Muscle Metabolism in an Alzheimer Mouse Model

By

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ABSTRACT

Alzheimer's disease (AD), the most common form of dementia in the elderly, is a global issue affecting about 24 million individuals. Because AD is a systemic pathology dementia is not the only leading factor contributing to loss of independence in AD patients. AD may also impair skeletal muscle metabolism and function. Creatine (CR) supplementation may enhance skeletal muscle hypertrophy/mass and function in sarcopenia and muscular dystrophies, but has yet to be studied in AD. This study examined the effect of oral CR on muscle metabolism, in a triple-transgenic (3xTg) AD mouse model. Twenty-four, 3×Tg AD mice (~8 month-old) were randomly assigned to control (CON) or CR (3% w/w) diet. Bodyweights and feed intakes were measured throughout the 8-week study. Lower limb (quadriceps muscle; QM and gastrocnemius; GM) and upper limb muscles (triceps; TM) were collected to analyze levels of CR, total protein, DNA, RNA, amino acids (AA), adenosine triphosphate (ATP), adenosine diphosphate (ADP), total and phosphorylated p70 ribosomal S6 kinase (p70S6K). Data (mean ± SEM) were assessed by analysis of variance (ANOVA) and Fisher's least significant difference (LSD) post hoc test. In comparison to the CON group, CR supplementation increased CR content in both GM ($p=0.002$) and QM ($p=0.037$), with higher ($p=0.032$) ATP/ADP ratio in CR in comparison with CON in QM. A higher protein concentration ($p<0.0001$) was notable in GM of CR supplemented group vs. CON. Total branched-chain AA levels in QM increased 2-fold ($p< 0.0001$) in CR groups. Additionally, CR resulted in a higher ($p<0.05$) protein/DNA ratio; an index of muscle cell size, in both QM and GM for CR groups. The index of cell capacity for protein synthesis (RNA/DNA ratio) in GM was also higher ($p=0.001$) in CR groups. However, phosphorylation (activation) level of p70S6K, an integral component in protein synthesis signalling pathway, did not show any significant differences in female ($p=0.161$) and male ($p=0.292$) CR supplemented groups

compared with CON. To conclude, CR supplementation is capable of inducing muscle hypertrophy/growth parameters in the 3×Tg AD mouse model, thereby enhancing protein synthesis capacity in skeletal muscles, thus possibly promoting muscle function in AD.

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DEDICATION

This work is dedicated to patients with Alzheimer's disease, hoping a bright future without Alzheimer's disease and its consequences for the world

To my dearest husband, Navid for his limitless love, care and encouragement,

There are no words to express my deep appreciation of him.

To my mother, Nazi Nategh and my father, Fariborz Farshidfar for their endless support

And for teaching me the meaning of unconditional love.

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LIST OF ABBREVIATIONS

AA: Amino Acids

Aβ: Amyloid Beta

AD: Alzheimer's Disease

ADP: Adenosine Diphosphate

AGAT: Arginine-Glycine Amidinotransferase

ALA: Alpha-Linolenic Acid

AMDR: Acceptable Macronutrient Distribution Range

AMP: Adenosine Monophosphate

AMPK: 5'-Adenosine Monophosphate-Activated Protein Kinase

APP: Amyloid Precursor Protein

ATP: Adenosine Triphosphate

BCAA: Branched-Chain Amino Acids

CK: Creatine Kinase

CON: Control

CR: Creatine

DHA: Docosahexaenoic Acid

DNA: Deoxyribonucleic Acid

EAA: Essential Amino Acids

EPA: Eicosapentaenoic Acid

GAA: Guanidinoacetate

GAMT: Guanidinoacetate N-Methyltransferase

GM: Gastrocnemius Muscle

HPLC: High Performance Liquid Chromatography

IGF-1: Insulin-Like Growth Factor 1

IL-1β: Interleukin-1Beta

IL-6: Interleukin-6

IP: Intraperitoneal Injection

IU: International Units

LBM: Lean Body Mass

MAP: Mitogen-Activated Protein

MMSE: Mini Mental State Examination

MPD: Muscle Protein Degradation

MPS: Muscle Protein Synthesis

MRFs: Myogenic Regulatory Factors

mTOR: Mammalian Target of Rapamycin

NFT: Neurofibrillary Tangles

OD: Optical Density

PCr: Phosphocreatine

PITC: Phenyl Isothiocyanate

PI3K: Phosphatidylinositol 3-Kinase

PUFA: Polyunsaturated Fatty Acids

PVDF: Polyvinylidene Difluoride

p70S6K: p70 Ribosomal S6 Kinase

QM: Quadriceps Muscle

RDI: Recommended Dietary Intake

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

TEA: Trimethylamine

TM: Triceps Muscle

TNF-α: Tumor Necrosis Factor-Alpha

3×Tg-AD: Triple Transgenic Mouse Model of AD

CHAPTER I. LITERATURE REVIEW: OVERALL INTRODUCTION

A substantial increase in life expectancy over the past two centuries means a greater proportion of the senior population in almost all countries around the world (1). Currently, in 2015, the number of people aged 60 years or older is about 901 million, representing 12% of the total world's population (2) and the median age of Canadians is 40.5 years, and 16.1% of the Canadian population is aged 65 years or older (3). Since the first of the baby boomers born shortly after World War II reached the age of 65 years in 2011, we can expect to see an accelerated growth rate in this sub-population (4). It is projected that this number will increase over 10 years, reaching 16.1% of the whole population in 2024 (5). Since aging is accompanied by decline in physical, psychological, biological and cognitive functions, impaired ability to adapt to stressors generally occurs in aged people (1, 6).

Alzheimer's disease (AD) is an irreversible neurodegenerative disease and the most common form of dementia in older adults affecting globally about 24 million individuals (7). In 2011, the number of people living with AD in Canada was 747,000, and it is expected to double during the following two decades (8). Similar trends are also seen in other westernized societies, for example, over 5 million of the 6.8 million cases of dementias in the USA have been diagnosed with AD (9). Overall, aging is a major risk factor for AD, wherein the incidence of the disease rapidly increases after the age of 65 years (10). Hence, as the elderly population continues to increase globally the number of AD cases is expected to double by 2050, reaching up to 1.5 billion people (9). This dramatic increase in AD patients will eventually result in an extensive challenge for health, social and financial networks (7).

The main pathological characteristics of AD in the brain are significant loss of neurons, extracellular accumulation of amyloid β (Aβ) plaques and intraneuronal neurofibrillary tangles (NFT) of hyperphosphorylated tau protein (11, 12). These events result in a chronic, progressive

decline in memory and cognitive functions, leading to clinical symptoms ranging from inability to remember recent events in the early stages of the disease to failure to speak, confusion, lack of language recognition, comprehension problems and long-term memory loss in the later phases of the disease (12, 13). Subsequently, as the disease advances, patients become more dependent on others for assistance to perform activities of daily life (7, 12). However, dementia is not the only leading factor contributing to loss of independence in AD patients. Evidences indicate that AD is a systemic pathology resulting in dysfunctions in various tissues including its effect on skeletal muscle. For instance, muscle problems, *i.e.*, losses of muscle mass and function, may be prevalent among AD patients (14, 15). Although the underlying cause for muscle problems in AD is unknown, hallmark pathological features such as Aβ plaque and NFT are also found in skeletal muscle in AD (16-18). Animal models such as the triple transgenic mouse model of AD (3×Tg-AD) can be used to investigate underlying mechanisms contributing to pathological features of the disease. The 3×Tg-AD was developed that overexpresses mutant amyloid precursor protein (APP), presenilin protein and tau protein; all thought to be linked to AD as these mutations lead to Aβ deposition, elevated intracellular calcium levels and hyperphosphorylated tau, all classic pathological features of the disease (19). Comparison of 3, 6 and 12-month old 3×Tg-AD mice with age-matched control animals revealed that the functionality of skeletal muscles was impaired even in 3-month old 3×Tg-AD animals compared with their control littermates (20). In addition, more Aβ accumulated in skeletal muscles of these 3×Tg-AD mice as the animals became older (20). Since muscle dysfunction in AD patients is never considered a major feature of the disease that requires further treatment, the underlying mechanisms causing skeletal muscle abnormality remain largely unidentified. However, decreased muscle mass or function consequently results in reduced physical activity, which in

turn exacerbates the problem and leads to progressive loss of skeletal muscle mass and function as an outcome of disuse atrophy (21).

Strategies aimed at enhancing muscle mass and function, are critical to increase functionality and quality of life in the elderly population including those with AD. Metabolically, change in muscle mass is determined by net muscle protein balance between muscle protein synthesis (anabolism) and degradation (catabolism). During phases of negative protein balance, degradation outpaces synthesis and proteins are lost. Thus, changing the metabolic pathways in the skeletal muscles toward more anabolism and less catabolism of the proteins may have beneficial roles in improving muscle mass and function in elderly (22, 23).

In addition to muscle dysfunction related to AD, sarcopenia can also affect muscular function in older adults (24, 25). Sarcopenia is a progressive degenerative disorder affecting ≥ 40% of older adults over the age of 70 years (26). It is characterized by involuntary muscle loss leading to functional disability, weakness, and frailty in the elderly (27). Numerous mechanisms has been suggested as potential contributors to sarcopenia onset, including anorexia of aging, protein imbalances, and oxidative stress (28, 29). While consensus preventive and management approaches for muscle dysfunction in AD and sarcopenia are not yet clearly defined, it is unquestionable that nutrition plays a critical role. More widely studied, potent nutrients used to attenuate muscle mass and function loss in older adults are protein/AA, vitamin D and calcium, antioxidants, omega-3 fatty acids and CR. One of these strategies is supplementation of patients with CR, which is a nitrogenous compound with vital roles in energy metabolism of the cells, particularly of the muscles. In fact, more than 95% of the human body's stored CR is located in skeletal muscles (30). CR is endogenously synthesized from arginine and glycine or exogenously acquired through dietary sources, mainly from meat and fish, or supplement. Ingestion of CR-rich foods or supplement helps the body to compensate for CR losses, which occurs through the

kidneys (31). Formerly, CR has been mainly applied by professional athletes involved in high-intensity exercises (32). However, even in the absence of exercise CR is capable of enhancing muscle mass and function by stimulating the anabolic pathways in these tissues (33, 34). The precise mechanism by which CR exerts an anabolic stimulus is not still clearly understood. It has been shown that CR can promote the differentiation of myogenic cells through activation of protein kinase B (Akt/PKB) and p70 ribosomal S6 kinase (p70S6K), two key proteins involved in phosphatidylinositol 3-kinase (PI3K)/PKB/mammalian target of rapamycin (mTOR) pathway (a key pathway implicated in skeletal muscle protein synthesis) (35). This effect was distinct from the effect of CR on cell osmolarity (35), which was previously supposed to play a major role in CR impact on the cells (36). Therefore, CR may be effective in stimulating protein synthesis inside skeletal muscle cells.

The following chapters, chapter II, III and IV, comprise 3 review articles, which provide broad literature reviews regarding muscle metabolism in AD, nutritional supplementation for sarcopenia in older adults (which also could be applied to some of the muscle dysfunctions seen in AD), and potential mechanism of actions of CR supplementation in skeletal muscles, respectively.

In chapter II, the existing literature is reviewed to summarize the available information linking AD and skeletal muscle dysfunction, which includes exploring the potential that some common mechanisms may underpin the dysfunctions in the brain and muscle in AD.

Since nutrition plays a central role in attenuating muscle mass loss in aging population, chapter III seeks to summarize current knowledge about some of the key nutritional supplements, such as protein and AA, vitamin D and calcium, antioxidants, and omega-3 fatty acids, offering a promising perspective in the management of impaired muscle mass and function in older adults with sarcopenia and presumably AD associated muscular dysfunction.

Finally, CR as one of the most popular nutritional supplements is reviewed in a separate chapter. Sources, transport and regulation of CR as well as beneficial and therapeutic applications of CR supplementation are summarized in chapter IV. Particularly, this report seeks to review studies addressing the mechanisms of action of CR supplementation on skeletal muscle metabolism; primarily its effect on muscle growth/hypertrophy.

At the end of these chapters we are able to describe why muscle dysfunction occurs in AD, what nutritional supplements can be helpful for an older adult with muscle problems and why CR is of interest in this study.

CHAPTER II. LITERATURE REVIEW: SKELETAL MUSCLE DYSFUNCTIONS IN ALZHEIMER'S DISEASE

A version of this chapter will be submitted for peered-review publication. ie

2.1 INTRODUCTION

AD, the most common form of dementia in the elderly, is a global issue affecting about 24 million individuals (7). For example, over 5 million of the 6.8 million cases of dementias in the USA have been diagnosed with AD (9). Overall, aging is a major risk factor for AD, wherein the incidence of the disease rapidly increases after the age of 65 years (10). Hence, as the elderly population continues to increase globally the number of AD cases is expected to double by 2050, reaching up to 1.5 billion people (9). This dramatic increase in AD patients will eventually result in substantial health, social and financial burdens (7).

The disease is a complex disorder, but the main identified pathological characteristics are significant loss of neurons due to extracellular accumulation of Aβ plaques and intraneuronal NFT of hyperphosphorylated tau protein in the brain (11, 12). These events result in a progressive decline in memory and cognitive functions, leading to clinical symptoms ranging from inability to remember recent events in the early stages of the disease to failure to speak, confusion, language recognition, comprehension problems and long-term memory loss in the later phases of the disease (12, 13). Consequently, as the disease progresses, patients become more dependent on others for assistance to perform activities of daily life (7, 12). However, dementia is not the only leading factor contributing to loss of independence in AD patients.

Evidences indicate that AD is a systemic pathology, with recognized clinical features such as abnormal weight loss and cachexia (14, 24, 37, 38).

Clinical and animal studies have indicated that the pathophysiology of AD may include morphological, biochemical and functional changes to skeletal muscle (14, 15, 39). Since skeletal muscle, the most abundant tissue in the body, encompassing 40-50% of the body's weight, is considered to be critical to survival and health (40), any impairments in this tissue should be of concern as these may exacerbate the AD state leading to more rapid decline in the health of AD patients. Indeed, in humans, skeletal muscle is responsible not only for mobility but also for various metabolic functions including the consideration as a key regulator of systemic aging (41, 42). In fact, the mortality rate and pathogenesis of many age-related diseases are said to be directly associated with the functional status, metabolic demand and mass of skeletal muscle (42). Decreased muscle mass or function consequently results in reduced physical activity, which in turn exacerbates the problem and leads to progressive loss of skeletal muscle mass and function as an outcome of disuse atrophy (21). Overall, the underlying mechanisms causing skeletal muscle abnormalities in AD patients remain largely unidentified. The objective of this review is to evaluate the existing literature and summarize the available information linking AD and skeletal muscle dysfunction, including exploring the potential that some common mechanisms may underpin the dysfunctions in the brain and muscle in AD.

2.2 PATHOGENESIS OF AD: MANIFESTATION IN CENTRAL AND PERIPHERAL SYSTEMS

There are various hypotheses that have been suggested in attempts to understand the pathophysiology of AD in the brain, with formations of Aβ plaque and NFT as the two most studied areas. First, APP is widely studied because it serves as a precursor for Aβ protein, which

is implicated as a hallmark feature in the pathogenesis of AD. APP, encoded by the *APP* gene on chromosome 21 at location 21q2l.3 (OMIM#104760), is an integral transmembrane protein that under physiological conditions helps neurons to maintain their normal function, including its roles as a growth factor for fibroblasts (43), as a regulator of calcium homeostasis in neurons (44) and adhesion for cell-cell binding and to the extracellular matrix (43, 45, 46). Although the underlying mechanisms of APP in regulating physiological activities are still controversial (47), its function in the pathogenesis of AD has been widely investigated. According to the amyloid hypothesis, there are two enzymes; α-and β-secretases, that are capable of cleaving APP at different locations near its carboxyl terminal to generate alternative APP products: P3 peptide or Aβ, the latter the main feature of AD **(Figure 2.1)**. Specifically, in the non-amyloidogenic pathway, sequential processing of APP first by α-secretase and then γ-secretase produces a large soluble APPα (sAPPα) peptide and a small P3 peptide. Usually, proper functioning of α-secretase prevents APP from being proteolyzed successively by β-γ secretase activity. In the amyloidogenic pathway, sequential cleavage of APP by β- and γ- secretase results in the production of sAPPβ and Aβ peptide (**Figure 2.1**). This hydrophobic Aβ peptide may range from 36 to 43 AA in length, with Aβ40 as the most abundant produced species followed by the less abundant Aβ42; the most germane isoform for the pathogenesis of AD (48). Accumulation of the abnormally formed fragment of APP, *i.e.*, Aβ, in the neurons results in the formation of amyloid plaques in the brain, which over time leads to dysfunction, shrinkage and death of neurons (49-51). The Aβ peptide is generally produced by neurons, glial cells, vascular endothelium and myocytes in the brain, however research indicate that it is also synthesized by peripheral tissues such as arteries, platelets, liver and skeletal muscle (52). Thus, the contribution of these tissues to

the circulating plasma Aβ level may influence AD pathology both inside and outside the brain (52).

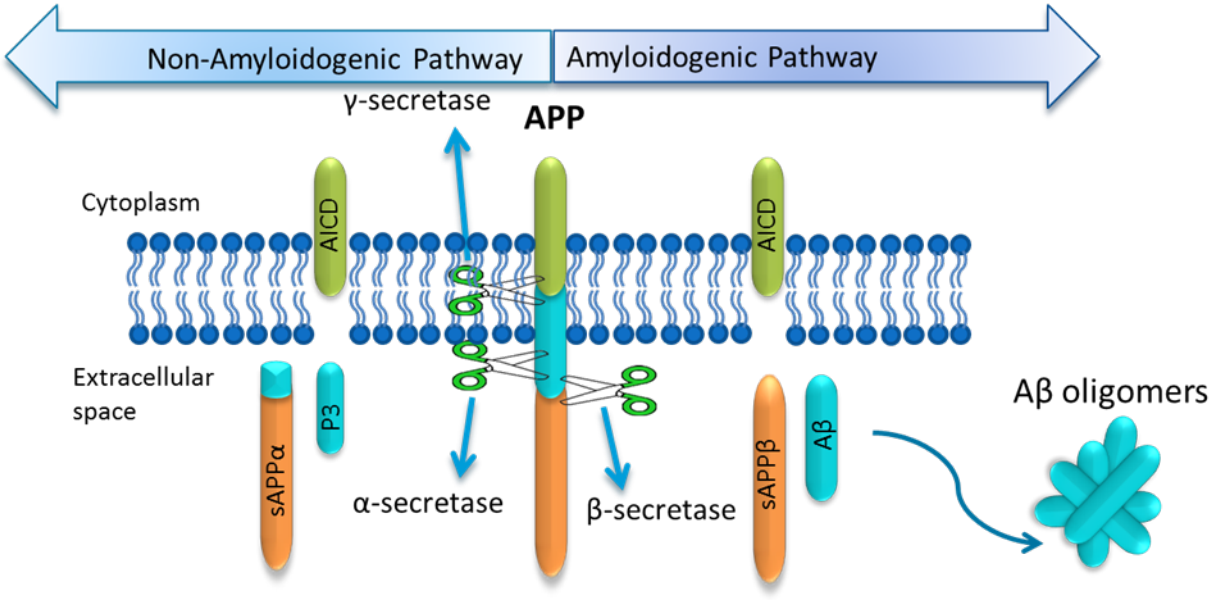

Figure 2.1. Schematic view of amyloid precursor protein (APP) cleavage by alpha, beta and gamma secretases and production of amyloidβ (Aβ). Non-amyloidogenic pathway results from the sequential processing of APP by α- and γ-secretes to produce a large soluble APPα (sAPPα) peptide and a small P3 peptide. Conversely, the amyloidogenic pathway involves cleavage of APP by β-and γ- secretases, resulting in the production of sAPPβ and Aβ. The Aβ units aggregate to form Aβ oligomers, the main constituent of amyloid plaques found in the brain. APP indicates Amyloid Precursor Protein; Aβ, Amyloid β; AICD, APP Intracellular Domain; sAPP, Soluble APP.

Another proposed hypothesis that greatly contributes to current understanding of the pathogenesis of cognitive decline in AD patients is the role of tau protein. Tau proteins are microtubule-associated stabilizing proteins that regulate transports in the axons of the neurons within the central and peripheral nervous system (53, 54) . In its normal state, tau protein is a highly soluble cytoplasmic microtubule-binding protein, with six main isoforms, ranging from 352 to 441 residues, identified in the central nervous system (55, 56). Hyperphosphorylation of tau protein and its accretion within neurons result in the formation of neurofibrillary tangles

(NFTs), which supposedly alters axonal transport and signal transduction pathways (54, 57) (**Figure 2.2**).

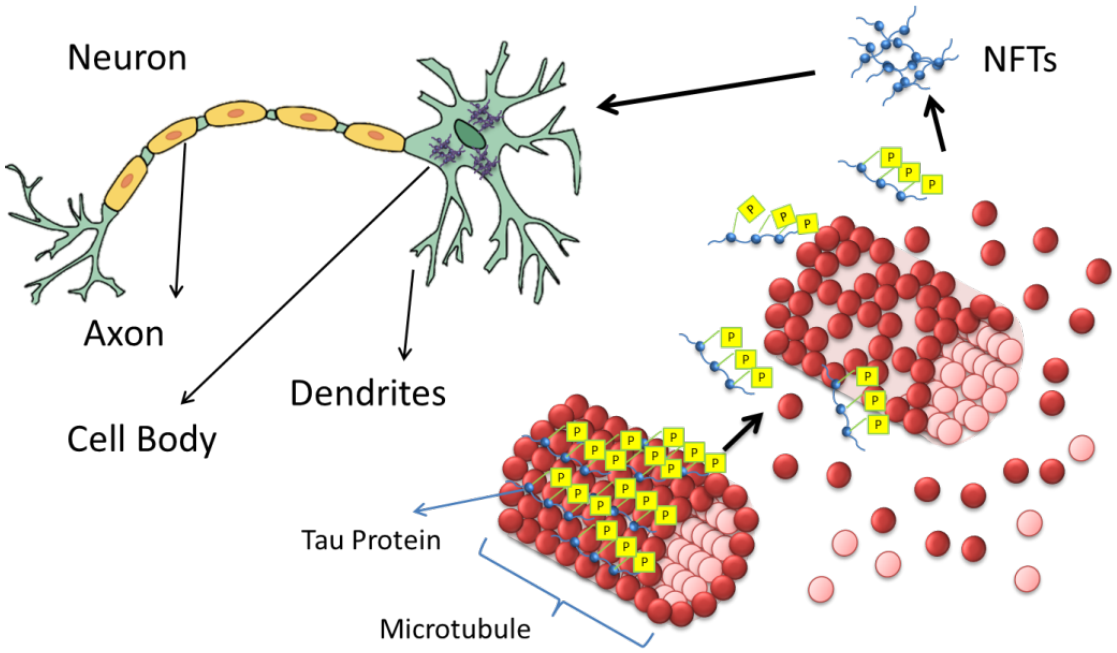

Figure 2.2. Hyperphosphorylation of tau protein and formation of neurofibrillary tangles. Tau protein is a microtubule-associated protein regulating transports in axons of the neurons. Hyperphosphorylation of tau protein and its accretion within the neurons result in the formation of neurofibrillary tangles (NFTs), which is supposed to alter the axonal transport and signal transduction pathways.

In the progression of the pathology of AD, Aβ peptide and NFTs coexist, where Aβ accumulation contribute to the hyperphosphorylation of tau protein (58, 59). The occurrence of neurofibrillary changes in the brain results in the formation of various lesion types and allows AD classification into different stages of progression (60). However, whether it is the toxic effect of NFTs or loss of normal tau protein function that results in the pathophysiological events of AD in the brain remains controversial (61). Furthermore, in the peripheral nervous system, increased phosphorylation of tau protein and its accumulation as NFTs in motor neurons have been demonstrated in a transgenic mouse model for familial forms of AD. Theses alterations

within the motor neurons in the peripheral nervous system were accompanied by functional deficits in the motor activities of the animals (18). In addition to animal models, NFTs aggregation in the spinal cord has also been reported in AD patients (62, 63). Of note, not only does tau phosphorylation affects motor function, it may also contribute to weight loss (55). Thus, tau protein dysfunction has the capacity to influence cognitive and non-cognitive features of AD.

Beyond deposits of Aβ peptide plaques and tau protein dysfunctions, other metabolic alterations also contribute to the pathophysiology of AD in the brain and peripheral systems. Oxidative stress and mitochondrial dysfunction are also united themes which contribute to cellular energy depletion and injury in neurodegenerative diseases and aging (64). Several studies have suggested a pivotal role of mitochondrial dysfunctions in the pathogenesis of various aging-related disorders including AD (65-67). Decreased mitochondrial functionality not only leads to less adenosine triphosphate (ATP) production, but also contributes to greater production and release of reactive oxygen species (ROS) from the mitochondria (67). ROS are a major inducer of programmed cell death, apoptosis, and autophagy, resulting in myocyte loss and/or atrophy (67). Likewise, a widespread abnormality in mitochondrial energy metabolism occurs in AD, not only in the neurons and brain tissue, but also in platelets, lymphocytes and fibroblasts (68). Overall, cellular oxidative stress and defect in ATP production lead to vulnerability of the body's cells to oxidative damage to the lipids, apoptosis and cell death (68). In support of this hypothesis, Kanamori, Nishimaki (69) found a truncated gene product in the intermembrane space of the mitochondria, and its reduced activity resulted in a considerable decline in the activity of complex I and IV of the electron transport chain in the mitochondria (69). In addition, Nunomura, Perry (66) demonstrated that oxidative damage is an early event that occurs in the process of neurodegeneration in AD. In summary, dysfunction of the energy

production system within the cells along with the production and accumulation of Aβ and NFTs in peripheral nervous system including neuromuscular junctions are all involved in the pathogenesis of AD in the brain and peripheral tissues, however there is still uncertainty about the contributing roles of these theories in the establishment and progression of AD.

2.3 MANIFESTATION OF DEFECTS IN SKELETAL MUSCLE OBSERVED IN AD

Ongoing research propose that AD is a systemic illness resulting in numerous peripheral manifestations, including weight loss, dysphagia, incontinence, seizure and muscular dysfunction (70). Indeed, muscle mass loss, muscular dysfunction and loss of muscle strength are some often observed non-cognitive features of AD, which may be associated with cognitive impairment and predict frailty, institutionalization and death in AD patients (14). The preceding sections summarize the available evidence related to AD effects on body composition and strength.

2.3.1 AD Alters Body Weight and Composition, Manifesting in Decreased Skeletal Muscle Mass

Weight loss can occur as a natural consequence of aging (71), however it is more prominent and frequent in AD patients (72, 73). More than 2 decades ago, Wolf-Klein and Silverstone (74) reviewed the literature on evidence of weight loss in AD. At that time 8 articles were published, all supporting a reduction in bodyweight in AD patients (74). During recent years more researchers have examined the relationship between body weight and altered body compositions in AD. To assess the association between annual changes in body mass index (BMI) and risk of developing AD Buchman, Wilson (38) followed 820 older adults for a mean period of 5.6 years. During this time close to 20% (151 individuals) developed AD. By controlling for confounding factors such as age, gender and education, each 1 unit less of BMI at baseline was accompanied by more than 5% increase in the risk AD. In addition, a decline of 1

unit/year in BMI was also associated with 25% increase in the risk of AD. Similar associations were observed between cognitive decline and baseline BMI as well as annual change in BMI (38). Similarly, Renvall, Spindler (75) demonstrated that the body weights of AD patients were lower ($p<0.02$) than those of cognitively normal individuals (55.7±7.1 kg vs. 66.7±11.9 kg, respectively). Although Buchman and others (37, 75) considered weight loss a common non-cognitive clinical feature in AD, researchers such as Buffa, Mereu (24) failed to show any BMI differences between AD patients and healthy individuals. Alternatively, they showed that AD patients have lower lean body mass and higher fat mass compared to healthy older adults (14, 24). In general, the relationship between AD and change in body composition appears to be predominantly related to decline in lean body mass (*i.e.*, sarcopenia). Sarcopenia is an age-related involuntary loss of skeletal muscle mass, quality and function (strength/performance, dynapia), leading to weakness and frailty in older adults (14).

During all stages of AD, especially the intermediate and late stages, patients may experience involuntary weight loss; however the decline in body weight may start as early as the preclinical stages of AD and during the mild cognitive impairment period (76, 77). A cross-sectional, case-control study by Burns, Johnson (14) demonstrated that lean body mass in patients with early AD was lower than individuals with no signs of dementia ($F=7.73$, $p=0.006$). In fact, after controlling for age and sex, the global cognitive function (a composite score on multiple cognitive tasks including memory test) was related to lean mass, which was measured by dual energy x-ray absorptiometry (DXA) ($\beta=0.12$, $p=0.007$). Body weight and BMI of demented and non-demented individuals did not show any significant differences ($p=0.10$ and $p=0.38$, respectively). Women and men revealed the same pattern in the fat-free mass loss in association with AD. Furthermore, a positive relationship was observed between whole brain

volume; mainly white matter volume, and lean body mass (β=0.20, $p<0.001$), suggesting that lean body mass loss has a greater correlation with AD than weight loss. There were no associations between body fat mass and any of these parameters, including cognitive performance, whole brain volume and white matter volume (14). In older adults, aged 70 years and more, body composition is related to cognitive function. Having lower muscle mass, *i.e.*, sarcopenia, or greater fat mass, or both, are all connected to lower cognitive performance. Although the research performed by Dvorak and Poehlman (78) revealed no association between cognitive status and appendicular muscle mass in AD patients, cognitive functioning based on the score for the Wechsler Adult Intelligence Scale, Third Edition (WAIS-III) Digit Symbol-Coding module in individuals with merely sarcopenia or obesity decreases 4.2 and 1.5 points, respectively, relative to the healthy reference group. However, sarcopenic obesity has a greater impact on cognition that reduces 7 points from functioning score comparing to healthy individuals. This indicates that change in body composition in elderly is related to altered cognition (79).

2.3.2 AD Alters Skeletal Muscle Strength

In addition to muscle mass loss, as muscle strength decreases the rate of decline in global cognitive function increases (80). In a prospective cohort study conducted by Raji, Kuo (15), having poor cognitive status (determined by the Mini Mental State Examination, MMSE score<21) was positively associated with lower skeletal muscle strength (measured by handgrip muscle strength test). MMSE is a 30-point cognitive screening questionnaire, which can differentiate normal cognitive status, mild, moderate and severe cognitive impairment from each other (81). Even after considering the effect of confounding variables such as very low MMSE scores (severe cognitive impairment or MMSE score<15), the relationship between poor

cognition and reduced strength in muscles remained constant. Therefore, decline in either muscle mass or strength should be considered as a clinical manifestation of AD.

As AD progresses, patients become entirely dependent on others to assist them with performing activities of daily living. While the role of dementia in functional decline of patients is not deniable, motor disturbances resulting from both AD's pathology and aging process makes the problem even more complicated. Evidence suggests that muscle dysfunctions may be quite prevalent in AD population (14, 80), however in the majority of cases these dysfunctions are never considered as clinical features of AD (82) that require further treatment. One possible reason for this oversight is that patients who are already diagnosed with AD lose their ability to localize their pains and discomforts or describe them to their caregivers, family members and physicians (83), therefore the muscle problems remains obscure within the disease setting. In this regard, partial or complete unawareness of deficits in AD can be defined as anosognosia, an impaired ability to recognize the presence or appreciate the severity of deficits in sensory, perceptual, motor, affective, or cognitive functioning, which may result in diagnostic delay and challenge in caring (83, 84). In addition, age-associated decline in muscle mass and strength commonly occurs concurrently with myopathy of AD (14), which makes it difficult to differentiate between these two pathologic conditions. The inability to move independently because of imbalance and incoordination and decline in cognitive status leads to immobility in AD patients and consequently muscle mass loss and dysfunction. Khodeir, Conte (85) conducted a study in 50 randomly selected individuals with probable dementia of the Alzheimer type. The result of this study revealed that immobility and cognitive dysfunction are associated with loss of lean mass (85). In general, decreased energy intake and physical inactivity in AD patients have

been shown to lead to reduced muscle mass in the extremities, which in turn impairs the level of activity in an older adult with AD (78).

On the other hand, being physically active is also important in mitigating the cognitive decline. Souza and his colleagues demonstrated that 8 weeks of swim training in male mice, prior to intracerebroventricular injection of Aβ peptide (400 pmol/mouse), resulted in reducing Aβ induced cognitive dysfunction and preventing oxidative stress and neuroinflammation (86). Furthermore, human studies also revealed that physical fitness and activity are capable of protecting and ameliorating cognitive impairment (87, 88). As Burns, Johnson (14) demonstrated, in the early stages of AD, physical function is significantly lower compared with age and sex-matched non-demented individuals. In fact, white matter volume was found to be directly related to physical activity (β=0.12, p=0.009) (14).

In summary, the evidences suggest that physical activity and cognitive function have complementary effect on each other, wherein being physically active leads to protection from or reduction of cognitive impairment. Having healthy skeletal muscles would also contribute to the preservation of physical activity, which would successively help to sustain muscle mass. Thus, the overall outcome is increased quality of life in an older adult due to the benefits of physical activity.

2.4 MECHANISMS ASSOCIATED WITH MANIFESTATION OF SKELETAL MUSCLE DYSFUNCTIONS IN AD ARE LINKED TO BRAIN PATHOPHYSIOLGICAL FEATURES OF AD

Unfortunately, to date, little attention has been given to many of the non-cognitive signs and symptoms of AD, thus the mechanism of actions of these remains unclear. However, as

highlighted in the preceding sections, dysfunction in the brain and skeletal muscle may emerge from common mechanisms (39).

2.4.1 Chemosensory Dysfunction and Decreased Appetite in AD

Impaired chemosensory commonly occurs in AD, leading to decreased appetite and anorexia in AD (89, 90). Decline in food intake as a consequence of mental and physical impairments in senescent population, especially in those with dementia, results in inadequate intake of nutritious elements (91). Altered taste and smell, agitation and behavioral disorders, medications and their adverse effects, decreased independency in self-feeding all contribute to diminished dietary protein and caloric intake in patients with dementia (92). In addition to decreased energy intake, higher energy expenditure is also noted in AD patients, resulting in energy imbalance in the body (93). The consequence of this energy derangement is loss of muscle mass (94). After all, as the body's largest protein store, skeletal muscle plays a crucial role in whole-body protein metabolism by acting as a central reservoir for AA to maintain protein synthesis in vital tissues and organs during dietary restraint (40). That is, metabolically, change in muscle mass is determined by net muscle protein balance between muscle protein synthesis (MPS) (anabolism) and degradation (MPD) (catabolism). During phases of positive protein balance synthesis outpaces degradation and protein synthesis augments the development of muscle mass. The inverse event happens in periods of negative balance, herein muscle catabolism outpaces synthesis and thus proteins are lost resulting in reduced muscle mass (22, 23). Thus, the alterations in taste and smell due to AD lead to decreased energy intake resulting in a negative skeletal protein balance manifested as decreased lean muscle mass.

2.4.2 Cholinergic System Dysfunction and Decrease in Motor Neurons in Skeletal Muscle in AD

Substantial research established the "cholinergic hypothesis of AD" as a pathophysiological feature in the disease (95, 96). Acetylcholine is the principal neurotransmitter of brain cholinergic neurons and contributes to cognitive and behavioral functions. Early studies indicate that presynaptic markers of the cholinergic system appear to be uniformly reduced in AD patients as illustrated by reductions in acetylcholinesterase activity and acetylcholine synthesis in the brain, which are strongly correlated with the degree of cognitive impairment in AD patients (95). Two types of acetylcholine receptor family, nicotinic and muscarinic receptors, respond to acetylcholine in the brain (97) and are affected in AD (98-100). It has been demonstrated that stimulation of muscarinic receptors in βAPP transgenic mice leads to a shift to non-amyloidogenic pathway in the brain and a decrease in Aβ level (100). In addition, Aβ has a high affinity for binding to nicotinic receptors in the brain and thus impairing the neurotransmission of acetylcholine and consequently cognitive dysfunction in AD (101). Furthermore, it is believed that skeletal muscle dysfunction associated with abnormal body weight loss detected in AD patients may result from abnormal regulation of the cholinergic system (39). Monteiro-Cardoso, Castro (39) showed that regulation of skeletal muscle activity by acetylcholine may be compromised in 3xTg-AD mice as a decrease in maximal acetylcholinesterase activity detected early in the pre-symptomatic stage and throughout the disease progression. Deficient acetylcholinesterase activity was detected in skeletal muscle of 3xTg-AD mice at 3 months of age; pre-symptomatic stage of AD, prior to deposition of Aβ in the brain, indicating early changes in skeletal muscle associated with AD-like pathology (39).

Besides the functions in the brain, the acetylcholine-mediated system also determines channel opening at neuromuscular junctions of skeletal muscles (102). Acetylcholine functions in the neuromuscular junction as a neurotransmitter responsible for converting the electrical signal of the neurons to the chemical signal of the muscles and thus generating a muscle contraction (103). Any disturbance in the acetylcholine function results in a disconnection between the neuron and the muscle fiber, impairing the contractility of the muscle (103). Disturbance in cholinergic system is implicated in neurodegenerative diseases manifesting motor dysfunction such as Parkinson disease (104). It has been reported that as high as one third of AD patients suffer from at least one motor sign which are commonly associated with cognitive decline and mortality (105). The motor signs implicated with AD may include gait disturbance, bradykinesia/hypokinesia, rigidity in neck, upper and lower limbs, and tremor (105). For instance, it has been shown in a clinical study that 40% of patients had recognizable gait disturbances (106). In general, assessment of the peripheral nervous system in AD reveals some abnormal features including decrease in the number of functioning motor neurons in skeletal muscles of AD patients (107, 108). Muscle biopsies from these patients were compatible with motor neuron disorders such as anterior horn cell disease or peripheral nerve dysfunction (108). Results from animal studies also indicated that homozygote transgenic mice for AD, which are deficient for tau protein, have weakness in their muscles (measured by wire-hanging test) compared with wild-type animals, the difference which is also detectable between heterozygous mice and the wild-types (109). Having muscle weakness in both homozygous and heterozygous mice indicates that the loss of function in tau protein results in myogenic dysfunction. However, the responsible mechanism is still not clear (109). In addition to tau-deficient mice models, in other types of AD transgenic mice, axonal injuries and motor disturbances are also detectable.

Wirths, Weis (110) demonstrated that in a double-transgenic mouse model, expressing mutant APP and presenilin 1, axonopathy, characterized by axonal swellings, spheroids, axonal demyelination and ovoids, is detectable in the spinal cord. Furthermore, Aβ peptide deposition in motor neurons precedes amyloid plaque formation (110). Moreover, defect in sensorimotor feedback loops, which alters the corticospinal excitability, along with oxidative damage to proteins and lipids in the peripheral tissues results in motor unit loss, axonal atrophy and demyelination of neural fibers (111). These data indicate that motor deficits in AD may start even before the initial manifestation of cognitive impairment in AD patients. In addition, the deleterious impact of the disease pathology on motor neurons can be an initiative mechanism that along with other factors such as negative energy and protein balances, lead to myopathy and muscular dysfunction.

2.4.3 Production and Accumulation of Amyloid β also occur in Skeletal Muscle

The Aβ peptide, which is generally produced by neurons, glial cells, vascular endothelium and myocytes in the brain, is also synthesized by peripheral tissues such as arteries with atherosclerosis, inactivated platelets, liver and skeletal muscle (52). Since Aβ possesses toxic properties, not only in the central nervous system but also in the periphery (112), and the deposits of this abnormally formed APP derivative are found in various tissues beside the brain, such as skeletal muscles (52), it is reasonable to expect non-cognitive manifestations in AD patients. The cellular mechanisms responsible for generating Aβ and its pathological depositions inside cells was demonstrated by Gabuzda, Busciglio (113) to be due to the depletion of ATP from cells, resulting in altered proteolytic cleavage of APP by β-secretase **(Figure 2.1)**, and increased Aβ inside the cells. These researchers found that by inhibiting the mechanisms responsible for cellular oxidative energy production a significant increase in Aβ was observed

inside the cultured cell line (113). In addition, under normal physiological conditions Aβ is produced in low quantities during the transport of APP through the secretory pathway of the cell. Inhibition of this pathway also resulted in the overproduction of Aβ (113). On the other hand, Aβ is also capable of inducing oxidative damage to cells. The free radical-associated oxidative insult to the proteins increases as a consequence of Aβ accumulation in cells, suggesting a neurotoxic role for Aβ (114). These properties of Aβ initiate a vicious cycle inside neurons: oxidative stress leads to increased burden of Aβ, which induces mitochondrial damage, oxidative stress and cell death (114, 115).

As shown by Beckett, Studzinski (116), the levels of Aβ in both brain and skeletal muscle of APP knock-in mice is higher than their wild type littermates (116). Therefore, contribution of skeletal muscles to circulating plasma Aβ level may influence AD pathology both inside and outside of the brain (52). The amount of Aβ in skeletal muscle has been shown to positively correlate with age (17). Because skeletal muscles account for over 40% of an adult's body weight an increase in Aβ in this tissue may result in its buildup in blood and then brain. Accumulation of Aβ in skeletal muscle can partially explain its role in muscle pathologies in AD (17). Using a 3xTg-AD mouse model, Monteiro-Cardoso, Castro (39) showed that an early contributing factor to impaired skeletal muscle function in AD may be related to the accumulation of Aβ in the muscle, which may precede other contributors such as mitochondrial dysfunction. As Mukhamedyarov, Grishin (16) explained in their study, accumulation of Aβ in skeletal muscle can lead to a disturbance in resting membrane potential of myocytes. In their study of frogs, Mukhamedyarov, Grishin (16) found that an increase in the concentration of Aβ in muscle depolarizes the plasma membrane of the skeletal muscle fiber in a dose-dependent manner. That is, as Aβ level increases there was a more rapid decrease in membrane potential

and also faster restoration of membrane potential to its initial value (16). In addition, Aβ also impairs contractions in frog muscle, it results in a decrease in the amplitude of contraction in response to chemical stimuli (acetylcholine receptor agonist) and elongation of the relaxation period in electrically-induced contraction (112). Likewise, in a double transgenic mouse model of AD (APP/PS1), the generation of electrical impulse (electrogenesis) within skeletal muscle fiber was significantly impaired compared to wild-type animals. In these transgenic rodents, Aβ accumulation resulted in significant depolarization and ionic imbalance within muscle fibers, which contributed to motor dysfunction (117). Furthermore, accumulation of similar biochemical deposits of Aβ and phosphorylated tau protein in the skeletal muscle fibers of patients with sIBM suggest a role for these compounds in the pathogenesis of myopathies in neurological disorders.

2.4.4 Age-Related Muscle Disorders such as Sporadiac Inclusion Body Myositis (sIBM)

Although age-related disorders such as AD and sarcopenia express different clinical manifestations, it seems that they share some common underlying pathophysiological features. Since both conditions co-occur in older adults, it can be speculated that a shared mechanism may have been responsible for the initiation and progression of these age-associated diseases. In a case report by Roos, Vesterberg (118), a 65-year old woman with evidence of sporadic inclusion body myositis (sIBM); an age-related muscle disorder resulting in weakness and atrophy of the distal and proximal muscles (119), showed an insidious deterioration of brain function during 6 months from the first manifestation of the disease. Further investigations by electroencephalography (EEG), memory tests, MMSE and an assessment by a clinical psychologist confirmed the diagnosis of AD in this patient. In fact, similar to AD, biochemical deposits including Aβ peptide and phosphorylated tau protein accumulate in the skeletal muscle fibers of patients with sIBM, which suggests a common etiology between sIBM and AD (118).

Similarly, Levacic, Peddareddygari (120) reported a case of a 73-year old woman with sIBM, who after 3 years from initial diagnosis started to reveal cognitive decline with difficulties in memory, speech, and attention leading to the diagnosis of AD (120). Therefore, simultaneous presentations of myopathy and AD with the same pathological features; presence of decreased lean body mass and muscle function, along with peripheral nervous system associated motor disturbances in AD patients, suggests that there might be some linking mechanisms relating muscle problems to cognitive impairments in these patients.

2.4.5 Mitochondrial Dysfunction Leading to Widespread Abnormalities in Energy Metabolism

Mounting evidences support the theory that impaired energy metabolism of the cells, as a result of mitochondrial dysfunction and oxidative damage may play a fundamental role in the pathogenesis of aging-associated disorders such as neurodegenerative diseases and myopathies (39, 65-67, 121, 122). To study the consequences of intracellular Aβ accumulation in skeletal muscle, Boncompagni, Moussa (122) used a muscle creatine kinase (MCK)- βAPP transgenic mouse model to show that elevated Aβ levels in skeletal muscle results in severe structural and functional alterations in mitochondria. Similarly, Monteiro-Cardoso, Castro (39) study's results indicated that the increase in Aβ levels in skeletal muscle of 3xTg-AD mice may precede and contribute to skeletal muscle mitochondrial dysfunction (39).

Another mitochondrial associated-mechanism linking AD and myopathy is related to parkin. Kitada, Asakawa (123) discovered a new mutation on chromosome 6 (6q25.2-q27) that was responsible for the pathogenesis of autosomal recessive Parkinson disease, therefore the product of this gene was named "Parkin" (123). Parkin is abundantly found in the brain, especially in the substantia nigra region, but has also been isolated from other tissues such as

skeletal muscle (123). Two years after the identification of parkin, Shimura, Hattori (124) revealed that parkin functions in the cell as an enzyme involved in ubiquitin ligation of proteins, thus mediating the degradation of selected proteins. The identity of these proteins remained to be determined by further researchers (124). Mutation in *parkin* gene and loss of its activity lead to the accumulation of the impaired mitochondria inside the cell, resulting in cell damage and finally neurons death (125). Under normal physiological conditions parkin is located in the cytosol of the cells. Upon mitochondrial damage and initiation of oxidative stress, parkin translocates to the outer membrane of the mitochondria, inducing its clearance by autophagic eliminating mechanisms. This phagocytosis prevents further release of reactive oxygen species from the mitochondria and thus prevents cell death (126). Therefore, parkin acts as a quality control system for mitochondria. Furthermore, it has been shown that increase in the levels of parkin inside skeletal muscle cells protect the myocytes against mitochondrial toxins associated insult and accumulated Aβ (127). In vitro studies of cultured skeletal muscle cells from parkin knock-out mouse, demonstrated that the lack of parkin results in Aβ buildup in muscle cells, suggesting a role for parkin in degrading Aβ (127). Likewise, parkin also decreases in the brain of patients with AD (128). Crossbreeding of transgenic mice for AD, APPswe/PSEN1DE9 (APP/PS1), and parkin transgenic mouse overexpressing parkin in the brain (Parkin OE), resulted in diminished load of Aβ in the brain and improvement in behavioral abnormalities (including locomotion, exploratory and anxiety-like behavior) in their offspring (APP/PS1/Parkin OE) (129). Thus, based on the insights gathered about parkin from the studies conducted to date, controlling the levels of parkin in cells could be considered as a promising target for AD and myopathy treatment.

2.4.6 Perturbation of mTOR Signaling

Perturbations of mTOR signaling pathway; plays a central role in cell growth, cell-survival and protein synthesis, have been implicated in AD (130). The mTOR pathway is a conserved serine/threonine kinase involved in many signal transduction pathways within the cell, regulating cell growth and homeostasis (131). mTOR is categorized as two protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), the major difference between these two complexes is having either the raptor or rictor subunit, respectively. mTORC1 is sensitive to the inhibitory effect of rapamycin (132), a macrolide antibiotic which helped in identifying the *TOR1* and *TOR2* genes in rapamycin-resistant yeast (133). While activation of mTORC1 further phosphorylates/activates p70S6K and protein synthesis, its inhibition activates autophagy machinery to eliminate damaged components and save energy for stressful conditions (130). Thus, due to the central role of mTOR in several signaling pathways, it is implicated in the pathogenesis of diseases in which growth and homeostasis are compromised, *e.g.*, cancer (134), aging (135), myopathies (136) and AD (130).

The mTOR complex was showed to be involved in the regulation of the phosphorylation of tau protein (137). As discussed earlier in this review, accumulation of hyperphosphorylated tau protein in the form of NFTs is a critical event in the pathogenesis of AD (57). It is widely believed that the phosphorylation and degradation of tau protein is regulated by mTOR (137-140). In an in vitro study by An, Cowburn (138), the phosphorylated level of downstream target of mTOR (p70S6K) was increased in neurons prior to the accumulation of NFTs and this level was associated with the total level of tau protein as well as the hyperphosphorylated form of tau protein (138). In a transgenic mouse model overexpressing mTOR, the hippocampal level of tau protein was 1.5 fold higher than their wild-type littermates, mostly due to hyperphosphorylated forms of tau protein. Further analysis in these rodents revealed that a reduction in autophagic

regulatory mechanisms as a result of mTOR hyperactivity is the main cause for the increase in the level of hyperphosphorylated tau (137).

The activation of p70S6K and mTOR is also essential for skeletal muscle hypertrophy as it is required for the initiation of a series of mRNA translation, which encodes protein necessary for protein synthesis. Moreover, mTOR is implicated in protein synthesis and degradation in myopathies (141, 142). Similar to what occurs in AD, increased activation of mTOR and the subsequent hyperphosphorylation of p70S6K have been observed in aged mice (143), suggesting a role for mTOR in the pathogenesis of sarcopenia and other age-related muscle abnormalities. Further inactivation of proteolytic pathways within skeletal muscle did not result in any improvement in muscle mass, and in contrast, it led to muscle pathology (143). Similarly, mTOR activity increases in denervated muscle fibers, resulting in muscle atrophy. Subsequent inhibition of mTORC1 in denervated muscle fibers alleviates the mTOR-induced atrophy in the muscles (131). Overall, the involvement of mTOR in pathogenesis of AD and myopathy is thought to provide evidence that it might be a potential therapeutic target for both AD and myopathy.

2.4.7 Insulin Resistance and Hyperinsulemia

Increase in visceral fat mass and adiposity in aging leads to insulin resistance and a rise in circulating levels of insulin, which in turn results in a higher risk of AD (144). Data from the literature suggest a greater prevalence of risk of dementia and cognitive impairment in patients with type 2 diabetes mellitus, perhaps due to the increased levels of insulin in their body as a result of insulin resistant (145). The identification of insulin receptors in the hippocampus, a part of the brain involved in recollection of events, provided evidenced for a role of insulin in memory function (146). An investigation by Levine and Crimmins (79) indicated that associations between both sarcopenic and nonsarcopenic obesity and cognitive function are

influenced by insulin resistance (79). Furthermore, it has been shown that insulin-degrading enzyme (IDE), which breaks down insulin, also has a role in regulation of Aβ peptide. High levels of insulin in the body compete with Aβ for IDE, resulting in a derangement in Aβ metabolism and its accumulation in neuronal tissue (147). In addition, trafficking of APP and Aβ to the plasma membrane from the trans-Golgi network, a major cellular site for Aβ production, and decline in intracellular levels of Aβ is accelerated by insulin. Thus, insulin and hyperinsulinemia promote extracellular accumulation of Aβ by increasing its secretion from the neurons and reducing its degradation by IDE (148). Furthermore, hyperphosphorylation of tau protein, which supports the cytoskeleton of neurons, can occur as a consequence of impaired insulin signaling in the brain, resulting in the formation of NFT and pathophysiologic events of AD (149).

It is also well established that insulin is an important regulator of muscle metabolism, promoting anabolism by increasing amino acid uptake and protein synthesis in the muscle cells. Insulin resistance results in decreased activity of anabolic pathways, promoting degradation of proteins by activation of catabolic pathways which consequently leads to muscle mass loss and impaired functionality of muscles (150). Thus change in insulin sensitivity may have multi-system effects in the body, on one hand affecting body composition and reducing muscle mass by disrupting the anabolic stimulus on muscle function (151), and conversely influencing the metabolism of Aβ, causing its build-up in the brain, with resulting cognitive malfunctioning (147).

2.5 CONCLUSION

Evidences indicate that AD is a systemic disease with a broad spectrum of manifestations, including skeletal muscle dysfunctions, which may present as losses of muscle

mass, strength and function. Thus, the negligence in assessment of muscle abnormalities in the setting of AD may have a huge impact on AD patients. After all, skeletal muscle, the most abundant tissue in the body, is responsible for mobility and many metabolic functions in the body. Hence, abnormalities and dysfunction in skeletal muscle may contribute to AD progression, leading patients to become more dependent on others to perform activities of daily living, and to the mortality rate for AD. Although the underlying cause for skeletal muscle dysfunction in AD remain largely unknown, growing evidences indicate direct association between cognitive dysfunction and skeletal muscle dysfunction in AD, which may emerge from shared mechanisms. Targeting treatment options toward these linking mechanisms may result in alleviation of both cognitive and muscular symptoms in AD. Thus it is important to continue to study the role of skeletal muscle in AD in order to elucidate its impact on AD progression.

CHAPTER III. LITERATURE REVIEW: NUTRITIONAL SUPPLEMENTATIONS AND ADMINISTRATION CONSIDERATIONS FOR SARCOPENIA IN OLDER ADULTS

Reprinted from Nutrition and Aging, Vol number 3, Farnaz Farshidfara, Veronika Shulgina and Semone B. Myrie, Nutritional supplementations and administration considerations for sarcopenia in older adults, 147–170, Copyright (2015), with permission from IOS Press

3.1 INTRODUCTION

In 1989 Irwin Rosenberg coined the term "sarcopenia", which is derived from Greek roots, *i.e.*, *sarx*: flesh and *penia*: loss (152). Rosenberg's intent was to use the term sarcopenia to provide a distinct classification for age-related muscle mass loss in order to increase awareness of this condition's impact on aging (27). Sarcopenia is a common geriatric condition defined as an age-related involuntary loss of muscle mass and function (strength or performance), leading to weakness and frailty (27, 153) and greater risk of falls and functional disability in older adults (154). Globally, as the percentage of older adult continues to be higher in the population, the number of people with sarcopenia will increase dramatically. Although the prevalence of sarcopenia is higher in individuals older than 60 years old, accounting for 5 to 13% of adults in this age category, it may begin as early as the forth decade of life (155). Worldwide, 40% of the elderly population over the age of ≥70 years old suffers from sarcopenia, representing more than 50 million people. This number is expected to rise to 500 million people by the year of 2050 (26). The health problems associated with aging cause health systems to face mounting cost

pressures and shortages of health care providers (1). In addition, sarcopenia is associated with major co-morbidities including obesity (156), osteoporosis (157), metabolic syndrome (9) and type II diabetes (158). Thus, due to its debilitating nature and a strong predisposition to multiple comorbidities in the elderly population, the research in the area of sarcopenia is expanding exponentially.

Although the etiology of sarcopenia is still not clearly understood many investigations have highlighted roles of both genetic and environmental factors in contributing to the development of this condition. Current understanding of possible etiologic factors associated with sarcopenia can be classified as outlined in **Figure 3.1** (28, 29, 159). While consensus preventive and management approaches for sarcopenia are not yet clearly defined, it is unquestionable that nutrition plays a critical role (160-162). A large body of literature supports the principal role of nutrition as a part of life style modification in maintaining and/ or increasing muscle mass and functionality (162, 163). However, decline in food intake as a consequence of physiological changes or mental and physical impairments in senescent population leads to malnutrition (91).

Hence, it may be necessary to supplement older adults with additional nutrients in order to attenuate age-related loss in muscle mass and function resulting from sarcopenia (164, 165). For instance, protein is one of the key nutrients required to maintain adequate muscle mass, however, in elderly subjects, both inadequate nutritional intake and impaired adaptation of skeletal muscle to utilize AA may prevent adequate muscle mass maintenance (166). Currently, nutritional guidelines for older adults recommend getting 10-35% of daily calories from protein, which may translates to 25-30 g of protein per meal, 3 times per day (167).

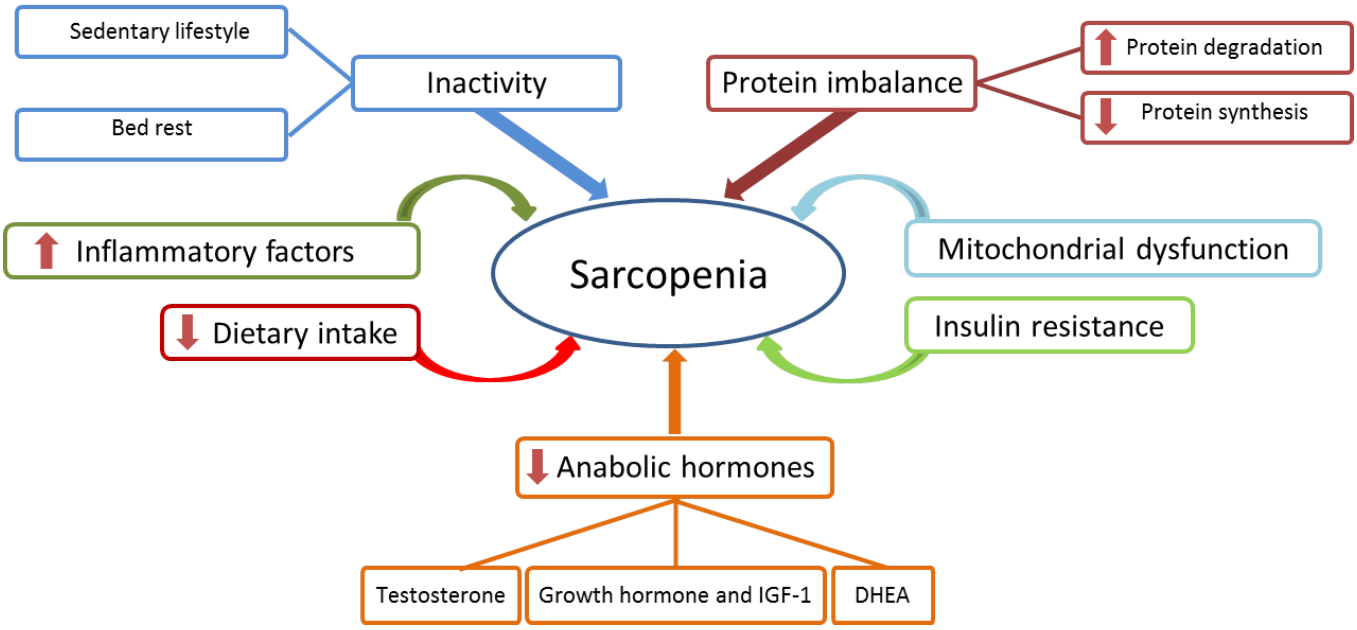

Figure 3.1. Possible Etiological Factors Associated with Sarcopenia. Sedentary life style and bed rest in older adults are the major causes of inactivity, which leads to loss of the skeletal muscle mass and atrophy. Inadequate food intake and malnutrition result in impaired skeletal muscle maintenance. Furthermore, mitochondrial dysfunction, insulin resistance and inflammation are regarded as common linking factors associated with sarcopenia. Decreased level of anabolic hormones, resistance to anabolic stimulus, decreased muscle protein synthesis and increased muscle protein degradation are among the most important etiologic factors resulting in sarcopenia in older adults. DHEA indicates dehydroepiandrosterone; IGF-1, Insulin-like growth factor 1.

However, despite these seemingly adequate nutritional recommendations, research shows that even in a supervised settings such as in long term care facilities, which should allow for more adherence to nutrition guidelines, more than 50% of older adults are malnourished (168). Malnourishment in older adults is in part related to age-related impairments in the digestive system including the impact on food digestion and nutrient absorption capabilities. Therefore, it appears reasonable to review the assessment of nutrient absorption capabilities of frail older adults in efforts to establish optimal nutritional guidelines and route of delivery to determine the

most viable form of nutritional supplementation (dietary vs. pharmacological) options that would enhance the nutritional status in elders. The aim of this paper is to review key sarcopenia-attenuating nutrients and to distinguish for each identified nutrient whether a dietary or a supplemental form is the most effective therapy for nutrient delivery in sarcopenic older adults.

3.2 IMPAIRED DIGESTION IN OLDER ADULTS IMPACT NUTRIENTS INTAKE – A KEY CONSIDERATION IN PREVENTION AND MANAGEMENT OF SARCOPENIA

Outside the central nervous system, the gastrointestinal tract (GI) contains the largest number and most complex system of neurons. The GI is also closely associated with glandular organs (liver, pancreas, gall bladder and salivary glands), autonomic and sensory neurons, and the vasculature. Therefore, the integrated activity and interaction between all of these cell types are of key importance to proper digestion and absorption of food matter and the subsequent nutrients. However, the physiology of aging involves changes to GI cells and function, potentially disturbing neuronal interaction and thus diminishing nutritional adequacy of the diet (169). The impairments in the GI system in the elderly start in the mouth. Weakening of oral muscles with aging might diminish swallowing and chewing ability and thus reduce adequate nutrition. In addition, loss of teeth, ill-fitted dentures, and dry mouth further diminishes the initial step of digestion, which starts in the mouth and requires adequate masticatory ability to form bolus of food prior to swallowing (170). A recent study by Hiramatsu *et al.* (171) compared swallowing and chewing ability in a group of healthy older adults (OAs, n=23, age~76 y) with a group of younger healthy adults (YAs, n=23, age~29 y). The results of the study showed that pre-meal and post-meal tongue pressures were significantly higher in YAs than in OAs. The numbers of swallows were significantly higher in YAs than OAs for both pre-meal ($p<0.01$) and

post-meal ($p<0.001$). The time interval from the initiation to the beginning of swallow after the meal was longer compared to before the meal ($p<0.05$), and was longer in OAs compared to YAs ($p<0.001$). These findings further confirmed that physiological changes due to aging, such as reduced muscle tone in the pharynx and esophagus increases the duration of swallowing. Moreover, functions involved in bolus formation (mastication, tongue mobility, and lip closure) seem to deteriorate with age, prolonging the meal time and resulting in oral muscles exhaustion and thus decreased swallowing ability (171). Furthermore, slower gastric emptying and delayed proximal gastric accommodation to a meal are also observed in the elderly. These aforementioned factors contribute to anorexia of aging, including loss of appetite and early satiation, resulting in reduced nutrient availability in the elderly, which contributes to loss of skeletal muscles and thus aggravating chances of developing sarcopenia (172). Moreover, most recent research suggests that the skeletal muscle protein synthetic response to dietary food intake is impaired in older adults. This proposed anabolic resistance is now regarded as a key factor in the etiology of sarcopenia (173).

All in all, older adults seem to suffer from a number of impairments of the GI system which interferes with nutrients utilization and thus, along with other aging associated impairments such as increased oxidative stress and inflammation, prevents adequate muscle maintenance, which results in malnutrition and sarcopenia.

3.3 NUTRITIONAL SUPPLEMENTATION IN SARCOPENIA

3.3.1 Protein and Amino Acids

Skeletal muscles serve as the largest reservoir for total body protein storage, comprising more than 50% of the total body protein content (161). Skeletal muscle proteins are under a

continuous cycle of synthesis and break down, resulting in constant renewal of muscle protein content (174). In younger adults there is a balance between MPS and MPD, which leads to preservation of muscle mass and inhibition of lean tissue loss. However, aging is associated with diminished muscle anabolic response to dietary proteins, which is defined as anabolic resistance (175). In addition, age-related adverse factors cause derangement in the degradation-synthesis equilibrium; thus contributing to sarcopenia (176). Therefore, inadequate intake of protein and other nutrients as a result of decreased appetite, chewing or swallowing difficulties, decreased gastric emptying, physical and mental impairments, medications-nutrient interactions, presence of multiple diseases, and altered hormonal responses can contribute to impaired synthesis of proteins in skeletal muscles (91). However, recent research indicates that ingestion of 35g of whey protein shows an increased rate of de novo muscle protein synthesis when compared to ingestion of 10-20g of whey protein. Therefore, it appears that anabolic resistance can be partially overcome by increasing protein intake in older adults to higher than 10-35% of the acceptable macronutrient distribution range (AMDR) (173).

Several studies have been conducted to date to examine the impact of supplementation of proteins and/or different amino acid mixtures on muscle mass and function (**Table 3.1**). Research shows that ingestion of a high-protein containing diet will triggers skeletal MPS, which will be maintained for up to 5 hours following ingestion (177). There is an inverse relationship between protein intake and appendicular lean mass loss, *i.e.*, for an older adult being in the highest quintile of protein intake (18.2% total energy, 1.1 g/ kg/day) is associated with 39% less appendicular muscle mass loss ($p<0.05$) (178). Researchers have also shown that different sources of protein have different effects on MPS. Wherein ingestion of high quality proteins such as whey and casein have been shown to be more effective in mitigating muscle mass loss in

sarcopenia than soy proteins (179, 180). In addition, some studies show that protein intake, when combined with physical activity such as resistance exercise, is capable of reducing muscle mass loss in the elderly more efficiently than merely protein ingestion (181). Overall, high quality proteins and AA have been shown to be effective in attenuating age-related muscle mass loss. Nevertheless dosages, combination, timing and duration of protein or amino acid supplements, and their association with exercise, are all factors requiring further studies in order to establish optimal therapeutic strategies to attenuate age-related muscle loss and decrease the number of older adults suffering from sarcopenia.

AA are organic compounds composed of two functional groups: amine ($-NH_2$) and carboxylic acid (-COOH) (182). They are building blocks of proteins, as well as energy substrates and signalling molecules in protein metabolism (183). Research shows that supplementation with 8-15g/day of essential amino acids (EAA) results in improved lean body mass (LBM) and muscle strength (161, 164). Among the various EAA, the branched-chain AA (BCAA), especially leucine, have been shown to have regulatory roles in enhancing MPS and reducing MPD (184, 185). It has been said that more than 20% of the total protein content of the diet is comprised of BCAA (186). Since the liver does not possess any aminotransferase enzyme for metabolizing BCAA, these AA reach the blood and then skeletal muscles almost with the same concentrations that they appeared in the diet (187). Thus, supplementation with BCAA, especially leucine, can potentially be of great importance to skeletal MPS (188, 189). Leucine is capable of inducing protein synthesis via the activation of mammalian target of rapamycin (m-TOR) complex-1 signalling pathway, which plays a central role in cell growth, cell-survival and protein synthesis. The mTOR pathway is a conserved serine/threonine kinase involved in many signal transduction pathways within the cell, regulating cell growth and homeostasis (131). The

mTOR system is categorized as two protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Maintaining and controlling protein synthesis are the primary roles of mTORC1, whereas the role of mTORC2 is less clearly perceived (190). While activation of mTORC1 will further phosphorylates/activates p70S6K and protein synthesis, its inhibition activates autophagy machinery to eliminate damaged components and save energy for stressful conditions (130). Thus, due to the central role of mTOR in several signaling pathways, it is implicated in the pathogenesis of diseases in which growth and homeostasis are compromised, *e.g.*, cancer (191), aging (135), myopathies (136) and sarcopenia (192). The activation of p70S6K and mTORC1 is also essential for skeletal muscle hypertrophy as it is required for the initiation of a series of mRNA translation, which encodes protein necessary for protein synthesis (**Figure 3.2**). Moreover, mTOR is implicated in protein synthesis and degradation in myopathies (141, 142). Increased activation of mTOR and the subsequent hyperphosphorylation of p70S6K have been observed in aged mice (143), suggesting a role for mTOR in the pathogenesis of sarcopenia and other age-related muscle abnormalities.

Research shows that both acute and chronic supplementation with EAA in the amount of 10 g and in combination with 3.5 g leucine are capable of enhancing MPS (65, 188). Since the leucine content of dietary proteins is a determinant of post-prandial MPS, investigating the effect of various protein sources on MPS in regard to their leucine content is of great interest in research. Luiking *et al.* (180) investigated whether there was a difference in the effects of a supplement high in whey protein and leucine or a dairy product-containing supplement on skeletal MPS in healthy older adults. The results of this study revealed that the experimental group, who received 20 g whey protein and 3 g leucine-enriched product had a higher protein fractional synthesis rate (FSR) compared with the control group who ingested 6 g milk protein

with the same calorie content (180). Similarly, the effects of soy and whey protein, combined with resistance exercise, on the levels of p70S6K phosphorylation were compared in thirteen healthy older men (179). Although at 2 hours post exercise the levels of phosphorylated p70S6K increased in both whey and soy supplemented groups compared with baseline ($p < 0.001$), the level of phosphorylated p70S6K remained elevated for 4 hours post exercise ($p < 0.001$) only in the 30 g whey protein supplemented group. These results showed that the effect of whey protein on protein synthesis signalling pathways in skeletal muscle was more prolonged than soy protein, possibly due to higher BCAA content of whey protein and thus higher stimulation of mTOR signaling pathway. However, more research is needed to better understand why different proteins have different effects on mTOR pathway stimulation and the subsequent MPS.

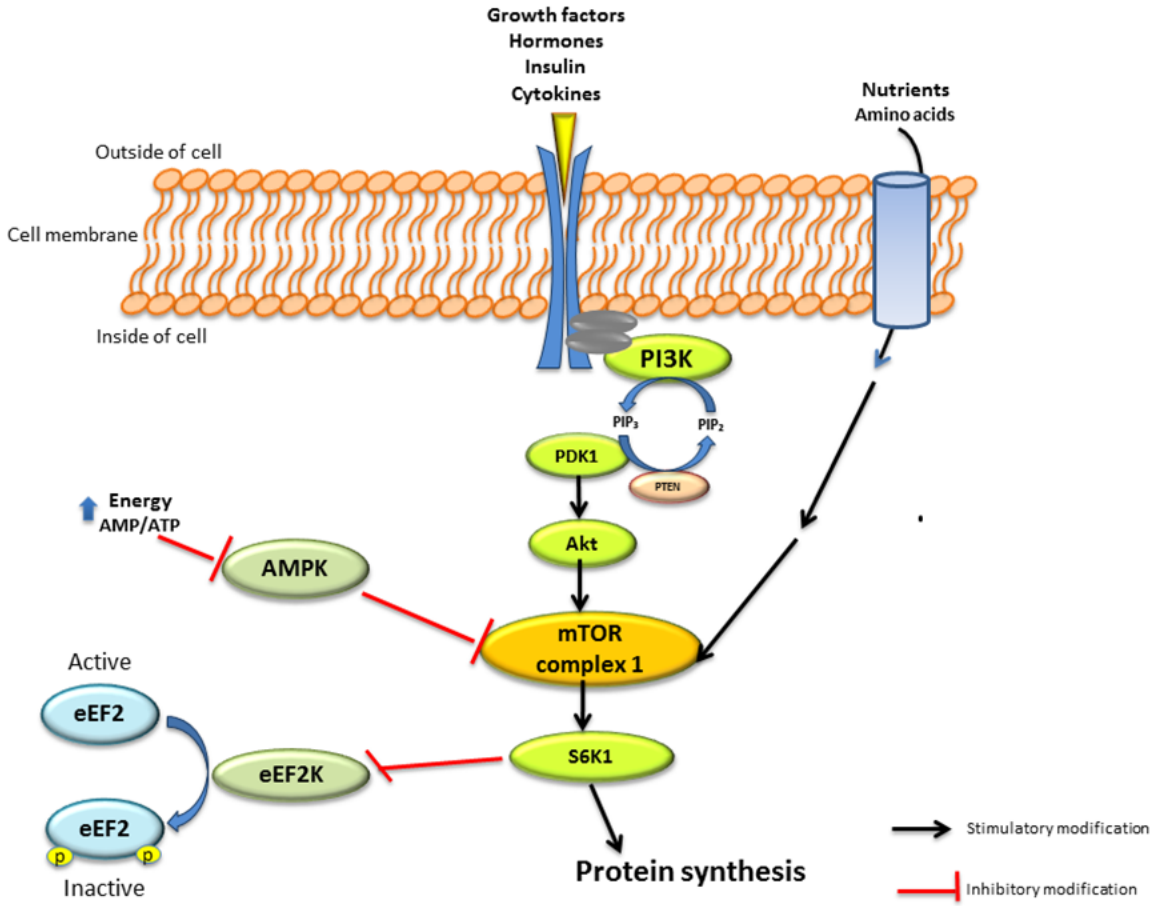

Figure 3.2. Simplified view of mammalian target of rapamycin (mTOR) signalling pathway and role of AA and leucine in skeletal muscles protein synthesis. mTOR is a conserved serine/threonine kinase involved in many signal transduction pathways within the body, regulating cell growth and homeostasis. The mTOR pathway is activated by insulin, growth factors and AA. Activation of mTOR results in the phosphorylation of specific proteins that ultimately phosphorylate and activate p70 ribosomal S6 protein kinase (p70S6K), which triggers a cascade of responses that subsequently results in protein biosynthesis. 5' adenosine monophosphate-activated protein kinase (AMPK) is also a key regulator of cellular energy homeostasis. AMPK can sense the cellular energy level and down-regulate the cellular pathways that consume ATP in case of decreased cell energy content. Eukaryotic elongation factor 2 (eEF2) in its active form results in activation of overall translation elongation in protein synthesis. PIP_2 indicates phosphatidylinositol 4,5 bisphosphate; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; PTEN, phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase; PDK1, 3-phosphoinositide dependent protein kinase-1; eEF2K, eukaryotic elongation factor 2 kinase; p, phosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate.

Aleman-Mateo *et al.* (193) conducted a study with 40 subjects, including men and women over 60 years of age with sarcopenia. They examined the impact of a diet rich in protein from ricotta cheese (15.7 g protein /day, including EAA at 8.6 g/day) on total appendicular skeletal muscle mass and muscle strength. Unfortunately, the results of the study did not reach statistical significance. In fact, diet rich in ricotta cheese did not have any impact on body composition and strength in elderly with sarcopenia. The authors suggested that perhaps higher amount of dietary protein is needed to elucidate an increase in muscle mass in older adults, *i.e.*, 2 g/kg/day, while this study only provided ~0.5 g/kg/day. Another important factor to consider is that this study used total lean body mass increase as an indicator of effectiveness of protein supplementation. Addition analysis on hand grip strength provided a clear tendency toward significance in the ricotta supplemented group ($p=0.06$), indicating increased muscle strength. As a final remark, the authors found that some participants experienced difficulty consuming the entire portion of 210 g of ricotta cheese due to an early satiety. This finding is consistent with previous findings on early satiety in the elderly, especially following a high protein meal (172).

The results of these studies suggest that older adults' digestive capabilities should be taken into account prior to prescribing protein supplementation. Moreover, due to substantial differences between younger adults and older adults' digestive systems, additional research is required in order to select the most efficient way for AA delivery to skeletal tissue in order to achieve the highest impact on MPS. For instance, Milan *et al.* (194) compared postprandial plasma AA concentrations between a group of older adults (n=15, 60-75 y) and younger adults (n=15, 20-25 y) following a high protein mixed meal (49.8 g protein). The results of the study showed that older adult digest and absorb protein within a mixed meal slower that younger adults. The authors suggested that delayed in AA absorption may delay or suppress protein synthesis in senescent muscle (194). Therefore, the type of protein and the context of the meal have to be modified to fit the needs of declining digestive functions of older adults. Hence, providing proteins and EAA, especially leucine, in forms of pharmacological supplements may appear as the most feasible way to achieve optimum protein/AA intake in older adults.

The impact of leucine on protein synthesis has been shown in a large body of literature (*e.g.*, **Table 3.1**); however, the effective dose by which leucine exerts its role on protein synthesis in the elderly is still not clearly understood. Animal studies indicate that increasing the BCAA-rich whey protein content of the diet from 12% to 18% (12% whey=13.8 g leucine/kg diet; 18% whey=21.1 g leucine/kg diet) results in attenuating the LBM loss in aged rats. In regards to human studies, most recent research show that administration of ≥30-40g of whey protein to older adults results in direct stimulation of protein synthesis through mTOR signaling pathway (192). Although these studies (**Table 3.1**) provide valuable sources of information on the impacts and effective dose of whey and leucine supplementation in attenuating muscle mass

and function losses in the elderly, there is still no agreement in the literature on the optimum level of whey and leucine intakes for treatment of sarcopenia.

In summary, supplementations with protein and AA, especially leucine, are promising strategies targeted at increasing MPS and attenuating age-related sarcopenia. However the exact effective and safe dosage is still unclear, but current suggestions include: ≥30g whey protein/day, BCAA-rich protein at 2g/kg/day, or to include whey protein as 18% of total daily caloric intake (**Table 3.1**). In addition to currently obscure protein/AA dosages, the exact route of delivery is questionable given the physiological impairments in the digestive system that occur with aging, which can significantly impact an individual's nutritional status. Therefore, supplementation with high quality protein in its elemental form of AA (especially BCAA) may be a more effective way for optimum MPS stimulation as it by-passes digestive step that appears to be problematic for older adults. Furthermore, on a cautionary note, although higher protein consumption is recommended to attenuate muscle mass loss in sarcopenia, potential adverse effects associated with higher than 0.8 mg/kg daily protein intake should not be overlooked. For instance, bone disorders and imbalance in calcium homeostasis, renal dysfunction, predisposition to cancer and coronary artery disease are some of the adverse effects mentioned in the literature as consequences of higher than RDA protein consumption (195). Future studies looking more in depth in the proper dose and timing of administration of protein and AA are required to establish a practical guideline for prevention and management of sarcopenia in the elderly.

3.3.2 Vitamin D and Calcium

Increase rates of longevity and adiposity in the general population result in an increased number of musculoskeletal system disorders such as sarcopenia and osteoporosis; both contributing to frailty and increased risk of fractures due to falls in older adults. It is important to

mention both muscle and bone diseases as they appear to be linked in both direct function and tissue cross-talk (**Figure 3.3**). Skeletal muscles and bone mass are constantly involved in each other's regulatory pathways, resulting in mutual co-dependency (196, 197). One of these mutual regulators appears to be vitamin D. Research findings showed that vitamin D-responsive hormones that are produced by bone tissue are activated in the presence of vitamin D and in return have positive effects on MPS. Similar association is seen with vitamin D-responsive factors produced by muscle cells, resulting in positive effect on bone tissue (198). Therefore, both muscle and bone strengths have to be considered in order to provide optimum treatment and preventative measures for older adults with sarcopenia.

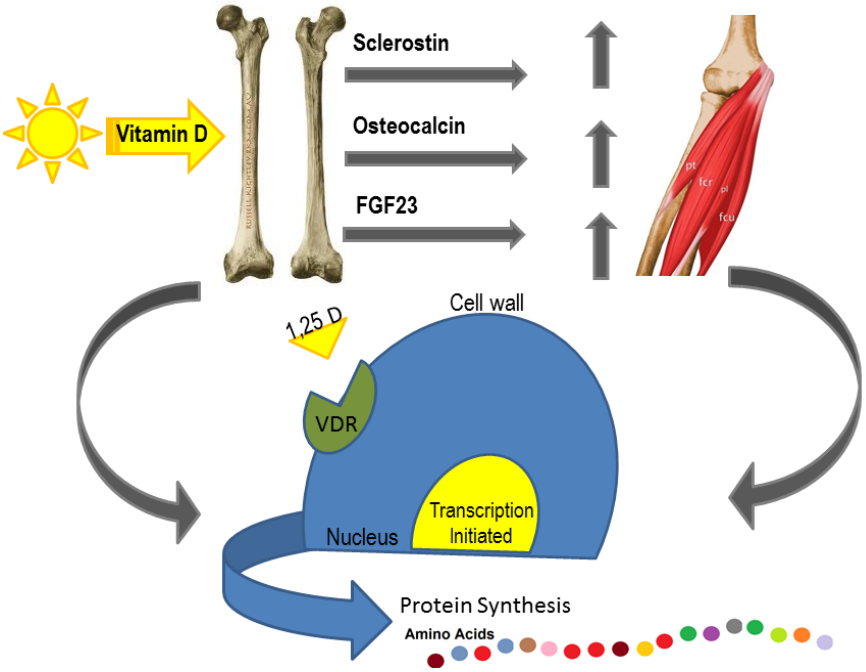

Figure 3.3. Muscle-bone cross-talk and vitamin D receptor. Sclerostin is secreted by mature osteocytes in response to vitamin D activation, and it increases bone mass which in response has a positive effect on certain muscles; however, the mechanism is still unknown. Osteocalcin is produced by osteoblasts and regulated by vitamin D. Osteocalcin has a potential effect in

muscles by altering mitochondrial function and insulin sensitivity. FGF23 (fibroblast growth factor 23) is a vitamin D responsive hormone produced by bones and has positive effects on cardiac and smooth muscles. Vitamin D Receptor (VDR) can be found in both skeletal and muscle cells, and is activated by 1, 25 Vitamin D (1,25 D) and contributes to initiation of protein synthesis through bone-muscle cross-talk mechanism (196, 199).

Following the discovery of vitamin D receptors in human muscle cells, it has been identified that vitamin D may induce MPS through direct initiation of transcription and indirect muscle-bone cross talk mechanism (198). In addition to anabolic effects on muscles, vitamin D may also have a role in moderating inflammation in skeletal muscles. Choi *et al.* (200) found that vitamin D supplementation in exercised rats decreased inflammatory cytokines, interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) genes expressions, and increased vitamin D receptor protein expression in skeletal muscles (200). A number of studies have concluded that supplementation with vitamin D might improve muscles function and bone strength, thus preventing sarcopenia, frailty, and decreased the risk of falls in the elderly (**Table 3.2**). For instance, Mastaglia *et al.* (201) demonstrated that the strength and function of lower extremities were better preserved with serum hydroxyl-vitamin D level ≥ 20 ng/ml. Indeed, lower vitamin D level in the blood was found to be associated with frailty in older adults (202). A meta-analysis by Bischoff-Ferrari *et al.* (203) indicated that vitamin D supplementation at a dose of 700-1000 mg/day in older adults was associated with 19% reduced risk of falling. In addition, the results from the Fourth Korea National Health and Nutrition Examination Survey (KNHANES IV) in 2009 demonstrated that the serum level of 25-hydroxyvitamin D had positive correlations with total and appendicular lean mass ($R=0.142$ and $R=0.157$, respectively) and negative correlations with total fat mass and appendicular fat mass ($R=-0.188$ and $R=-0.197$, respectively) ($p<0.001$ for all). In fact, sarcopenia in older Koreans was inversely associated with serum vitamin D concentrations (204). However, further gender-specific analysis of the KNHANES IV data

revealed that blood vitamin D levels tended to have a negative association with sarcopenia only in women 50 years of age or older (205). In contrast, Dupuy *et al.* (206) conducted a cross-sectional study to determine whether muscle mass was influenced by vitamin D intakes in 1989 community-dwelling older French women (~80 y). Low intake of vitamin D was estimated based on self-reported data and defined at <70 μg/week based on recommended dietary intake (RDI) of vitamin D. Although in this study low muscle mass revealed a significant, positive correlation with obesity, malnutrition and decreased handgrip strength, no association was observed between muscle mass and dietary vitamin D intake (206).

In addition to vitamin D status and intake, the effect of calcium intake on body composition in older adults has been reported in the literature. Data from the KNHANES IV study indicated that the daily calcium intake had a positive (rho = 0.281) (all $p<0.001$) and negative correlation (rho = -0.140, $p<0.001$) with skeletal mass and total body fat mass, respectively. Sarcopenic older adults had a lower daily consumption of calcium (≤278 mg per day) in comparison with participants without sarcopenia (207). Furthermore, calcium status is influenced by blood vitamin D levels, since 1,25-dihydroxyvitamin D, the active form of vitamin D in the body, binds to vitamin D receptors in the gut and stimulates production of proteins involved in the transluminal transport of calcium in the intestine (208).

In summary, although, the mechanism of muscle-bone cross-talk is poorly understood, its existence has been prior suspected. Evidences indicate that sarcopenia might be associated with vitamin D deficiency, however this is all based primarily on observational studies as reviewed above and summarized in Table 2.2. Unfortunately, to date there has been no clear randomized clinical trials showing the benefits of vitamin D for sarcopenia. Nevertheless, supplementation might be effective for protection against loss of musculoskeletal unit and the subsequent

complications, such as falls, which lead to immobility and further decrease in lean muscles mass (i.e. muscle atrophy due to immobility). Evidence suggests that 700-800 international units (IU) of vitamin D in combination with calcium supplement reduces the risk of non-vertebral and hip fractures by approximately 13-26% (209-211). Since natural vitamin D requires sun exposure for activation, which may not be feasible in many situations, thus pharmacological supplementation with its active form may be the most efficient way to achieve optimum vitamin D status for most elders.

3.3.3 Antioxidants

Oxidative stress is a condition resulting from an imbalance between increased free radical production and decreased cell protection mechanism; known as anti-oxidative capacity. Since free radicals are unstable and have high chemical reactivity, they can wreak havoc on a wide spectrum of biomolecules including DNA, lipids and proteins. Amongst the various free radicals, reactive oxygen species (ROS), which are derived from oxygen, are of utmost importance in biological systems because of their major production site in the cells, *i.e.*, mitochondria (212). As cellular respiration organelles, mitochondria consume approximately 90% of the oxygen inside the cells and overproduction of ROS can have destructive effects on mitochondria and cells resulting in mitochondrial mediated apoptosis or cell death (213). Indeed, the impact of free radicals on aging process was described as early as 1950's by Denham Harman (214) where he enunciated that intracellularly produced free radicals are capable of damaging cell components such as DNA and RNA, leading to aging and aging-associated degenerative alterations (214). For more information on the generation of ROS and counteraction of cells to oxidative stress during aging the readers are referred to comprehensive literature review such as by Finkel et al. (215).

Among various proposed mechanisms for sarcopenia, impairment in mitochondria and increased oxidative stress are strongly supported by the literature (67, 216-218). Aging results in reduced content and bioenergetics of mitochondria within skeletal muscle and consequently, oxidative phosphorylation capacity will be diminished in myocytes (67, 217). Decreased functionality of mitochondria, not only leads to less adenosine triphosphate (ATP) production, but also contributes to greater production and release of ROS from mitochondria (67). ROS are a major inducer of programmed cell death, apoptosis, and autophagy, resulting in myocyte loss and/or atrophy (67). Sullivan-Gunn *et al.* (219) assigned 58 mice into four age categories; 6, 12, 18 and 24 months of age and fed them with standard chow and water under controlled conditions. At 14 months of age, representing middle age, the survival rate of these mice started to drop, whereby 60% of 18-month old animals survived, and half of them died in the 24 months of age group. In addition, skeletal muscle mass loss or sarcopenia was observed at 18 months of age. These mice also showed an increase in ROS levels and a decrease in anti-oxidant enzymes levels in skeletal muscle, indicating a possible role of oxidative damage in sarcopenia (219). Cross sectional studies in human subjects also demonstrated that older adults with sarcopenia consume below the RDA for antioxidant nutrients compared to those without sarcopenia (220). Hence, strategies aiming at reduction of ROS production or detoxifying them may contribute to reversing the devastating consequences of these compounds.

Some researches in the field of sarcopenia have been focused on oxidative stress and treatment strategies using antioxidants. Marzani et al. (221) conducted a study to assess the impact of an antioxidant mixture consisting of rutin, vitamin E, vitamin A, zinc and selenium on anabolic response of aged muscle to stimulatory effect of leucine supplementation. In this study adult (8 months, $n = 32$) and old (20 months, $n = 33$) male rats were each assigned to one of two

experimental protocols to receive either a control diet supplemented (Aox+) or non-supplemented (Aox-) with the antioxidant mixture. After 7 weeks, fast-twitch epitrochlearis muscles were dissected and further incubated in incremental concentrations of leuicne. In Aox- groups, addition of leucine increased protein synthesis in adult rats and to a lesser extent in old rats. No significant alteration was observed in adult rats supplemented with the antioxidants; however, MPS response to leucine was enhanced as a result of antioxidant supplementation in old rats. In this situation, improvement in protein synthesis in old rats was not mediated through activation of p70S6K, but thought to be intervened by a reduction in inflammatory markers, such as plasma α_2 macroglobulin, which showed a decrease in old rats after antioxidant supplementation (221). Although, the effect of antioxidants on MPS (activation of elements of protein synthesis pathway) and inflammatory biomarkers were assessed in this study, the exact mechanism by which antioxidants induce their impacts on the muscle cells was not investigated. In addition, alteration in muscle mass and/or function in aging due to antioxidant supplementation are also areas that require further examination.

Animal and human body possesses endogenous antioxidant compounds and enzymes, *e.g.*, glutathione, superoxide dismutase and catalase, to defend the body against oxidative attack (222). In addition, various exogenous dietary sources such as vitamin C (223), vitamin E (224), carotenoids (225) and polyphenols (226) also can compensate the body's anti-oxidant requirements (227, 228). The ability of antioxidants to scavenge ROS prevents further damage to the cell structures notably mitochondria (229). Ryan *et al.* (165) supplemented 28 aged rats either with or without vitamin C and E and demonstrated that these vitamins lowered the indices of aging-associated oxidative damage. Similarly, Bobeuf and colleagues (230) demonstrated that supplementation of older rats with vitamin C (l-ascorbic acid, 2% by weight) and E (dl-alpha-

tocopheryl acetate, 30000 mg/kg) combined with resistance exercise for 4.5 weeks increased appendicular fat free mass after 6 months of study period; however these researchers could not detect any significant change in oxidative damage parameters.

Carotenoids; organic pigments abundantly found in plants and vegetables, are supposed to have an inverse association with muscle dysfunction and disability in older adults (231). Follow-up study of 628 elderly men and women aged 65 and older for 6 years revealed that lower plasma concentrations of carotenoids were accompanied by greater risk of poor hip, knee and grip muscle strength (231). Similar results regarding the relationship between plasma carotenoid levels and hip, knee and grip strengths were also attained from elderly women who participated in a cross-sectional study; in fact, higher carotenoids levels were correlated with better strength measures (232). These observational studies demonstrate that carotenoids may have protective effect against aging-associated muscle function loss. However, further clinical controlled studies are required to demonstrate the beneficial roles of carotenoids in attenuating sarcopenia in older adults.

In addition to carotenoids, vitamins C and E, the antioxidant effect of other dietary nutrients such as resveratrol has been investigated in the literature. Resveratrol is a member of polyphenolic compounds, which are known for their anti-oxidative and anti-inflammatory properties. Skin of red grapes is the major natural source of resveratrol, but it can also be found in peanuts and berries (233). Resveratrol is present in plants and fruits in a small amount, therefore in order to reach the pharmacological doses effective for health related conditions, resveratrol is extracted from *Polygonum cuspidatum* (knotweed) as a nutritional supplement (234). Investigators claimed that polyphenols and resveratrol have beneficial preventive and therapeutic effects on a number of health conditions such as obesity, diabetes,

hypercholesterolemia, cancer, Alzheimer's disease and aging associated disorders (233). In an *in vitro* study with C2C12 myoblast cells, resveratrol hindered increased production of ROS induced by transforming growth factor-β1 (TGF-β1), a cytokine promoting fibrosis in muscles (235). Other *in vitro* studies also demonstrated that resveratrol is capable of reversing muscle mass loss induced by inflammation, mainly the pro-inflammatory cytokine TNF-α, through regulation of Akt/mTOR/FoxO1 signaling pathway, which enhances MPS required for muscle structure and function. Akt (protein kinase B) is a kinase capable of controlling both MPS, via phosphorylating and activating of mTOR, and MPD, by inactivating of transcription factor Forkhead Box O1 (236). In addition, intra-gastric administration of resveratrol with a dose of 100 mg/kg/day to 8 month-old rats for 12 weeks decreased ROS level in subsarcolemmal and intermyofibrillar mitochondria and increased antioxidative capacity as measured by total superoxide dismutase (SOD), glutathione peroxidase and catalase enzymes activity levels in tibialis anterior muscle (237). Nevertheless, there are some discrepancies in the literature regarding antioxidative effect of resveratrol. When Barger et al. (238) supplemented 14 month-old male mice with 4.9 mg/kg/day for approximately 16 months, markers of oxidative damage to DNA and RNA [8-hydroxy-29-deoxyguanosine (8-OHdG) and 8-hydroxyguanosine (8-OHG)] and the marker of lipid peroxidation (F2-isoprostanes) in soleus and extensor digitorum longus muscles did not change significantly as a consequence of resveratrol supplementation (238). Although the duration of this study was long enough to give rise to any beneficial effects of resveratrol, the provided dosage might not be sufficient to initiate the antioxidative impacts of resveratrol. Ingestion of higher doses, 12.5 mg/kg/day, of resveratrol for 21 days in older rats (34 month-old), who received hind limb suspension for 14 days during the course of the study, was shown to affect oxidative stress markers by increasing catalase and manganese SOD (MnSOD)

activities and decreasing lipid peroxidation and hydrogen peroxide contents in gastrocnemius muscle (239). Although Jackson et al. (239) examined the gastrocnemius muscle as a whole, without having concern about different fiber types, a later study by the same research group (240) demonstrated that slow-twitch, gycolytic gastrocnemius muscle fibers are more responsive to resveratrol than fast-twitch, oxidative fibers in gastrocnemius muscle. Supplementing old (27 month-old) male rats with 50 mg/kg/day resveratrol for 6 weeks enhanced sirtuin 1 (SIRT1), a histone residue deacetylase which is supposed to be associated with increased longevity and aging suppression (241), in slow-twitch gastrocnemius muscle fibers. However, the resveratrol supplementation regimen did not have any impact on oxidative stress markers and muscle mass (240). Nonetheless, the increase in SIRT1 activity was also shown in a study conducted by Zheng et al. (237) that has been discussed earlier in this section.

To assess the effect of resveratrol in aging-associated muscle mass loss, Bennett *et al.* (242) applied hind limb suspension in combination with 125 mg/kg/day resveratrol supplementation for 14 days in 32 month-old male aged rats. Although resveratrol was not effective in preventing the muscle mass loss during loading period, it improved type II, fast-twitch muscle fiber size and muscle mass during the recovery period. Wang *et al.* (243) used an interleukin-10 (IL-10), an anti-inflammatory cytokine, knockout mouse model to investigate the effect of the grape seed extract (consisting of >95% flavonols), which is a by-product of wineries and grape juice producing industries. This mouse model exhibit chronic inflammation, accelerated muscle mass loss and frailty. Supplementing these animals with resveratrol for 12 weeks ameliorated muscle mass loss, reduced protein degradation and rectified the inhibition of anabolic signaling pathway (243). Although resveratrol revealed a protective effect against oxidative damage by reducing autophagy and apoptosis, some researchers claim that it was not

able to attenuate muscle mass loss in sarcopenia (244). In fact 10-month resveratrol supplementation of middle-aged mice was effective in preserving the fast-twitch muscle fiber function and age related oxidative stress, however it could not prevent muscle mass loss in these animals (244). Although the result of this study in regard to the effect of resveratrol on muscle mass was in agreement with Joseph et al. (240), different fiber types were responsive to the beneficial effects of resveratrol supplementation in these studies. Therefore, it is not clear whether the effect of resveratrol is fiber-specific and if so, which fiber types are more responsive to this treatment. More research is required to elucidate the mechanism and role of resveratrol in attenuating muscle mass and function loss in elderly.

Overall, antioxidants provide a promising venue for combating muscle mass loss in sarcopenia, since increased oxidative stress is an inseparable part of this disorder and many other aging-associated conditions. Antioxidants are found in plenty of foods, primarily in vegetables and fruits. It is well known that the bioavailability of antioxidants from vegetable and fruit sources is optimum to maintain defenses against oxidative stress in healthy young people. As stated earlier in this review, a number of digestive misalignments prevent older adults from optimal nutrient utilization. However, in the case of antioxidants, it appears that older adults benefit from dietary vegetable and fruit consumptions as much as do younger adults. For instance, Kim, *et al.* (154) performed a cross-sectional examination of the KNHANES IV study, comprised of 823 men and 1,089 community-dwelling women aged ≥65 years, to assess the relationships between vegetables and fruits consumptions and the effects on sarcopenia. The results of the study showed that after adjustment for all covariates, higher frequency of vegetables, fruits and combined fruits and vegetables consumption was accompanied by lower

risk of sarcopenia among older men. For women, the risk of sarcopenia decreased as the number of servings of fruit per day increased

Although, without a doubt, dietary antioxidant intake is superior to synthetic antioxidants, some older adults might be forced to resort to synthetic supplementation. A number of studies indicated that there is a difference in bioavailability between different synthetic antioxidants. For instance, pharmacokinetic studies indicate that carotenoids are less bioavailable in the form of a supplement, while synthetic and food-derived vitamin C is relatively comparable (245, 246). Therefore, unless indicated otherwise, it appears prudent to use dietary therapy rich in vegetables and fruits in efforts to combat sarcopenia-induced oxidative stress. However, given the anorexia of aging, more research is needed to identify if some older adults might be experiencing significant difficulty consuming sufficient amounts of vegetables and fruits to obtain their beneficial health effects. Also, it will be prudent to identify if preparatory techniques such as mincing/puree could help to increase vegetables and fruit consumption in the elderly. Furthermore, more research is required to determine if supplementation with antioxidants could be beneficial adjuvant therapy to AA and proteins for enhancing or maintaining muscle mass and function in aging populations.

3.3.4 Omega-3 Fatty Acids

The etiology of sarcopenia is still not clearly understood, however a number of factors have been proposed as potential contributors to sarcopenia. One of these possible mechanisms is the increased circulating cytokines and pro-inflammatory markers (**Figure 3.1**), which are associated with the aging process (161, 247, 248). Dysregulation of inflammatory pathways during aging is suggested to play a major role in the pathogenesis of various age-related disorders such as cardiovascular events, Alzheimer's disease and sarcopenia. In fact, elevated

levels of pro-inflammatory cytokines like IL-6, interleukin-1β (IL-1β) and TNF-α, as well as decreased anti-inflammatory cytokines, as a consequence of aging initiates a cascade of events, ultimately resulting in impaired response of skeletal muscle to anabolic signals (247). In younger muscles, anabolic stimuli affects MPS, outpacing it from MPD and subsequently resulting in increased muscle mass (249). Nevertheless, anabolic resistance in aged muscle deranges this equilibrium between MPS and MPD, which leads to gradual loss of muscle mass (249). It has been shown that the presence of even low-grade inflammation in aged rats results in irresponsiveness of post-prandial MPS to nutritional stimuli, whereas control non-inflamed counterpart rats have increased MPS after food intake (250). In addition, long-term prevention of inflammation with a non-steroidal anti-inflammatory drug (*e.g.*, ibuprofen) reduces the inflammatory biomarkers and cytokines levels such as fibrinogen, α_2-macroglobuline, IL-6 and IL-1β leading to enhanced MPS and diminished MPD in aged rats (251).

Omega-3 fatty acids are polyunsaturated fatty acids (PUFA) and essential nutrients with anti-inflammatory properties. Oily fish like salmon is a common source of omega-3 fatty acids, while plant oils such as canola and flaxseed oil also contain omega-3 fatty acids (252). A large body of evidence supports the beneficial effects of these fatty acids in various clinical conditions including cancers, cardiovascular diseases, inflammatory disorders and cognitive impairments (253). Omega-3 fatty acids exert their anti-inflammatory activity through several mechanisms including reduced leukocyte chemotaxis, decreased production of eicosanoids from arachidonic acid, and reduced T-cell reactivity (253). Therefore, it has been speculated that older adults with sarcopenia would also benefit from omega-3 fatty acids, since inflammation is one of the underlying pathophysiological events during the disease process (160, 248). Smith *et al.* (160) conducted a study with 16 healthy older adults, aged 65 years or more, to investigate whether

supplementation with omega-3 fatty acids for 8 weeks had any impact on the rate of MPS and anabolic signaling. In this randomized controlled trial, subjects were assigned to either omega-3 fatty acid or corn oil (control) group. The measurements were done at basal, post-absorptive conditions and after administration of hyperaminoacidemic-hyperinsulinemic clamp, before and after supplementation with omega-3 fatty acids and corn oil. The results of this study revealed that hyperaminoacidemic-hyperinsulinemic clamp induced a rise in MPS in both groups ($p<0.01$); however only omega-3 fatty acids could augment this increase ($p=0.01$). Furthermore, similar positive effects on muscle mTORC1 and p70s6k activity, which are involved in anabolic signaling pathways in muscles, were observed in omega-3 fatty acids supplemented group ($p=0.07$ and $p<0.05$, respectively). Overall, the data from this trial suggests a possible role of omega-3 fatty acids in overcoming the metabolic resistance in skeletal muscle and attenuating muscle mass loss in older adults (160). Nevertheless, the effect of omega-3 fatty acids on muscles has been studied beyond the scope of increasing MPS and muscle mass. In fact, consumption of omega-3 fatty acids can improve muscle function by enhancing muscle strength and performance (254-256). Another study by Smith *et al.* (256) demonstrated that supplementation with omega-3 fatty acids [four 1 g pills per day, providing 1.86 g eicosapentaenoic acid (EPA; 20:5n–3)/day and 1.5 g docosahexaenoic acid (DHA; 22:6 n–3)/day] for 6 months not only increases thigh muscle volume, but also enhances hand grip strengh and average isokinetic power (256). Similarly, dietary assessments of nearly 3000 elderly men and women have shown positive associations between fatty fish consumption and grip strength in both genders (254).

As discussed earlier, the hypothesis behind the beneficial effects of omega-3 fatty acids on improvement of muscle mass (size) and function is primarily thought to be due to reduced

inflammatory processes and its associated biomarkers (247). To test this theory, Cornish *et al.* (248) recruited 51 older adults in a randomized double-blind trial. Subjects were almost equally assigned to one of two groups to receive either 14 g alpha-linolenic acid/day (ALA, an 18 carbon essential omega-3 fatty acid) through 30 ml of flax seed oil or iso-caloric corn oil (placebo). At the same time, both groups were completing a resistance-training program. After 12 weeks, male subjects in the ALA-supplemented group demonstrated a significant reduction in blood concentration of IL-6 (62 ± 36% decrease; $p = 0.003$). However, male subjects in the placebo group and women in both ALA and placebo group did not show any significant changes in IL-6 concentrations. Likewise, while the trend of change in blood concentration of TNF-α was parallel to IL-6, it failed to reveal any statistical significant difference. In addition, resistance training with or without ALA supplements was effective in enhancing muscle thickness ($p=0.05$), muscle strength ($p=0.006$) and LBM ($p<0.01$), and ALA had showed minimal effects on these parameters. The researchers speculated that the lack or minimum effect of omega-3 fatty acid in this study in enhancing muscle mass can be due to the supplemented form, *i.e.*, flax seed oil versus omega-3 in fish oil (248). Nevertheless, the potential influence of ALA in lowering plasma IL-6 level in older men is promising for future directions toward the treatment of sarcopenia.

Although consumption of fatty fish and plant-based rich sources appear to be reasonable strategies to achieve adequate omega-3 fatty acids supplementation, research shows that dietary intake of omega-3 fatty acids in older adults might be insufficient (252). Therefore, more research is needed to identify barriers preventing older adults from consuming substantial amounts of omega-3 fatty acids from natural sources. Currently, research shows that consumption of ≥1.27 g/day of omega-3 fatty acids or ≥1 servings of fatty fish per week is

associated with higher bone mineral density and increase in grip strength (257). Thus, dietary and/or supplemental forms of omega-3 fatty acids both appear as useful and effective ways to improve sarcopenia-related functional impairments in older adults.

3.4 CONCLUSION

With the rapid growth in the older adult population, sarcopenia is becoming increasingly prominent as a major health problem, contributing to enhanced frailty and debility. Sarcopenia is a severe debilitating condition with a progressive, frequently irreversible nature. A broad range of investigations is required to understand the pathophysiology of this condition in order to find feasible treatment approaches to combat this age-related disorder. Without a doubt, nutrition plays a central role in attenuating muscle mass loss in aging population, with some research to date highlighting the roles of proteins and AA, vitamin D and calcium, antioxidants and omega-3 fatty acids as some of the most promising nutritional strategies to manage sarcopenia. To optimize these nutritional strategies, one should also consider the physiological impairments that seemed to accompanied aging such as early onset of satiety in combination with prolonged chewing might significantly diminish the amount of dietary nutrients consumed by an elderly person. Therefore, in efforts to prevent the onset of the sarcopenia, early continued nutritional therapies should be considered for older adults in forms of both pharmacological and dietary interventions. There is also a paucity of information regarding combination nutritional therapies which includes protein/AA, vitamin D and calcium, omega-3 fatty acids and antioxidants. Overall, more research is needed to establish exact age of supplementation initiation, exact amounts for the nutrients of interest, combinations of nutrients, and optimum form and schedule of administration.

Reference	Objective(s)	Subjects	Study design	Exercise protocol (yes or no)	Study duration	Measured variable and the outcome
Human studies						
Tieland *et al*. **(258)**	To determine the combined effect of protein supplementation and prolonged resistance training on muscle mass, strength and function in frail older adults.	n=62 (21 male and 41 female), frail elderly≥65 y	Randomized, double-blind, placebo-controlled trial Milk protein concentrate, 30 g/day or placebo	Yes	24 weeks	In milk group: • Lean mass ↑ • Fat mass ↑ • Muscle strength ↔ • Physical performance ↔
Casperson *et al.* **(188)**	To determine whether supplementation of healthy elderly with low-volume of leucine during a 2-week period can increase protein synthesis in individuals who habitually consume protein close to the RDA	n=8 (5 male and 3 female), healthy and sedentary older adults, 68 ± 2 y	Clinical trial Leucine, 4 g/meal and 3 meals/day	No	2 weeks	• Muscle phenylalanine concentrations ↔ • Muscle free phenylalanine enrichments ↔ • Post-absorptive MPS ↑ • Phosphorylated mTOR ↑ • Phosphorylated P70S6K ↑ • Phosphorylated 4E-BP1 ↑
Aleman-Mateo *et al.* **(193)**	To examine the impact of a diet rich in protein from ricotta cheese on total appendicular skeletal muscle mass and muscle strength	n=40 (17 male and 23 female), healthy older adults, 76 ± 5.4 y	Randomized, single-blind, placebo-controlled trial Habitual diet plus ricotta cheese 210 g/day (15.7 g protein/day, including EAA at 8.6 g/day) or habitual diet	No	3 months	• Percentage of relative change in total appendicular skeletal muscle mass: no difference between ricotta cheese and control group • Muscle strength in ricotta cheese group ↑ • Fasting insulin level in the men in the ricotta cheese group ↓

			without ricotta cheese			
Farnfield *et al.* **(259)**	To assess the effect of whey protein and RE on the phosphorylation of mTOR and downstream signaling proteins in the skeletal muscles of young and old men	n=31, healthy men (n=16 young men, 18-25 y and n=15 older men, 60-75 y)	Randomized, double-blind, placebo-controlled trial Whey protein (WP) containing drink (26.6 g AA per serving, including 3.66 g leucine) or placebo	Yes	12 weeks	• ↑ Strength in all subjects following RE • ↑ Phosphorylated mTOR in WP for both older and younger subjects • ↑ Phosphorylated P70S6K, eIF4G, and 4E-BP1 in WP for older subjects • Older subjects failed to maintain the increased phosphorylation levels of signalling proteins
Chale *et al*. **(260)**	To assess the chronic effects of whey protein concentrate (WPC) and resistance training on muscle mass, strength and function in mobility-limited older adults	n=80 (34 male and 47 female), mobility-limited older adults 70-85y	Randomized, double-blind, placebo-controlled trial WPC, 40 g/day containing 20 g protein or an isocaloric control	Yes	6 months	No significant difference between WPC and control groups, however in both groups: • Total body lean mass ↑ • Total muscle cross sectional area (CSA) ↑ • Muscle strength ↑ • Muscle performance ↑

Wall *et al*. **(261)**	To compare the effect of two casein containing meal with (PRO + LEU) or without (PRO) leucine on post prandial MPS response	n=24, healthy older men, 74.3 ± 1.0 y	Randomized, placebo-controlled trial Casein containing meal (20 g) with (PRO + LEU) or without (PRO) 2.5 g leucine	No	1 day	Greater muscle protein synthetic rate following PRO+LEU ingestion over the entire 6-hour post-prandial period
Dickinson *et al.* (262)	To assess the influence of ingesting a leucine-enriched beverage on myofibrillar protein synthesis, mTOR and amino acid transporter mRNA expression	n=15, healthy older men, 72 ± 2 y	Randomized, placebo-controlled trial 10 g EAA containing 3.5 g leucine or 10 g EAA containing 1.85 g leucine	Yes	2 days	In high leucine-supplemented group: • Skeletal muscle myofibrillar protein synthesis ↑ • Phosphorylated mTOR ↑ • Phosphorylated P70S6K ↔ • Phosphorylated 4E-BP1 ↔ • Expression of amino acid transporter mRNA ↑
Luiking *et al.* **(180)**	To investigate whether there is a difference in the effects of a supplement high in whey protein and leucine or a dairy product-containing supplement on MPS in healthy older adults	n=19 (9 male and 10 female), healthy older adults≥60 y	Randomized, double-blind, placebo-controlled trial High whey protein, leucine-enriched beverage(20 g whey protein, 3 g total leucine) or milk	Yes	1 day	In whey-leucine group: • Post-prandial FSR ↑ • Interaction of treatment and exercise in terms of muscle protein FSR – • Plasma post-prandial leucine and EAA concentrations ↑

			protein (6 g protein)			
Dirks *et al.* **(263)**	To assess the effect of protein supplementation in attenuating the muscle loss during a short period of muscle disuse	n=23, healthy older men, 69 ± 1 y	Randomized, placebo-controlled trial High whey leucine enriched (20.7 g protein including 10.6 g EAA and 2.8 g leucine) twice daily or control group	No	5 days	• Immobilization => ↓ CSA and ↓ muscle strength in both groups without difference between groups • No effect of protein supplementation on muscle mass and strength loss
D'Souza *et al.* **(192)**	To investigate the effects of resistance exercise (RE) and graded ingestion of whey protein on intramuscular content of EAA and BCAA as well as the phosphorylation level of P70S6K	n=46, healthy older adults, 60-75 y	Randomized, placebo-controlled trial A beverage containing either placebo, 10 g, 20 g, 30 g and 40 g of WPC	Yes	1 day	• RE => ↓Muscle BCAA • 30 and 40 g WPC => ↑ Muscle BCAA • Signficant positive correlation between Phosphorylated P70S6K level and dose of WPC at 2 hrs. post exercise • Signficant positive correlation between Phosphorylated P70S6K level and muscle leucine content

Daly *et al*. **(181)**	To assess the effect of a moderately high protein diet combined with progressive resistance training (PRT) on muscle mass, strength, function, blood inflammatory markers, blood pressure and lipids	n=100, healthy older women ≥60 y	Randomized, cluster, placebo-controlled trial Protein-enriched diet (PRT+Meat), 200 g raw red meat/day (~45g protein), 6 times/week or control diet	Yes	4 months	In PRT+Meat group (vs. control group): • Total lean tissue mass ↑ • Leg lean tissue mass ↑ • Muscle strength ↑ • Insulin-like growth factor ↑ • Interleukin-6 ↓ • No change in blood lipids or blood • pressure
Mitchell *et al*. **(179)**	To compare the effects of soy protein with whey protein on the level of P70S6K phosphorylation	n=13, healthy older men 60-75 y	Randomized, placebo-controlled trial 30 g soy or 30 g carbohydrate	Yes	1 day	Results were compared with previously published data from the ingestion of 30 g whey protein or placebo. • Phosphorylated P70S6K at 2 hrs. post exercise in both soy and whey groups ↑ • Phosphorylated P70S6K at 4 hrs. post exercise only in whey group ↑
Animal studies						
Zeanandin *et al.* **(264)**	To investigate the long-term effect of leucine supplementation on body composition, insulin signaling pathway and insulin sensitivity of muscles in aged rats	n=120 aged rats 18 months old at the start	Randomized, controlled trial Leucine-supplemented (4.45%) diet (LEU group) containing 15% protein	No	6 months	In LEU group: • No change of mTOR signalling pathway in muscle • ↑ mTOR signalling pathway in adipose tissue • ↑Insulin stimulated glucose transport in muscle • No change in glucose tolerance • No change in skeletal muscle mass

Vianna *et al.* **(265)**	To assess the long-term effect of leucine supplementation on body composition in aged rats	n=45, 6 month-old rats divided to 3 groups: euthanized at 6 month of age as baseline reference of metabolic status, control and leucine supplemented group	Randomized, controlled trial Diet supplemented with 4% leucine or control diet	No	40 weeks	• ↓ Body fat gain in leucine group • Similar body weight gain in control and leucine groups • No difference in lipid and glycemic profile between control and leucine groups • No effect of leucine on muscle RNA concentrations, total serum and muscle protein levels
Savary-Auzeloux *et al*. **(266)**	To assess the effect of leucine (LEU) and anti-oxidants (AOX) supplementation on attenuating muscle mass loss during immobilization in adult rats	n=265, Male Wistar rats aged 6-8 months	Randomized, controlled trial Experimental group, 13% casein +AOX (immobilization), casein+AOX +4.45% LEU (first 15 d of recovery), casein+leu for the rest or control diet, 13% casein without alanine	No	8 days unilateral limb immobilization followed by 10, 15, 20, 30 and 40 days of recovery	• ↓ Muscle mass in both groups as a result of immobilization • ↑ Muscle mass recovery in experimental group relative to control group • ↑ Post-prandial and post-absorptive MPS in experimental group

			(immobilization), with alanine (recovery)			
Mosoni *et al*. **(267)**	To compare the effects of 3 nutritional strategies (high protein, high leucine and anti-oxidative/anti-inflammatory diets) on lean body mass of aged rats	n=172 male Wistar rats, 16 months at the start and 22 months at the end	Randomized, trial 6 experimental diets: whey (12% protein), casein (12% protein), and high whey (18% protein), each diet either with or without an anti-oxidative/anti-inflammatory mixture	No	6 months	• ↓ Inflammation and oxidative stress as a result of anti-oxidative/anti-inflammatory mixture • ↑ Lean body mass only in high whey protein group

Table 3.1. Summary of human and animal studies (<10 years) depicting cause and effect between of protein and leucine supplementation and muscle variables in older adults

EAA, essential amino acids; mTOR, mammalian target of rapamycin; mRNA, messenger ribonucleic acid; P70S6K, p70 ribosomal S6 protein kinase; 4E-BP1, 4E-binding protein 1; RDA, recommended dietary allowance; MPS, muscle protein synthesis; FSR, fractional synthesis rate; BCAA, branched-chain amino acids; PRO, protein; LEU, leucine; eIF4G, eukaryotic translation initiation factor 4G.

Reference	Objective(s)	Subjects	Study Design	Exercise protocol (yes or no)	Measured variable and the outcome
Bischoff *et al*. **(203)**	To test the efficacy of supplemental vitamin D and active forms of vitamin D with or without calcium in preventing falls among older individuals.	8 articles n=2426 (81% female) ~80 y	Meta-analysis Oral dose of supplemental vitamin D was examined across double blind randomized controlled trials	No	Study Group: • Daily vitamin D doses of 700-1000 IU • ↓Risk of falls by 19% • ↓Risk of fall by 26% with vitamin D3 • The benefit was sustained for 12-36 months
Kim *et al*. **(204)**	To investigate whether vitamin D level is associated with sarcopenia in older Koreans.	n=1380 (male) n=1789 (female) ≥50 y	Epidemiological Serum 25(OH)D and PTH levels were measured	No	25(OH)D ↑ • Sarcopenia ↓ • Fat mass ↓ • Skeletal mass ↑ • No association between sarcopenia and PTH
Mastaglia *et al*. **(258)**	To analyze the relation between vitamin D nutritional status and muscle function and strength in a group of healthy women aged over 65 years.	n=90 (female), healthy elderly ≥65 y	Epidemiological Retrospective data was collected to analyse effect of sun exposure on muscle strength.	No	High Sun Exposure Group: • 25(OH)D >20ng/ml • Lower extremity strength and function ↑ • PTH level ↓
Tajar *et al*. **(202)**	To determine the association of serum 25(OH)D and PTH levels with the frailty status.	n=1,504 (male) 60–79 y	Epidemiological Completed questionnaire, performance measures, fasting blood sample	No	Frail Subjects: • 62.3% insufficient 25(OH)D (<50 nmol/l) • Age ↑ • PTH levels significantly associated with frailty ↑

Park *et al*. **(205)**	To test a hypothesis: vitamin D deficiency would be positively associated with sarcopenia in a gender-specific manner in adults aged ≥50 years, independent of other covariates and possible confounders, including body composition, blood tests, dietary intakes, and serum PTH levels.	n=2258 (male) n=3005 (female) ≥50 y	Epidemiological Health interview, a health examination, and nutrition surveys	Yes	Study group • Risk of sarcopenia ↑ by 1.46-fold by ↓serum 25(OH)D by 10 ng/mL (in women) • Exercise = no effect on sarcopenia • Sarcopenia =PTH ↑(in men)
Seo *et al* **(207)**	The aim of the study was to investigate whether dietary calcium intake is associated with sarcopenia in non-obese, older Korean adults.	n=592 (male) n=707 (female) non-obese, older adults, ≥60 y	Cross-sectional Nutrition survey used a 24-hour recall method	No	Dietary calcium intake ≤278 mg per day • Sarcopenia ↑ • Lean body mass ↑ • Fat mass ↓
De Souza Genaro *et al*. **(268)**	The aim of the present study was to investigate whether low vitamin D, high PTH, or both, are associated with sarcopenia.	Females: n=35 (sarcopenia) n=70 (without sarcopenia) ≥65 y	Randomized, control trial Dietary intake, laboratory measurements, grip strength, anthropometrics	No	Sarcopenia Risk ↑ • PTH (≥37.7 pg/mL) ↑ • Skeletal muscle mass ↓ • Dietary calcium ↓ • 25(OH)D ~20 ng/mL ↓

Table 3.2. Summary of recent human studies (≤10 years) depicting cause and effect between vitamin D and calcium supplementation and muscle variables in older adults

25(OH)D, 25 hydroxy vitamin D; PTH, parathyroid hormone; IU, international unit.

3.5 BRIDGE TO CHAPTER IV

CR is one of the most popular supplements currently used in athletic populations, and is of growing interest for utilization for clinical applications, such as sarcopenia (269), and also could be applied to some of the muscle dysfunctions seen in AD. CR is an ergogenic compound with vital roles in energy metabolism of the cells, particularly of the muscles (270). While many studies over the past few decades have shown that CR supplement has many beneficial effects on skeletal muscle physiology and metabolism, primarily to enhance muscle mass, the underlying mechanisms by which CR exerts its beneficial effects are poorly understood. The following chapter provides an overview of CR metabolism, *i.e.*, sources, transport and regulation, beneficial effects and therapeutic applications. Special attention is focused on the mechanisms of action of CR supplementation on skeletal muscle metabolism.

CHAPTER IV. LITERATURE REVIEW: CR SUPPLEMENTATION AND SKELETAL MUSCLE METABOLISM- REVIEW OF THE POTENTIAL MECHANISM OF ACTIONS

A version of this chapter will be submitted for peered-review publication.

4.1 INTRODUCTION

Since the identification of CR (α-methylguanidino-acetic acid) in 1832 by Michel Eugene Chevreul, knowledge about this nitrogenous organic acid has increased remarkably, and is currently one of the most popular supplements among athletes (32, 271). CR has a key role in energy metabolism of the body's cells, particularly of the muscles. In fact, up to 94% of the human body's stored CR is located in skeletal muscles (270), with an average concentration range of 90 to 160 mmol/kg dry muscle mass (272-274). Formerly, CR has been mainly applied by athletic populations involved in high-intensity exercises (32, 275). Currently, the use of CR supplementation goes beyond the utilization as an ergogenic aid for sports, and is now actively studied as a therapy for attenuating loss or enhancement or regeneration of skeletal muscle mass and function in older adults with aging-related disorders such as sarcopenia (276-279), for muscular dystrophies (280-282) and other non-skeletal muscle clinical situations (283-285).

It widely accepted that resistance exercise is a major stimulus for muscle growth/hypertrophy (286), and most studies that investigate the muscle anabolic effects of CR usually use CR supplement in conjunction with resistance training (275, 277, 287). However, even in the absence of exercise, CR has been shown to be capable of enhancing muscle mass by stimulating the anabolic pathways in these tissues (33, 34). Some studies have demonstrated that CR supplementation in patients with cast-induced immobilization could improve muscle mass

and strength (288, 289). Overall, there is a growing interest among researchers to investigate the benefits of CR supplementation in clinical and geriatric medicine, in part because of its potential effects on muscle metabolism.

Although CR supplement is well known to have many beneficial effects on human skeletal muscles in healthy and disease states, there is a paucity of information underlying the mechanisms of action. This review seeks to explore and summarize the available information outlining the mechanisms related to how CR may exerts its beneficial effects in skeletal muscle, particularly focusing on the effects on muscle growth/hypertrophy.

4.2 CR METABOLISM– SOURCES, TRANSPORT AND REGULATION

CR is endogenously synthesized in the liver, kidney and pancreas from arginine, glycine and methionine or exogenously acquired through dietary sources, mainly from meat, poultry and fish, or supplement. Endogenous CR synthesis is primiarily an inter-organ process, which starts with the production of guanidinoacetate (GAA) in the kidney and completed in the liver by formation of CR (270, 290, 291) (**Figure 4.1**). However the pancreas is also a contributor to CR synthesis, with the presence of both CR production enzymes: arginine-glycine amidinotransferase (AGAT) and S-adenosylmethionine- guanidinoacetate N-methyltransferase (GAMT), both expressed in this tissue (292). CR is transported to high energy demanding organs, *e.g.*, brain, heart and skeletal muscle, by the CR transporter; CreaT (SLC6A8) (8, 293). This sodium dependent transporter is responsible for the uptake of CR against a high concentration gradient (8, 293, 294). The electrogenic and active transport of CR occurs with at least two molecules of Na^+ and one Cl^-. Hormones (*e.g.*, insulin) enhance CR transport and activate the $Na^+ + K^+$-ATPase (294, 295). Once CR is inside the cell, it exists in both free and phosphorylated form (phosphocreatine, PCr). The conversion of CR to PCr is a reversible

reaction which is catalyzed by the enzyme creatine kinase (CK) (296). PCr contributes to about 60-70% of the muscle total CR and because of its polarity is unable to cross the cell membrane, resulting in trapping of CR inside the cells (297). The remainder of the intracellular CR is in the free from. Both forms are spontaneously and irreversibly degraded into creatinine, which is excreted via the kidneys (**Figure 4.1**). Every day, in the human body,~1–2 g of CR is irreversibly converted to creatinine (298); thus ingestion of CR-rich foods or supplement helps the body to compensate for CR losses. For the average omnivorous adult, about half the body's CR store is replenished exogenously through the diet at an amount of about 1-5 g/day (31, 299, 300).

CR supplementation has been shown to have several effects on skeletal muscle metabolism including impact on muscle fiber composition, and muscle growth/hypertrophy. Specific effects include increasing glycogen stores and lean muscle mass. Overall, the CR-induced effects on muscle metabolism leads to improvements in bioenergetics and physical performance as measured by power output and resistance training, which may translate to improvement in muscle strength.

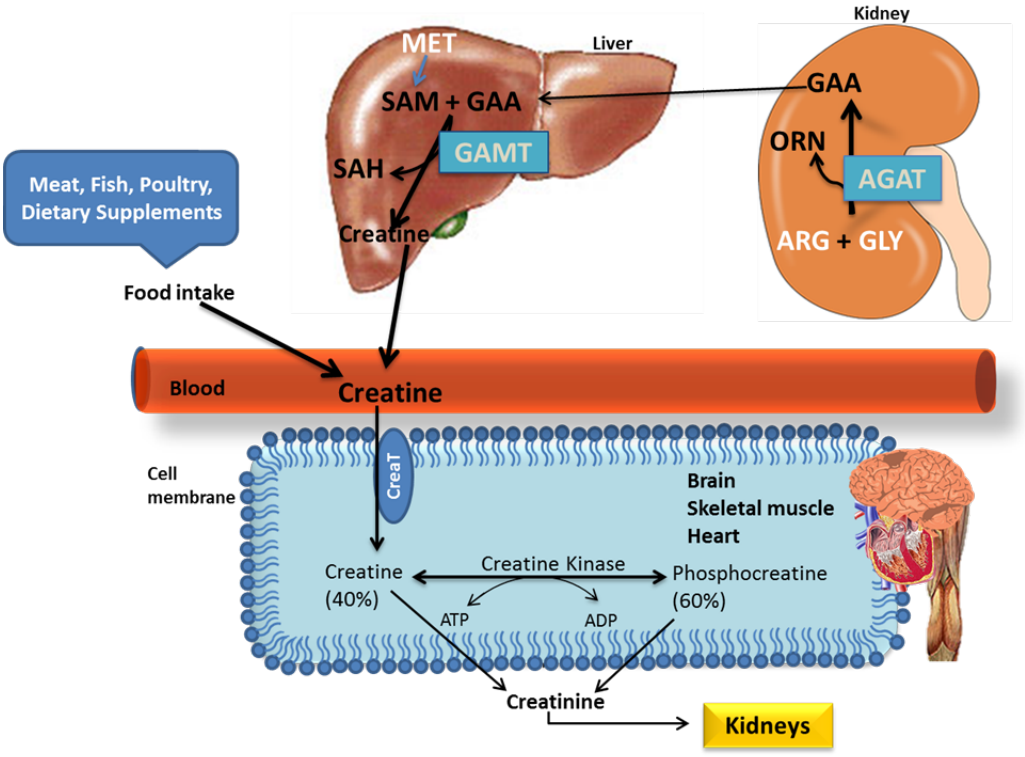

Figure 4.1. Schematic view of CR metabolism and transport in human. CR is endogenously synthesized in the human body from the AA arginine (ARG), glycine (GLY) and methionine (MET). First, ARG and GLY form guanidinoacetate (GAA) and ornithine (ORN) through a reversible reaction, catalyzed by the enzyme arginine-glycine amidinotransferase (AGAT), which is very active in the kidneys. Second, the enzyme, S-adenosylmethionine-guanidinoacetate N-methyltransferase (GAMT), which is the mostly active in the liver, transfers a methyl group (donated from MET) from S-adenosylmethionine (SAM) to GAA to form S-adenosylhomocysteine (SAH) and CR. CR is also provided through exogenous sources (diet or supplement). Once entered into the bloodstream, CR is transported to high demand organs, *e.g.*, brain, heart and skeletal muscle. The CR transport protein, CreaT (SLC6A8), facilitates the uptake of CR in these tissues. Inside the cell, CR exists in both free and phosphorylated form (phosphocreatine, PCr), both of which are spontaneously and irreversibly degraded to creatinine and excreted via the kidneys. ATP indicates adenosine triphosphate; ADP, adenosine diphosphate.

4.3 BENEFICIAL AND THERAPEUTIC APPLICATION OF CR SUPPLEMENTATION ON SKELETAL MUSCLE METABOLISM

4.3.1 Older Adults and Age-related Loss of Muscle Mass and Function

While the impact of CR supplementation on enhancing muscle mass, function and strength in young adults is probably undebatable (287, 300, 301), there is a need to understand whether CR is effective for older adults to help combat loss of muscle mass and function (*i.e.*, age-related sarcopenia). CR supplementation may be required in elders to observe any significant impact on muscle mass and function. Candow, Little (302) demonstrated that 10 weeks of CR (0.1 g/kg) supplementation, in conjunction with resistance training (3 d/wk), improved lean body mass and decreased muscle protein catabolism in older men (59-77 yrs) compared to placebo. Similar results in terms of increased muscle performance and strength as a consequence of 12 weeks of CR (5 g/d) ingestion combined with resistance training (3 d/wk) were observed in older women (64.9 +/- 5.0 yrs) (303). Although many researchers claim that the effect of CR is only detectable when combined with exercise (304), it has been shown that even in the absence of exercise CR is capable of enhancing muscle mass by stimulating the anabolic pathways in the muscle tissues. Gotshalk, Kraemer (305) found that LBM, muscle strength and performance were enhanced in older women (58-71 yrs) as a result of 7 days of CR supplementation (0.3 g/kg). In this study, subjects did not receive any physical training or exercise and only supplemented with CR or placebo. Hespel, Op't Eijnde (288) and Johnston, Burke (289) demonstrated that CR supplementation in patients with cast-induced immobilization could improve muscle mass and strength. Nevertheless, there are still some controversies as to whether CR is beneficial for an elderly population to help reduce muscle mass loss (30, 279). Overall, the role of CR in prevention of sarcopenia still remains to be determined.

4.3.2 Muscle Disorders

CR is widely used as an adjuvant therapy for many myopathies because of its well-known capability to induce strength and lean body mass gain (298). Myopathies are primary skeletal muscle disorders with many subdivisions, which cause weakness and disability in patients. The underlying defect may be either due to alteration in contractile apparatus or impairment of energy pathways. The research to date strongly suggests that several months of CR monohydrate ingestion at a dose between 0.075 to 0.1 g/kg/day has positive effects both on strength and fat-free mass in patients with muscular dystrophy (282). In addition, CR has the best effect on dystrophinopathies and myotonic dystrophy type 2. Whereas, patients with myotonic dystrophy type 1 and facioscapulohumeral dystrophy have demonstrated no significant effect from supplementation (281, 306), which emphasizes that the magnitude of beneficial effects may differ among different subgroups of myopathies. In the future more clinical studies should focus on these effects and also make a comparison between conventional treatments for myopathies (*i.e.*, corticosteroid) with high adverse effects and CR as a nutritional supplement (282, 298, 300). In children with muscular dystrophies, CR can increase strength and fat free mass (274). In addition, according to the investigation of Bourgeois, Nagel (307), CR can decrease accumulation of fat in the body and improve body mass index during maintenance chemotherapy in children who suffer from acute lymphoblastic leukemia.

4.4 CR SUPPLEMENTATION INCREASES MUSCLE MASS – POTENTIAL MECHANISMS OF ACTION

As briefly reviewed above, CR supplementation is often reported to increase body mass in many populations. There are two main explanations provided for this increase: 1) intramuscular water retention owing to the osmotic loading properties of CR, 2) direct stimulation of myofibrillar

protein synthesis pathway. The contributions of other potential mechanisms may include CR effects on satellite cell activity and cellular energy status.

4.4.1 Cellular Hydration, Resulting from the Osmotic Effect of CR, Serves as an Anabolic Signal for Protein Synthesis

CR supplementation is well known to induce rapid/short-term increase in muscle mass (308-310), which is most likely due primarily to intramuscular water retention owing to the osmotic loading effect of CR (36, 311, 312). This osmotic state leads to immediate increase in body weight; primarily increased body water content (311), and this cellular hydration state has been suggested to be a major anabolic signal for protein synthesis for more sustained increase in muscle mass (36), however the mechanism for this skeletal muscle hypertrophy is not clear for the case of CR supplement. In the early 1990's Häussinger, working primarily with hepatocytes, postulated that cellular hydration state is an important factor controlling cellular protein turnover (313, 314). Cellular hyper-hydration state was thought to decrease catabolism of glycogen, glucose, RNA and protein while simultaneously stimulating glycogen, RNA, DNA and protein (314-316). The underlying mechanism by which changes in cellular hydration status (osmosensing) leads to protein synthesis is now believed to start with the integrin system, at least in hepatocytes (317). Reviewing available studies on the topic of cellular hydration and protein synthesis, Häussinger hypothesized that cellular swelling leads to integrin G-protein-mediated activation of kinases such as tyrosine kinase that then activates stress response proteins like mitogen-activated protein (MAP) kinases, which initiates a cascade of downstream phosphorylation events that leads to increase protein synthesis and gene transcription (316, 317).

While principles of Häussinger's cellular hydration theory were primarily conducted with hepatocytes, erythrocytes and astrocytes (316), it is believed that the concept also applies to the

intramuscular osmotic effect of CR supplementation. Indeed, it has been postulated that CR-induced cellular swelling may be the starting point for anabolic signals (318), which may affect factors such as increased expression of myogenic transcription factors (288, 319-322). The anabolic effects of CR have been demonstrated in the absence of exercise stimulus as shown in several *in vitro* studies (**Table 4.1**). Using muscles from chick embryos, Ingwall and colleagues (321, 322) showed that CR supplementation stimulated the synthesis rates of myosin heavy chain and actin, two major contractile proteins; however there was no significant difference in overall total protein synthesis. However, Fry and Morales (323) found that although CR supplementation increased intracellular total CR this had no effect on total protein synthesis or myosin heavy chain synthesis. Later works showed that CR supplementation, in combination with resistance exercise, can elicit an anabolic response by upregulating skeletal muscle specific genes through increased expressions of myogenic regulatory factors (MRFs) such as MRF4 and myogenin in humans (288, 319), which may play a role in increasing myosin heavy chain (320-322). Unfortunately, most of these studies did not directly address osmosis/cellular hydration factor related to CR supplementation, thus it is unclear of the role of cellular hydration under these circumstances.

4.4.2 Direct Activation of Protein Synthesis through Activation of Components in the mTOR Pathway

More recent works have focus on the direct effect of CR on protein synthesis. *In vivo* studies in humans using isotopic technique found no direct effect of CR on protein synthesis due to short term CR supplementation (324, 325). Parise, Mihic (324) used ^{13}C-leucine kinetics to measure MPS rate in healthy individuals given CR supplementation for 8-9 days, and found this did not increase MPS, but it did reduce leucine oxidation in some individuals (men but not

women); which the authors stated is suggestive of anti-catabolic action for some protein. Similarly, also using ^{13}C-leucine kinetics, Louis, Poortmans (325) found that CR ingestion for 5 days had no effect on MPS in healthy males.

Conversely, *in vitro* studies showed that addition of CR to differentiation medium of C_2C_{12} murine skeletal myogenic cells lead to hypertrophy of myotubes (35, 326). Louis, Van Beneden (326) found that this CR-induced myotube hypertrophy was in part mediated by overexpression of insulin-like growth factor 1 (IGF-1) and MRFs (326). Further work by this group showed that CR can promote the differentiation of myogenic cells through activation of Akt/PKB and p70S6K, two key proteins involved in PI3K/ PKB/mTOR pathway; a key pathway implicated in skeletal MPS (35) (**Figure 4.2**). This effect was shown to be distinct from the effect of CR on cell osmolarity (35), which was previously purported to play a major role in CR impact on the cells (36). In the cellular hydration signaling of protein synthesis theory, Häussinger and colleagues also showed that in some instances increased cellular osmolality (cellular swelling) may activates specific components of the protein synthesis pathway such as p70S6K; however it was acknowledged that crosstalk between hyperosmotic signaling and mTOR pathways is poorly understood (327). However, in the case of CR, the work of Deldicque, Theisen (35) suggested that CR effect on cellular osmolality does not appear to be involved in directly activating components of the protein synthesis pathway. However, all these studies are *in vitro* and may not accurately portrait the situation in a multiple system organism.

In general, mTOR is a conserved serine/threonine kinase involved in many signal transduction pathways within the body, including regulating protein synthesis and cell growth and homeostasis (131, 286). In fact, CR supplementation has also been implicated in activation of mTOR in non-skeletal tissues (328). Cunha, Budni (328) found that CR treatment in mice

increased phosphorylation of Akt and p70S6K. Other evidences also suggest direct relationship between CR and mTOR (329). Sumitani, Goya (329) found that LY294002, a pharmacological inhibitor of PI3K, and rapamycin inhibited insulin-induced differentiation of C_2C_{12} myoblasts, and during the process these inhibitors repressed muscle creatine kinase and myogenin gene transcription.

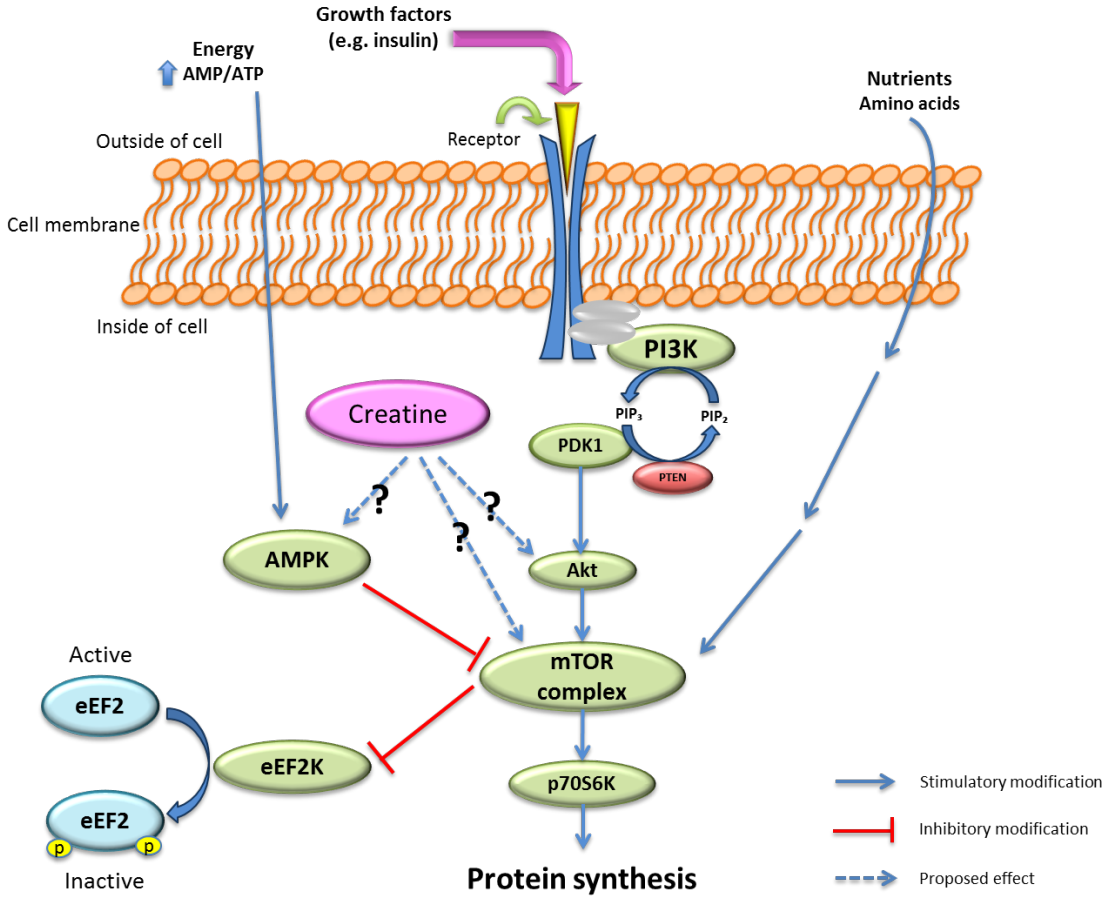

Figure 4.2. Simplified view of mTOR signalling pathway and possible role of CR in skeletal muscles protein synthesis. mTOR is a conserved serine/threonine kinase involved in many signal transduction pathways within the body, regulating cell growth and homeostasis. The mTOR pathway is activated by insulin, growth factors and AA. Activation of mTOR results in the phosphorylation of specific proteins that ultimately phosphorylate and activate p70 ribosomal S6 protein kinase (p70S6K), which triggers a cascade of responses that subsequently results in protein biosynthesis. 5' adenosine monophosphate-activated protein kinase (AMPK) is also a key regulator of cellular energy homeostasis. AMPK can sense the cellular energy level and down-regulate the cellular pathways that consume ATP in case of decreased cell energy content. Eukaryotic elongation factor 2 (eEF2) in its active form results in activation of overall translation elongation in protein synthesis. It is postulated that CR may act through activation of Akt/PKB and p70S6K (35), or PI3K (328); all key proteins involved in PI3K/Akt/ mTOR pathway, or inhibition of AMPK. PIP_2 indicates phosphatidylinositol 4,5 bisphosphate; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; PTEN, phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase; PDK1, 3-phosphoinositide dependent protein kinase-1; eEF2K, eukaryotic elongation factor 2 kinase; p, phosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate.

4.4.3 Cellular Energy Status

Besides growth factors and specific nutrients such as AA, the rate of MPS is also regulated by the cellular energy status (286, 330). The 5'-adenosine monophosphate-activated protein kinase (AMPK) pathway is a sensor of cellular energy status, and is regulated by changes in the cellular level of AMP-to-ATP ratio. The activation of AMPK is known to inhibit activation of mTOR and thus protein synthesis (330) (**Figure 4.2**). Since the main known purpose of CR supplementation is to serve as a ready source of energy to cells this may also be a main regulator of MPS state.

The energy status of cells is maintained in part by PCr-CK energy system, which functions by supplying muscles with PCr, which can react with ADP and produce ATP (**Figure 4.1**). Although the PCr-CK energy system acts as an energy buffer for the cells and produces large amounts of ATP, the storage capacity of this system in skeletal muscles is quite small and after about 10-20 seconds of high intensity exercise, PCr is depleted rapidly. However, this capacity can be increased by oral ingestion of CR supplement. High dose of oral CR supplementation is well known to considerably increase muscle's concentrations of both free and phosphorylated form of CR (331), with ranges of increases from 20-50% following its use (272, 332). Studies have shown that CR supplementation may affect PCr/CR ratio, which affects AMPK activation (333, 334). The *in vitro* work of Ponticos, Lu (334) suggested that the activation of AMPK in muscle depends in part on high Cr and low PCr levels. Using L6 rat skeletal muscle cells, Ceddia and Sweeney (333) showed that CR supplementation (0.5 mM) resulted in about 5-fold increase in PCr and 9-fold increase in Cr; overall a decrease in PCr/Cr ratio, and about 2-fold increase in AMPK phosphorylation, but protein synthesis was not assessed. However, a study in healthy adult subjects (18-30 yrs old) indicated that CR

supplementation (15 g down to 2.5 g/d) following leg cast immobilization did not affect AMPK expression and phosphorylation (335). Overall, there is a scarcity of information to ascertain whether CR supplementation can directly affect protein synthesis through its impact on cellular energy capacity.

4.4.4 Effect on Satellite Cell

CR's ability to increase muscle mass is also thought to occur in part through its effect on satellite cell mitotic activity (318, 336, 337). This increased mitotic activity leads to increased expression of myogenic transcription factors, and subsequently protein synthesis/muscle mass. Olsen *et al.* (337) conducted a study on healthy, young, male subjects to examine the effect of CR ingestion in combination with strength training exercise on the activity of satellite cells and the concentration of myonuclei within a muscle fiber. Although strength training alone was able to increase the number of nuclei per each muscle fiber and the number and activity of satellite cells, CR supplementation augmented this effect, causing a higher increase in the proliferation of satellite cells (337). Similarly it has been shown that oral CR supplementation is able to impact satellite cell's proliferation by increasing their mytotic activity in the rat's skeletal muscle. This effect was noticeable only in those muscles that received overload prior to CR ingestion (336). However, it remains unclear how CR augments satellite cell mitotic activity.

4.4.5 CR Augments the Effect of other Anabolic Stimuli

Perhaps CR effects on muscle growth/hypertrophy are more to amplify the effect of well-known anabolic stimuli such as protein/AA and exercise (which trigger release of growth factors). For instance, Cribb *et al.* (338) reported that in young males, 11 weeks of resistance training combined with a protein-CR supplement increases lean body mass, muscle fiber cross

sectional area and contractile protein more than supplements of protein-carbohydrate or protein. CR supplementation in combination with exercise has been shown to increase growth factors and satellite cell mitotic activity (287, 337, 339). In a similar study conducted by Volek *et al.* (310), the effect of CR supplementation in combination with resistance exercise in increasing muscle fiber cross sectional area, muscle mass and performance was higher than control male, healthy subjects that only received exercise with placebo.

4.5 CONCLUSION

While it is continuously shown that CR supplementation can enhance muscle mass in athletic and other populations, the understanding of the underlying mechanisms is poor. The available evidence suggests that CR may exert its effect on skeletal muscle growth/hypertrophy through multiple mechanisms. To move forward in realizing the full benefits of CR supplementation requires a better understanding of the mechanisms of action of its effect on skeletal muscle metabolism, given that up 94% of CR is stored in this tissue.

Increased protein synthesis as a result of enhanced intracellular fluid volume or the direct effect of CR on protein synthesis signaling pathways, maintaining high cellular energy potential through PCr-CK system and augmented satellite cell proliferative activity in response to CR supplementation are suggested mechanisms of action for CR inside the muscle cells. These changes can occur with or without using other anabolic stimulants such as exercise and protein supplementation, however, the anabolic stimulatory impact of CR is more prominent when it is used in combination with those stimulants. Although there is a large body of literature investigating the impact of CR ingestion on muscle mass and function, finding precise underlying mechanism(s) responsible for the effect of CR remains to be elucidated.

Reference	Objectives	Study population	Type of study	Type, dose and duration of CR supplementation	Exercise (yes or no)	Gross effect(s) on muscle	Metabolic effect(s) on muscle
Ingwall *et al.* (1972) **(322)**	To investigate the effect of CR on synthesis of myosin heavy chain	Cell culture of 12-d chick embryo pectoral myoblasts and explants of breast muscle tissue from 14- to 15-d chick embryo	*In vitro* study	Not indicated, 5 mM for 1-4 days	Not applicable	Not applicable	• No effect on total muscle protein synthesis, ***but:*** • ↑ Synthesis of skeletal muscle myosin heavy chain both *in vitro* and *in vivo* → The response was concentration dependent over the range of 10-100 μM and only occurs in cells already synthesizing muscle protein, not the myoblasts
Ingwall *et al.* (1974) **(321)**	To determine the effect of CR on muscle actin, myosin heavy chain and total protein synthesis	Cell culture of 11- to 13-d chick embryo pectoral myoblasts	*In vitro* study	Not indicated, 5 mM for 7 days	Not applicable	Not applicable	• No effect of CR on total protein synthesis, ***but increase in specific muscle proteins:*** • ↑ Myosin heavy chain synthesis in response to CR • ↑ Actin synthesis in response to CR
Fry and Morales (1980) **(323)**	To investigate the effect of CR on synthesis of muscle specific proteins	Cell culture of 11- to 12-d chick embryo pectoral myoblasts	*In vitro* study	Not indicated, 5 mM for 5 days	Not applicable	Not applicable	• No effect of CR on myosin and total protein synthesis, ***but:*** • Inhibition of myosin and total protein synthesis by a CR analogue, 1-carboxymethyl-2-iminohexahydropyrimidine (CMIP) • ↑ 5,700 folds in extracellular CR → ↑ 20 folds in intracellular CR

Young *et al.* (1984) **(340)**	To determine the effect of CR on myosine heavy chain (MHC) protein and mRNA content in cultured muscle cells with steady state protein metabolism	Cell culture of 12-d chick embryo leg muscle	*In vitro* study	Not indicated, 0-1 mM for 4 hours	Not applicable	Not applicable	• ↑ Leucine incorporation into MHC by 30-40% in response to 0.2 mM CR • ↑ MHC mRNA content by 15% in response to 0.2 mM CR: much less than the increased rate of leucine incorporation → Possibility of the involvement of the other mechanisms in enhancing MHC synthesis • No effect of CR in rapidly differentiating muscle cell cultures
Hespel *et al.* (2001) **(288)**	To investigate the effect of CR on muscle volume, function and Myogenic transcription factors expression during muscle immobilization and subsequent rehabilitation	22 healthy young men and women (aged 20-23 yrs.)	Randomized, double-blind, placebo-controlled study	CR monohydrate, 20 g/d for 2 weeks (immobilization period), 15 g/d from week 1 to 3 (training period consisted of 4 unilateral knee extensions) and 5 g/d from week 4 to 10 (training period consisted of 6 unilateral knee extensions)	Yes	↓ Muscle CSA and maximal knee-extension power in both placebo and CR group → Faster recovery in CR group during rehabilitation period	• ↑ MRF4 protein expression in CR group after rehabilitation • Significant correlation between MRF4 protein expression and change in mean muscle fibre diameter • ↑ Myogenin protein expression in placebo group after rehabilitation
Parise *et al.* (2001) **(324)**	To investigate the effect of CR on lean body mass, muscle CR, PCr and ATP concentrations and indexes of	27 healthy men (n=13) and women (n=14) (23 ± 4 yr.)	Randomized, double-blind, placebo-controlled study	CR monohydrate, 20 g/d for 5 days and then 5 g/d for 3-4 days	No	No effect of CR on lean body mass	• ↑ Muscle total CR and PCr • No gender difference for muscle total CR, PCr and ATP • ↓ Muscle protein breakdown in men but not in women in response to CR • No effect of CR on muscle protein synthesis

	whole body protein turnover						
Willoughby *et al.* (2001) **(320)**	To determine the effect of 12 week CR and resistance training (RT) on fat and lean body mass, muscle strength, myofibrillar protein content and MHC expression	22 men (20.41 ± 1.73 yr.)	Randomized, double-blind, placebo-controlled	CR monohydrate, 6 g/d for 12 weeks	Yes	↑ lean body mass, thigh volume and muscle strength in response to CR	• ↑ Myofibrillar protein in response to CR • ↑ MHC-I and MHC-IIx in response to CR
Willoughby *et al.* (2003) **(319)**	To examine the effect of 12 week CR and RT on muscle CK (M-CK) and MRFs gene expression	22 men (20.41 ± 1.73 yr.) (Samples from previous study, Willoughby *et al.*, 2001, were studied)	Randomized, double-blind, placebo-controlled	CR monohydrate, 6 g/d for 12 weeks	Yes	No measured in this study, but measured in Willoughby *et al.*, 2001	• ↑ M-CK mRNA expression in response to CR • ↑ MRF-4 and Myogenin mRNA and protein expression in response to CR • ↑ Myo-D mRNA and protein expression in response to both CR and RT
Tarnopolsky *et al.* (2003) **(341)**	To evaluate the effect of acute and moderate-term CR on CR transporter (CreaT) gene expression in	Study1, acute effect: 27 healthy men and women (23 ± 4 yr.)	Randomized, double-blind, placebo-controlled	CR monohydrate: Study1: 20 g/d for 5 days and then 5 g/d for 3-4 days	Study1: No Study2: Yes	Not measured	• ↑ Total muscle CR content in all studies • No effect of acute CR loading on CreaT mRNA content • No evidence of CreaT downregulation in response to moderate-term loading of CR in young men

	young and elderly	Study2, moderate-term effect: 19 healthy men Study3, moderate-term effect: 15 men (67.8 ± 4.0 yr.) and 15 women (69.3 ± 6.3 yr.)		Study2: 10 g/d 6 d/week for 8 weeks Study3: 5 g/d 3 d/week for 14 weeks	Study3: Yes		• No evidence of CreaT downregulation in response to moderate-term loading of CR in elderly men and women
Louis *et al.* (2003) **(342)**	To assess the combined stimulatory effect of CR and resistance exercise on muscle protein synthesis breakdown	7 healthy men (21 ± 1 yr.)	Controlled, cross-over study	CR monohydrate, 21 g/d for 5 days and then 7 g added to the first oral feeding bolus at 1st hour of acute study (subjects were studied twice: before and after ingestion of CR)	Yes	Not measured	• ↑ Muscle total CR in response to the CR supplementation • No effect of CR on post-exercise leg blood flow • ↑ MPS in response to exercise and post-exercise feeding with no additional effect of CR • No effect of CR on any aspect of protein metabolism
Louis *et al.* (2003) **(325)**	To determine the effect of CR on muscle protein synthesis and breakdown in the fed state	6 healthy men (26 ± 7 yr.)	Controlled, cross-over study	CR monohydrate, 21 g/d for 5 days and then 7 g added to the first oral feeding bolus at 3rd hour of acute study (subjects were studied twice: before and after ingestion of CR)	No	Not measured	• ↑ Muscle total and free CR in response to the CR supplementation • No effect of CR on protein synthesis or breakdown in either post-absorptive or fed state
Louis *et al.* (2004) **(326)**	To assess the role of IGF-I and MRFs in CR related	C_2C_{12} murine myoblasts	*In vitro* study	Not indicated, 5 mM for 72 h	Not applicable	Not applicable	• Dose-response relationship between CR concentration and protein content of myotubes with the 5 mM having the maximal effect on protein content

	myotubes' hypertrophy						• ↑ Average diameter of myotubes in response to CR • ↑ IGF-I mRNA by 3.7 after 72 h of CR incubation • ↑ Transcription of MRFs in response to CR
Deldicque *et al.* (2005) **(339)**	To assess whether CR supplementation could: 1. ↑ *IGF-I* and *IGF-II* genes expression 2. ↑ phosphorylation state of p70S6K and 4E-BP1	6 healthy young men (age 23 ± 0.6 yr.)	Randomized, double-blind, placebo-controlled, cross-over study	CR monohydrate, 21 g/d for 5 days	Yes	Not measured	• ↑ Muscle total CR • ↑ Resting muscle mRNA expression for IGF-I and IGF-II • No cumulative effect of CR and exercise on IGF-I and IGF-II • ↑ Phosphorylation level of 4E-BP1 in CR vs. placebo at 24h post-exercise
Deldicque *et al.* (2007) (35)	To identify signaling cascades by which CR affects growth and differentiation of myogenic cells	C_2C_{12} murine skeletal muscle myoblasts	*In vitro* study	CR monohydrate, 5 mM for 72-96 h	Not applicable	Not applicable	• ↑ Infusion of myoblasts • ↑ Labeled methionine incorporation into sarcoplasmic and myofibrillar proteins • ↑ Expression of MHC-II, troponin T and titin • No effect of mannitol, taurine and β-alanine → Osmolarity independent mechanism of CR • Inhibitory agents of mTOR/p70S6K and p38 → Complete blockage of myogenic differentiation → No effect of CR in reversing this blockage → Potential involvement of mTOR/p70S6K and p38 in CR action • ↑ Phosphorylation level of Akt/PKB, GSK-3, p70S6K and p38

Deldicque *et al.* (2008) **(343)**	To evaluate the effect of CR on exercise induced enhancement in anabolic signaling and gene expression in muscle cells	9 healthy young men (21.7 ± 0.55 yr.)	Randomized, double-blind, placebo-controlled, cross-over study	CR monohydrate, 21 g/d for 5 days	Yes	Not measured	• ↓ Phosphorylation level of PKB at rest and ↓ phosphorylation of 4E-BP1 24h post-exercise • ↑ mRNA for collagen 1, GLUT4 and MHC-I at rest and MHC-II immediately after exercise • ↑ mRNA for MAFbx MHC-IIA, PPAR-γ coactivator 1-α and IL-6 and ↑ phosphorylation level for p38 immediately after exercise and independent of CR ➔ No effect of CR in enhancing anabolic signaling
Safdar *et al.* (2008) **(318)**	To assess the effect of CR on intramyocellular global and targeted gene expression and protein content	12 healthy men (26 ± 3 yr.)	Randomized, double-blind, placebo-controlled, cross-over study	CR monohydrate, 10 g/d for 3 days and then 5 g/d for 7 days	No	↑ lean body mass and total body water	• ↑ Total muscle CR content • ↑ Expression of 216 genes and ↓ expression of 69 genes in the skeletal muscle in response to CR • ↑ p38, MAPK, ERK6 and protein kinase Bα protein contents →↑ mRNA expression and protein content of kinases involved in osmosensing and signal transduction, cytoskeleton remodeling, protein and glycogen synthesis regulation, satellite cell proliferation and differentiation, DNA replication and repair, RNA transcription control and cell survival,

Table 4.1. Summary of studies on the effects of CR supplementation on muscle mass, highlighting possible mechanism of actions in regards to muscle protein synthesis

Reference	Objectives	Study population	Type of study	Type, dose and duration of CR supplementation	Exercise (yes or no)	Gross effect(s) on muscle	Metabolic effect(s) on muscle
Op 't Eijnde *et al.* (2001) **(344)**	To examine the effect of CR on muscle GLUT4 content and total CR and glycogen concentrations during muscle immobilization and subsequent rehabilitation	22 healthy young men and women (aged 20-23 yrs.)	Randomized, double-blind, placebo-controlled study	CR monohydrate, 20 g/d for 2 weeks (immobilization period), 15 g/d from week 1 to 3 (training period consisted of 4 unilateral knee extensions) and 5 g/d from week 4 to 10 (training period consisted of 6 unilateral knee extensions)	Yes	Not measured	• ↓ Muscle GLUT4 during disuse in placebo group, but not in CR group • ↑ Muscle GLUT4 during rehabilitation period in CR group, but not in placebo group • ↔ Muscle glycogen and total CR during disuse period • ↑ Muscle glycogen and total CR in CR group after 3 weeks of rehabilitation, but not in placebo group
Saab *et al.* (2002) **(312)**	To investigate the effect of CR on magnetic resonance (MR) transverse relaxation (T2) distribution, as an indicator of intracellular water (ICW), in skeletal muscle	15 healthy men	Randomized, double-blind, placebo-controlled	CR monohydrate, 20 g/d for 5 days	No	Not measured	• ↑ T_2 components → ↑ ICW

Vierck *et al.* (2003) **(345)**	To determine the effect of CR and other ergogenic compounds on myogenic satellite cell proliferation	Ovine satellite cells	*In vitro* study	CR monohydrate, CR pyruvate, 0.1%, 0.25%, 0.5%, and 1.0% w/v of basal media	Not applicable	Not applicable	• ↑ Myogenic satellite cell differentiation in response to CR monohydrate, but not other forms of ergogenic supplements
Powers *et al.* (2003) **(311)**	To assess the effect of CR on muscle CR concentration, body mass, total body water (TBW), extracellular water (ECW) and ICW volumes	32 healthy young men (n=16, 22.8 ± 3.01 yr.) and women (n=16, 21.8 ± 2.51 yr.)	Randomized, double-blind, placebo-controlled	CR monohydrate, 25 g/d for 7 days and then 5 g/d for 21 days	Yes	Not measured	• ↑ Muscle CR concentration in response to the CR supplementation • ↑ Body mass • ↑ TBW • ↔ ECW and ICW
Murphy *et al.* (2004) **(346)**	To assess whether CR has direct effect on contractile apparatus or the improvement in muscular performance is related to decreased ionic strength	Single. Mechanically skinned rat muscle fibers	*In vitro* study	3 different set of experiments: 1)↑ CR concentration 2)↑ CR concentration plus ↓ ionic strength 3)↑ CR and PCr concentration with no change in ionic strength	Not applicable	Not applicable	• No effect of ↑ CR alone on muscle contraction • After short term CR supplementation: ↑ CR concentration + ↓ ionic strength (as a result of intracellular water accumulation) → beneficial effect on contractile apparatus of the muscle fibers • After long term CR supplementation: return of CR/PCr ratio and ionic strength to the pre-supplementation level → Persistent beneficial effects on contractile apparatus probably due to ↑ ATP level associated with ↑ PCr level

van Loon *et al.* (2004) **(347)**	To determine the effect of CR on muscle glycogen stores and GLUT4 mRNA and protein contents	20 healthy young men	Randomized, double-blind, placebo-controlled	CR monohydrate, 20 g/d for 5 days and then 2 g/d for 37 days	No	Not measured	• ↑ Muscle total CR, PCr, free CR and glycogen content in response to CR loading dose, but not maintenance dose • ↔ GLUT4

Table 4.2. Summary of studies on the effects of CR supplementation on muscle mass, highlighting possible mechanism of actions in regards to non-protein synthesis effects

CHAPTER V. STUDY RATIONALE, OBJECTIVES AND HYPOTHESES

5.1 RATIONAL

AD mainly affects the elderly population who are already burdened with age-related health issues such as sarcopenia. Age-related loss of muscle mass or sarcopenia, as one of the most dramatic anatomic changes in elderly population, is strongly connected to decreased physical activity as a consequence of normal aging or disease condition (14). Research indicates that there is a direct link between upstream neurological changes and aging-associated muscle mass loss in the elderly (14). Furthermore, there is some evidence to suggest that the accumulation of Aβ plaques and intraneuronal NFT that occurs in the brain in AD may lead not only to cognitive problems, but also muscle impairments. Most recently Burns *et al.* (14) suggested that AD-related alterations in body composition might be predominantly related to loss of lean mass (*i.e.*, sarcopenia). That is, sarcopenia may be accelerated in the earliest stages of AD. Indeed, preliminary studies suggest that a sequence of events provokes loss of skeletal muscles in patients with AD (14, 16, 20, 24). Neuronal atrophy in the brain has been shown to cause less dopaminergic neurotransmission and thus impaired connection between neurons (111). Moreover, defects in sensorimotor feedback loops which alters the corticospinal excitability, along with oxidative damage to proteins and lipids in the peripheral tissues results in the loss of motor units, axonal atrophy and demyelination of neural fibers (111). Given the impact of AD and aging on muscle mass in AD and elderly populations it is critical to explore strategies aimed at enhancing muscle mass in order to increase functionality and quality of life. One of these strategies is supplementation of patients with CR, which is an ergogenic compound with vital roles in cell energy metabolism, particularly muscle cells (30). Formerly, CR research has been mainly focused on athletic populations (32), however there is a growing interest among

researchers to investigate the importance of CR supplementation in geriatric medicine as an aid to help attenuate aging-related disorders such as sarcopenia and AD. It has been shown that even in the absence of exercise CR is capable of enhancing muscle mass by stimulating the anabolic pathways in these muscle tissues (33, 34). Examples, Hespel *et al. (288)* and Johnston *et al.* (289) demonstrated that CR supplementation in patients with cast-induced immobilization could improve muscle mass and strength. Nevertheless, there are still some controversies as to whether CR is beneficial for elderly population to help reduce muscle mass loss (30, 279). Furthermore, there is a need to understand the underlying effects associated with altered muscle metabolism in AD and elderly populations.

5.2 OBJECTIVES

5.2.1 Overall Objective

The overall objective of this investigation is to determine the effects of CR supplementation on muscle metabolism in an Alzheimer mouse model.

5.2.2 Specific Objectives

To determine whether CR supplementation has any effect on:

I. Muscle protein and AA contents
II. Muscle cell size and protein synthetic capacity
III. Energy pool of the muscle cells
IV. Activation of elements of intracellular signalling pathways involved in the regulation of MPS.

5.3 HYPOTHESES

CR supplementation will:

I. Increase muscle mass as measured by increased protein and AA concentrations in fast twitch fiber muscle groups from the lower limb in CR supplemented group versus the control group.
II. Increase protein/DNA and RNA/DNA ratios as indexes of muscle cell size and protein synthetic capacity, respectively, in CR supplemented group versus the control group.
III. Increase muscle cell energy potential as measured by increased ATP/ADP ratio in CR supplemented group versus the control group.
IV. Activate/phosphorylate p70S6K as an element of intracellular signalling pathways involved in the regulation of MPS in CR supplemented group versus the control group.

CHAPTER VI. CR SUPPLEMENTATION IMPROVES SKELETAL MUSCLE ANABOLIC RESPONSE IN TRIPLE-TRANSGENIC ALZHEIMER'S DISEASE MOUSE MODEL

A version of this chapter will be submitted for for peered-review publication.

6.1 INTRODUCTION

Alzheimer's disease (AD), the most common form of dementia in the elderly, is a global issue affecting about 24 million individuals (7). The disease is a complex disorder, but the main identified pathological characteristics are significant loss of neurons, extracellular accumulation of amyloid beta (Aβ) plaques, and intraneuronal neurofibrillary tangles of hyperphosphorylated tau protein in the brain, resulting in progressive decline in memory and cognitive functions (12, 13). However, dementia is not the only leading factor contributing to dependency in AD patients. There is evidence to indicate that AD is a systemic pathology, with recognized clinical features such as abnormal weight loss and cachexia (14, 24, 37, 38). In fact, using a triple transgenic (3×Tg) mouse model of AD, a recent study suggests that derangements of skeletal muscle may be one of the earliest dysfunctions in AD, occurring at the pre-symptomatic stage prior to some hallmark pathophysiological changes such as deposition of Aβ in the brain (20). The 3×Tg-AD mouse model was developed to overexpress mutant amyloid precursor, presenilin and tau proteins, all thought to be linked to AD, as these mutations lead to Aβ deposition, elevated intracellular calcium levels and hyperphosphorylated tau (19). In the study by Monteiro-Cardoso

et al. (20), comparison of 3, 6 and 12-month old 3×Tg-AD mice with age-matched controls revealed that the functionality of skeletal muscles was impaired even in 3-month old 3×Tg-AD animals compared with their control littermates. In addition, more Aβ accumulated in skeletal muscles of 3×Tg-AD mice with age (20). Yet, despite these ongoing studies, muscle dysfunction in AD patients is often an overlooked feature of the disease, thus the underlying mechanisms causing skeletal muscle abnormalities in AD remain largely unidentified. However, age-related progressive loss of muscle mass, or sarcopenia, is thought to contribute to muscle dysfunction in AD (14, 24). Decreased muscle mass and/or function in sarcopenia consequently results in reduced physical activity, which in turn exacerbates existing muscle dysfunction and leads to progressive loss of skeletal muscle mass and function as an outcome of disuse atrophy (21).

Skeletal muscle mass may represent as much as 50% of human body weight and is fundamental for mobility and many metabolic functions (40). Thus, strategies aimed at maintaining or enhancing muscle mass and function are critical to increase functionality and quality of life in the elderly population, including those with AD. Skeletal muscle is a dynamic tissue wherein muscle proteins are constantly degraded and rebuilt (348); muscle mass is regulated by the balance between muscle protein synthesis (anabolism) and degradation (catabolism) (349). Changing the metabolic pathways in skeletal muscles toward more anabolism and less catabolism of proteins will be instrumental in improving muscle mass and function in the elderly with AD (22, 23). The main anabolic signaling pathway controlling skeletal muscle protein homeostasis is the stimulation of the mammalian target of rapamycin (mTOR) pathway, a highly conserved serine/threonine kinase system in mammals that is involved in many signal transduction pathways within the body, including regulating cell growth and homeostasis (131, 286). Factors such as nutrients, physical activity and anabolic hormones (e.g. growth hormones,

insulin like growth factor-1 (IGF-1) and androgens) are known stimulators of skeletal muscle protein synthesis via the mTOR pathway (350-352). Indeed, nutritional strategies are crucial parts of maintaining or enhancing muscle mass, and as such dietary supplements such as CR are studied for their ability to increase muscle mass and function.

CR, a nitrogenous compound, is endogenously synthesized from arginine and glycine or exogenously acquired through dietary sources, mainly from meat and fish, or supplement (274). A preponderance of evidence suggests that CR supplementation is a safe and effective strategy for attenuating muscle mass and strength losses in both health and disease conditions (269, 294, 353). CR has a vital role in energy metabolism of highly metabolically active tissues, particularly skeletal muscle, by maintaining a high cellular energy potential through ATP/ADP ratio in myofibers (354). In fact, more than 95% of the CR is stored in skeletal muscles (30). Ingestion of CR-rich foods or supplements help the body to compensate for CR loss, which occurs daily through the kidneys (31). CR supplementation has been shown to enhance skeletal muscle mass and function in older adults (302, 305) and muscular dystrophies (280, 353), however, this has yet to be studied in AD-related muscle dysfunction. Therefore, the overall objective of this study was to determine the effects of oral CR supplementation on muscle metabolism in the 3×Tg-AD mouse model. The specific objectives tested whether CR supplementation had any effect on: I) energy pool of the muscle cells, II) muscle protein and amino acid (AA) contents, III) muscle cell size and protein synthetic capacity, and IV) activation of elements of intracellular signalling pathways involved in the regulation of muscle protein synthesis.

6.2 MATERIAL AND METHODS

6.2.1 Animals and Diet

This study was approved by the University of Manitoba Animal Ethics Committee. Twenty-four 3×Tg-AD mice (mixed gender), about 8 months old at the start of the study, were assigned randomly to one of two treatment groups without control (CON, 0 CR) or with CR supplement (3%, w/w). For the CR group, CR monohydrate (Alfa Aesar, Ward Hill, MA, USA) was mixed with nutritionally complete semi-synthetic diet commonly used in nutrition research (**Table 6.1**).

Basal diet (g/kg)[†]	**CON**	**CR**
Casein	211.0	211.0
CR[¥]	0	30.0
Starch	283.5	283.5
Glucose (Dextrose)	200.0	193.0
Non-Nutritive Cellulose	50.0	50.0
Vitamin Mix	10.0	10.0
Mineral Mix	50.0	50.0
l-Cysteine	2.5	2.5
Choline Chloride	2.5	2.5
Inositol	2.5	2.5
Tert-butylhydroquinone	0.015	0.015
Canola Oil	165.0	165.0

Table 6.1. Experimental diets used in the study

[†]Diet Purchased from Dyets Inc. (Bethlehem, PA, USA).
[¥]CR is provided as CR monohydrate (Alfa Aesar, Ward Hill, MA, USA).

The 3×Tg-AD animals were obtained from an existing colony housed at the research lab of Albensi *et al*. (355). Animals were single-housed in standard cages at the O'Burrell laboratory (animal holding facility located at the St. Boniface Hospital Research/Albrechtsen Research

Centre, University of Manitoba) and were maintained on a 12-hour light/12-hour dark cycle at 22°C and humidity of 40%. During the 8-week period of the study, mice were fed an average of 6 grams feed/day. All animals had free access to drinking water throughout the study. Food intake was measured twice a week, and body weights of the animals were measured once a week during the course of the study. At the end of 8 weeks, all animals were anaesthetized with a mixture of ketamine (62.5 mg/kg intraperitoneal, IP) and xylazine (12.5 mg/kg IP), followed by decapitation.

6.2.2 Tissue Collection

Fast twitch fiber muscle groups from the upper limb (triceps) and lower limb (quadriceps, QM, and gastrocnemius, GM) were collected from the animals when they were sacrificed. Tissues were snap frozen in liquid nitrogen and stored at -80°C for further analyses of CR-associated muscle parameters. All samples were prepared in duplicates in each analysis.

6.2.3 Biochemical Analyses

6.2.3.1 Assessment of CR

Tissue CR concentration was determined by an enzymatic method using a kit (BioVision, Milpitas, CA, USA).

6.2.3.2 Protein, RNA and DNA determinations

Alkali-soluble protein, RNA and DNA were extracted from muscle tissues using methods from Forsberg *et al.* (356). Briefly, muscle tissue (~15 mg) was homogenized with 0.2 M cold perchloric acid and kept on ice for at least 10 minutes before centrifuging at 2800 × g for 5 minute. The supernatant was discarded, and the tight pellet was collected and re-suspended in 0.5 ml of 0.3 M sodium hydroxide and incubated for 1 hour in a 37°C water-bath and an aliquot used

for measurement of protein concentration, assessed by modified Bradford's method according to Kruger *et al.* (357). Briefly, 1 ml of Bradford Reagent (85% phosphoric acid, anhydrous ethyl alcohol, coomassie blue G and water) was mixed with 60 µl of 0.3 M sodium hydroxide and 60 µl of tissue sample homogenate, and the optical density (OD) of total protein was read at 450 nm and 595 nm.

For RNA determination, the remainder of the above 0.3 M sodium hydroxide aliquot of each tissue sample was mixed with 500 µl of 1 M perchloric acid and kept on ice for at least 30 minutes before centrifuging at 2800 × g for 5-minute intervals. The absorbance of the collected supernatant was read at 260 nm for RNA quantification, using a Thermo Scientific Nanodrop 2000 spectrophotometer (Wilmington, DE, USA). For DNA determination, the tight pellet was re-suspended in 500 µl of 2 M perchloric acid, and incubated for one hour in a 70°C water-bath, followed by centrifugation at 2800 × g for 5 minutes intervals. The absorbance of the collected supernatant was read at 260 nm for DNA using the Nanodrop.

6.2.3.3 Amino acid determination

AA concentrations in muscle tissue samples were determined by high performance liquid chromatography (HPLC) using the combined methods of Hariharan *et al.* (358) and Bidlingmeyer *et al.* (359). Briefly, 100 mg of tissue was homogenized in 3 ml of 2% perchloric acid and centrifuged at 3000 × g at 4°C for 15 minutes, and the collected supernatant was mixed with 3 ml of 2% perchloric acid, and this procedure repeated 3 times. To 3 ml of supernatant, 375 µl of 2 M potassium carbonate was added, centrifuged and filtered using 17 mm syringe filters. Fifty µl of 56 mM internal standard, norleucine was pipetted into 50 µl of each sample, and the mixture was vacuum-dried using the Speed Vac concentrator. The samples were vacuum-dried again after adding 10 µl of trimethylamine (TEA)-methanol-water (1:2:1, by vol).

To derivatize the samples, 20 µl of a fresh-made solution of phenyl isothiocyanate (PITC) reagent mix: water-TEA-ethanol-PITC (1:1:7:1, v/v), was added to each sample cocktail, vortex-mixed and allowed to stay at room temperature before vacuum-drying. The derivatized AAs were re-suspended in 500 µl of mobile phase A (0.14 M sodium acetate containing 0.5 ml/l TEA) adjusted to pH 7.5, and filtered into screw-top vials using 17 mm syringe filters and analyzed by HPLC system (Varian 920LC with ultraviolet detector, Agilent Technologies,Santa Clara, CA, USA). For each AA standard, 0.01 M solution was prepared and mixed isovolumetrically with 56 mM internal standard, norleucine. The same steps as the preparation of the samples were followed for the standards. Separation of AA was performed using 5 um ODS2, 250 × 4.6 mm Spherisorb column (Waters, Mississauga, Ontario, Canada) maintained at 35^{o}C. Mobile phases, A (0.14 M sodium acetate containing 0.5 ml/l TEA, pH 6.35), B (water) and C (acetonitrile), were set at a flow rate 1.0 ml/min with gradient program starting and ending at 97% phase A, 0% B and 3% C. Total run time for each sample analysis was 55 min.

6.2.3.4 Assessment of ATP and ADP

ATP and ADP were assessed by enzymatic methods using colorimetric assay kits (BioVision, Milpitas, CA, USA). Based on the supplier's instruction manual, samples were first deproteinized using deproteinization sample preparation kit from BioVision (Milpitas, CA, USA), followed by preparation of the samples and measurement of the OD at 570 nm.

6.2.3.5 Protein extraction and blotting

These procedures were performed as described previously by Doble and Kardami (360). Briefly, whole triceps muscle samples were grinded in liquid nitrogen using a mortar and pestle. Powdered samples were homogenized in an 350 µl ice-cold buffer consisting of (in mmol/l): 10 tris-hydrochloric acid (pH 7.4), 100 sodium chloride, 300 sucrose, 2 magnesium chloride, 1%

thiodiglycerol, 60 β-glycerophosphate, 10 sodium fluoride and 1:100 dilution of protease inhibitor cocktail (PIC), phosphatase inhibitor cocktails (PPICii and PPICiv) and 0.2 M sodium orthovanadate (NaOV) using a glass-Teflon homogenizer. After addition of equal volumes of 2X sodium dodecyl sulfate (SDS) buffer and inhibitors (PIC, PPICii and PPICiv) and boiling for 5 minutes, homogenates were centrifuged at 14,000 × g for 15 min at 4°C to remove residual material, the supernatants were collected, and the protein concentration was quantified using the bicinchoninic (BCA) assay. Proteins (25 μg/lane) were separated using SDS-polyacrylamide (7.5%) gel electrophoresis (SDS-PAGE) before electroblotting to polyvinylidene difluoride (PVDF) membrane. After blocking in 5% bovine serum albumin for 1 hour at room temperature, membranes were incubated overnight at 4°C for phospho-p70S6K Thr^{389} and total p70S6K (Cell Signaling, Danvers, MA, USA) antibodies. Anti-rabbit antibody was used as a secondary antibody, and the antigen-antibody complexes were visualized after adding an enhanced chemiluminescent (ECL-2) substrate. The quantification of immunoreactive protein bands was performed by densitometric analysis (BioRad, Mississauga, Ontario, Canada).

6.2.4 Statistical Analysis

GraphPad Prism software, version 6 (GraphPad Software, Inc., La Jolla, CA, USA) was used. Data were assessed by two-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc test to examine whether there were significant differences between CR-supplemented and CON groups and between females and males. The data were pooled and re-analyzed with student t-Test when there was no sex effect. All results are expressed as mean value ± SEM. Significance level was set at $p<0.05$ for all variables analyzed.

6.3 RESULTS

6.3.1 Body Weight and Feed Intake

Over the duration of the experiment, the body weights and food intakes of the male mice were significantly greater than those of the females; however, there were no significant differences in body weight and feed intake between CR supplemented and CON groups (**Table 6.2**). Overall, male mice gained between 8.6% and 9.2% body weight during the 8 weeks on the diets for the CON and CR supplemented group, respectively. Female mice on CON diet gained about 5.4% in body weight during the study, while those on CR supplementation loss about 3.6%, but this was not significant ($p>0.05$). All mice were in good health during the study, except for one male in the CR supplemented group that was euthanized at the end of week 7 due to prolapsed penis irresponsive to treatment, thus this animal was not included in the data analyses therefore only four CR supplemented males were available for this study.

Treatment group	**CON (n=12)**		**CR (n=12)**		**p-value (treatment effect)**	**p-value (sex effect)**
Gender / Parameter	**Female (n=6)**	**Male (n=6)**	**Female (n=8)**	**Male (n=4)**		
Initial body weight (g)	25.53 ± 1.89	37.35 ± 1.25	28.44 ± 1.52	35.98 ± 2.04	0.661	< 0.0001*
Final body weight (g)	26.65 ± 1.14	39.83 ± 2.06	27.60 ± 1.08	38.63 ± 3.21	0.943	< 0.0001*
Change in body weight (g)	1.39	3.23	-1.03	3.32	0.521	0.091
Daily feed intake (g/day)	3.19 ± 0.10	3.93 ± 0.13	3.80 ± 0.23	3.80 ± 0.23	0.558	< 0.0001*

Table 6.2. Body weights and feed intake of the animals

Data are presented as means ± SEM. Two-way Anova was used for data analysis.
*p-value<0.05 was considered significant.

6.3.2 Effect of CR Supplementation on Skeletal Muscles CR Levels

Supplementing 3×Tg-AD mice with CR at a dose of 3% (w/w) resulted in intramuscular increase in CR concentrations as seen in GM and QM (**Table 6.3**). On average, the concentration of CR in GM was 14% higher ($p<0.05$) in CR supplemented groups compared with CON. For the QM, CR concentration in the CR supplemented group only increased to 8% compared with CON ($p<0.05$).

Treatment group	**CON (n=12)**		**CR (n=12)**		**p-value (treatment effect)**
Gender / Parameter	**Female (n=6)**	**Male (n=6)**	**Female (n=8)**	**Male (n=4)**	
CR concentration (nmol/µl)					
Gastrocnemius muscle (GM)	0.27 ± 0.01	0.27 ± 0.01	0.32 ± 0.01	0.29 ± 0.01	0.002*
Quadriceps muscle (QM)	0.35 ± 0.01	0.33 ± 0.01	0.38 ± 0.02	0.34 ± 0.01	0.037*
ATP concentration (nmol/µl)	0.026 ± 0.010	0.049 ± 0.019	0.054 ± 0.023	0.048 ± 0.016	0.470
ADP concentration (nmol/µl)	0.090 ± 0.014	0.087 ± 0.019	0.097 ± 0.028	0.077 ± 0.007	0.227
ATP/ADP ratio	0.27 ± 0.10	0.32 ± 0.15	0.61 ± 0.19	0.59 ± 0.17	0.032*

Table 6.3. Skeletal muscle biochemistry: CR, ATP, ADP and ATP/ADP ratio in CON and CR supplemented 3xTg AD mice

Data are presented as means ± SEM. Two-way Anova was used for data analysis. No gender effect was observed.
*p-value<0.05 was considered significant.

6.3.3 Effect of CR Supplementation on Skeletal Muscles Protein Concentration and Cell Capacity for Protein Synthesis

Total alkali protein concentration, protein/DNA and RNA/DNA ratios in GM and QM are illustrated in **Figure 6.1**. The total alkali soluble protein concentration significantly increased ($p<0.0001$) in GM in response to CR supplementation (CR, F: 7.21 ± 0.16, M: 6.75 ± 0.19 vs. CON, F: 4.92 ± 0.30, M: 5.76 ± 0.46 mg protein/ mg wet tissue weight). In addition, muscle cell size, as measured by protein to DNA ratio, was greater in both GM ($p=0.002$) and QM ($p=0.005$) of CR supplemented groups (GM, F: 11.37 ± 0.74, M: 11.75 ± 1.55 and QM, F: 11.80 ± 0.52, M: 12.54 ± 0.50 mg protein/μg DNA) vs. CON (GM, F: 8.07 ± 0.41, M: 9.01 ± 0.76 and QM, F: 10.04 ± 0.50, M: 10.97 ± 0.41 mg protein/μg DNA). Furthermore, CR supplementation led to a significant increase ($p=0.001$) in RNA/DNA ratio, as an index of cell capacity for protein synthesis, in GM (F: 0.53 ± 0.04, M: 0.55 ± 0.09 in CR supplemented compared with F: 0.34 ± 0.02, M: 0.40 ± 0.03 in CON).

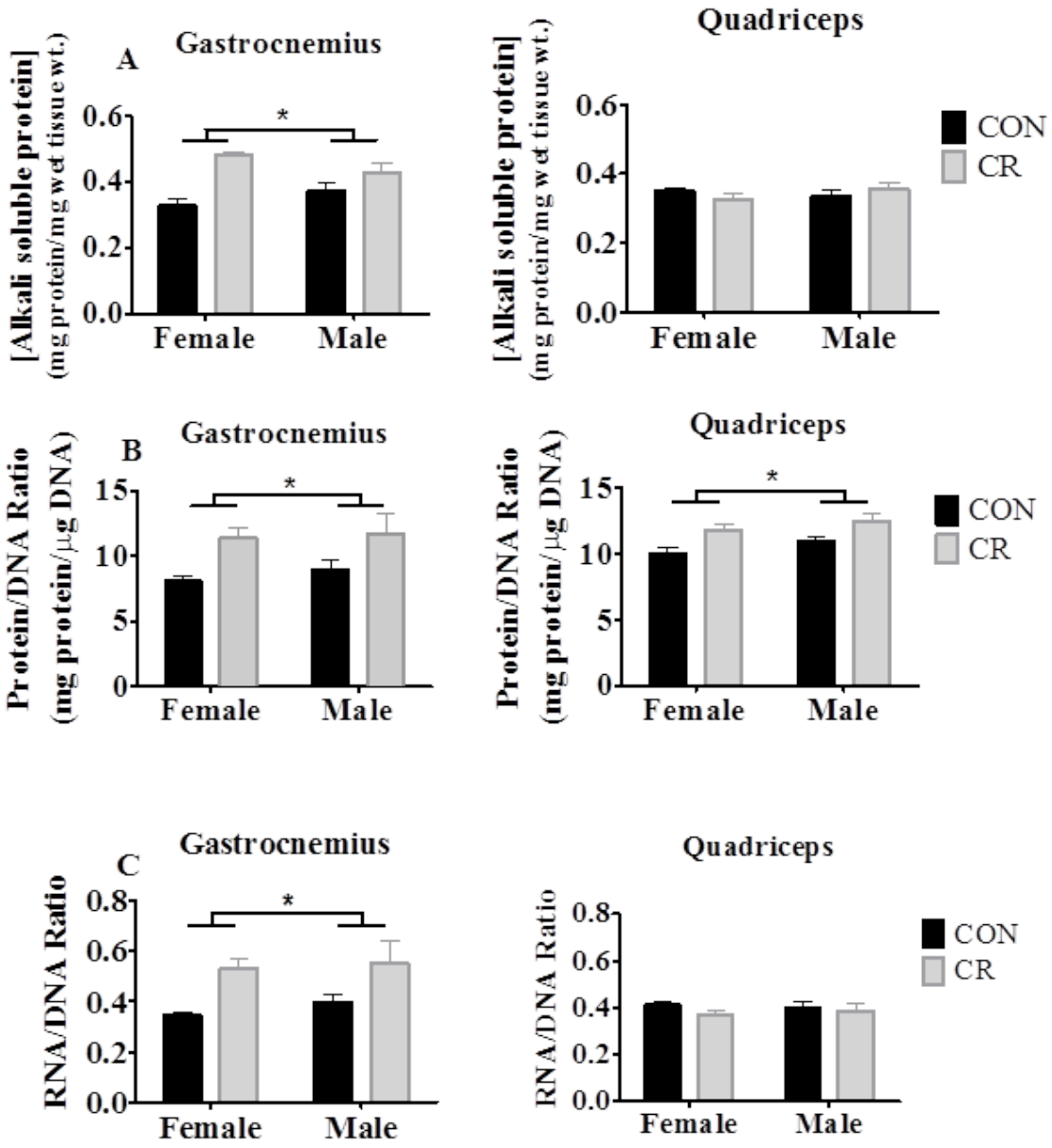

Figure 6.1. Muscle alkali soluble protein concentration (A), Protein/DNA ratio (B) and RNA/DNA ratio (C) in gastrocnemius (GM) and quadriceps muscles (QM) from 3xTg AD mice fed control (CON, n=12) and CR (n=12) supplemented diets, as indicated

Data are presented as means ± SEM. Two-way ANOVA was used for data analysis.
*$p<0.05$ compared between CR supplemented and CON.
wt. indicates weight; [ ] indicates concentration.

6.3.4 Effect of CR Supplementation on Skeletal Muscle AA Concentrations

Table 6.4 displays the AA concentrations (μmol/mg wet tissue) in QM in CR supplemented and CON groups.

Treatment group	**CON (n=12)**		**CR (n=12)**		
Gender / **AA (μmol/mg wet tissue)**	**Female (n=6)**	**Male (n=6)**	**Female (n=8)**	**Male (n=4)**	**p-value (treatment effect)**
Histidine	31.9 ± 6.0	37.0 ± 7.1	46.0 ± 3.5	53.6 ± 6.8	0.012
Valine	28.5 ± 2.9	35.7 ± 4.7	61.5 ± 2.8	62.2 ± 7.0	< 0.0001
Methionine	6.6 ± 1.7	5.8 ± 1.6	4.1 ± 0.7	11.2 ± 7.5	0.046
Isoleucine	18.5 ± 2.5	20.6 ± 2.4	24.9 ± 1.2	32.9 ± 2.5	0.001
Leucine	100.4 ± 16.8	122.0 ± 19.1	217.7 ± 11.4	218.4 ± 15.7	< 0.0001
Phenylalanine	18.8 ± 2.2	15.9 ± 1.3	16.5 ± 0.9	18.2 ± 2.4	0.404
Lysine	139.8 ± 11.2	144.0 ± 15.8	198.8 ± 12.8	182.6 ± 11.0	0.0004
Glutamic acid	6.2 ± 1.2	7.6 ± 2.5	12.5 ± 0.9	16.9 ± 3.3	0.0009
Serine	28.0 ± 7.6	36.2 ± 9.5	62.9 ± 6.8	64.2 ± 5.5	0.0002
Glycine	121.6 ± 31.1	136.5 ± 33.0	199.7 ± 9.8	220.6 ± 9.5	0.0014
Taurine	2307.4 ± 375.7	2783.2 ± 479.2	3414.6 ± 116.6	4160.7 ± 519.3	0.0027
Tyrosine	37.2 ± 7.7	34.2 ± 5.2	59.8 ± 7.3	66.6 ± 10.6	0.0008
Cysteine	9.9 ± 2.4	14.1 ± 3.0	27.2 ± 1.1	30.9 ± 2.1	< 0.0001
Aspartic acid	31.9 ± 4.9	29.0 ± 6.3	52.2 ± 2.6	55.9 ± 9.7	0.0002
Sum of BCAA	147.4 ± 20.2	178.2 ± 24.9	304.1 ± 13.5	313.5 ± 11.1	< 0.0001
BCAA/non-BCAA	0.056 ± 0.004	0.058 ± 0.004	0.074 ± 0.002	0.066 ± 0.007	0.0006
BCAA/Sum	0.053 ± 0.003	0.055 ± 0.003	0.069 ± 0.002	0.062 ± 0.006	0.0006
Sum	2870.5 ± 460.6	3410.3 ± 579.2	4396.8 ± 132.2	5201.9 ± 509.1	0.0007

Table 6.4. AA content of QM in CON and CR supplemented 3xTg AD mice

Data are presented as means ± SEM. Two-way Anova was used for data analysis. No gender effect was observed.
*p-value<0.05 was considered significant.

Following 8 weeks of CR supplementation, the concentrations of most AA significantly increased in the muscle, with an overall 50% enhancement ($p<0.05$) in total AA content in CR supplemented (4665 ± 209.9 μmol/mg wet tissue) vs. CON (3140 ± 362.1 μmol/mg wet tissue).

Specifically, significantly higher concentrations were found for almost all of the 14 individual AA analyzed; with the exception of phenylalanine, when comparing the CR supplemented vs. CON groups. In addition, the concentrations of all BCAA (leucine, isoleucine and valine) as well as the sum of BCAA increased (CR, 617.8 ± 16 vs. CON, 307.2 ± 9.5 μmol/mg wet tissue, $p<0.001$) as a consequence of CR supplementation. In particular, a profound and significant effect of CR on the level of leucine is noteworthy, showing an almost 2-fold increase ($p<0.05$) in CR supplemented (217.9 ± 8.8 μmol/mg wet tissue) vs. CON (111.2 ± 12.6 μmol/mg wet tissue). Taurine, which is the most abundant AA-like compound in the body and found in high quantity in muscles, also showed a 1.4-fold increase ($p<0.05$) in CR supplemented group (3663 ± 203.8 μmol/mg wet tissue) vs. CON (2545 ± 299.0 μmol/mg wet tissue).

6.3.5 Effect of CR Supplementation on the ATP/ADP Ratio of Muscle Cells

Comparison of absolute ATP and ADP concentrations in QM did not show any significant difference ($p>0.05$) between CR supplemented and CON groups (**Table 6.3**); however, CR supplementation significantly altered the energy status of the skeletal muscle by enhancing ($p<0.05$) the ATP to ADP ratio in CR supplemented group (0.60 ± 0.12) vs. CON (0.29 ± 0.08).

6.3.6 Effect of CR Supplementation on Phosphorylation Levels of Protein Synthesis Signalling Components: p70S6K

Relative levels of phosphorylated (phospho-threonine 389)- p70S6K, serving as an indicator of mTOR pathway activation, were not significantly different in male (0.34 ± 0.04, p=0.292) and female (0.19 ± 0.03, p=0.161) CR supplemented groups compared with CON (M: 0.30 ± 0.04 and F: 0.15 ± 0.02, **Figure 6.2A and 6.2B,** respectively) in triceps.

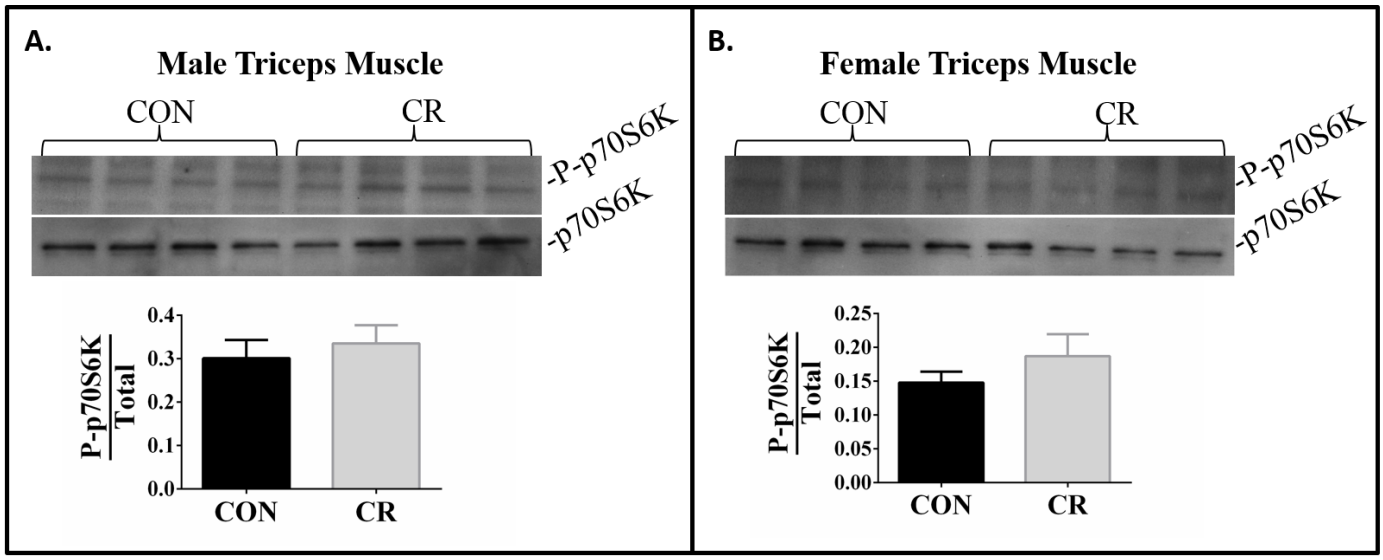

Figure 6.2. Relative levels of phosphorylated (P) p70S6K over total p70S6K, in (A) male, and (B) female triceps muscle, from 3xTg AD mice fed control (CON) and CR supplemented diets, as indicated. Each panel shows images of western blots for P-p70S6K, total p70S6K, and corresponding graphs (n=4), Bars show SEM

Data are presented as means ± SEM. T-test was used for data analysis. P-p70S6K; phosphorylated level of p70S6K.

6.4 DISCUSSION

The main findings of our study are that oral CR supplementation for 8 weeks to middle age (~8 month old) 3×Tg-AD mice significantly enhances intramuscular CR content, leading to improved myocellular energy potential, as measured by increased ATP/ADP ratio, and improved skeletal muscle hypertrophy and anabolism, as seen by: 1) increased muscle cells size, as measured by protein/DNA ratio, 2) enhanced RNA/DNA ratio (an index of skeletal muscle cell protein synthetic capacity); however western blot assessment of triceps muscle indicates this process was not associated with increased activation (phosphorylation) of p70S6K in the mTOR pathway, and 3) increases in muscle protein and free AA concentrations, notably BCAA. Overall this study indicates that CR supplement may improve skeletal muscle metabolism in middle-aged AD mice.

In this study, skeletal muscle CR content was increased up to a 14% in the CR group compared to CON. This result is in agreement with other studies that have shown that CR supplementation elicits an enhancement in CR content in skeletal muscles of both human and animals (331, 361-363). This increase in CR content in skeletal muscle can be up to 50% in humans (331, 361) and 55% in animals (364, 365), depending on the pre-supplementation concentration and type of muscle examined (331, 363, 364, 366). Having a higher proportion of slow-oxidative (Type I) than fast-glycolytic (Type II) fibers in skeletal muscles increases the likelihood of intramuscular CR accumulation as a result of CR supplementation due to lower total CR content and breakdown rate during muscle contraction in slow-oxidative fibers (363, 366, 367). The muscle groups that were used in this study consisted mostly of fast twitch (Type II) muscle fibers, which may partly explain the lower increase in muscle CR concentration observed in this study compared with other studies (363, 368). In addition, the 3xTg-AD animal

model used in this study is known to exhibit alterations in skeletal muscle metabolism (20) which have affected the level of responsiveness to CR ingestion, however this was not tested in this study.

The results of our study are indicative of an increase in protein synthetic capacity in muscle cells as well as an enhancement in cell protein content as a result of long-term CR ingestion. Increased protein may be the consequence of not only increased protein synthesis but decreased protein degradation, i.e. slower turnover (349). Having increased in both RNA/DNA ratio, an index of cell protein synthetic capacity, and protein content of muscle cells, CR possibly stimulates the production of proteins inside the cells by a mechanism that requires further investigation. The efficacious impacts of CR supplementation on muscle cells extends to an increase in muscle cell size as measured by protein/DNA ratio, providing more evidence regarding the favorable effects of CR ingestion on muscle mass and hypertrophy. The impact of CR supplements on enhancing muscle mass has been scrutinized in a large body of literature, showing that CR can enhance muscle mass in the presence and absence of exercise, in younger and older adults, and in health and disease conditions (32-34). Although studies on the impact of CR ingestion on muscle without performing physical activity is equivocal, even in the absence of exercise, CR has been shown to be capable of enhancing muscle mass by stimulating the anabolic pathways in this tissue (33, 34), which concurs with the results in our study.

The precise mechanism by which CR exerts an anabolic stimulus is not clearly understood, however, it has been shown that CR can promote the differentiation of myogenic cells through activation of mTOR signalling pathway (35). Therefore, CR may be effective in stimulating protein synthesis inside skeletal muscle cells. For instance, *in vitro* studies by Louis *et al.* (326) and Deldicque *et al.* (35) indicated that addition of CR to C_2C_{12} murine skeletal

myogenic cells lead to hypertrophy and promotion of differentiation of myogenic cells through activation of protein kinase B (Akt/PKB) and p70S6K, two key proteins involved in mTOR anabolic signalling pathway. However, the results of this study indicate that the difference between the activation levels of p70S6K in CON and CR groups was not significant. Since p70S6K is a very sensitive signalling pathway in the myocytes and can be affected by various stimuli at different time points (369, 370), this could be a possible explanation why we did not observe a significant difference at the time point used here. Future studies might address the state of p70S6K phosphorylation at various time points following CR supplementation.

To our knowledge, the current study represents the first report of the effect of CR supplementation on skeletal muscle AA profile. However, other studies have reported skeletal muscle AA profile response to dietary supplementations (371). Ishikura *et al.* (371) did not reveal any significant change in the concentration of most AA in skeletal muscles of rats supplemented with taurine for 2 and 3 weeks (371). However, the type of supplement, the duration of the study and the animal model that was used in that study was different from the current study. Overall, assessment of intramuscular AA are important for assessment of skeletal muscle AA metabolism as shown in our study, which is not possible to draw conclusions based only on plasma AA concentrations (372). The BCAAs, aspartic acid, glutamic acid and alanine (not measured in this study) are the only AA metabolized in skeletal muscle, thus significant increases in their intramuscular concentrations could be due to higher uptake or lower release from skeletal muscle (192, 371), the processes of which are tightly regulated (373).

While we could not measure muscle mass in our study through standard body composition assessment methods such as dual energy x-ray absorptiometry, we indirectly showed that CR was effective in increasing muscle fiber size by at least increasing protein

content of the muscle cells. This effect could partially be due to altered energy status in the muscle; the ATP/ADP ratio increased ($p<0.05$) in the CR supplemented group compared with control littermates. In the cytosol of the cells, creatine kinase (CK) transfers the high-energy bond phosphate group from cellular energy production site (mitochondria) to CR to form phosphocreatine (PCr), which serves as a spatial energy buffer for the cells that can react with ADP to produce ATP (294, 374). Needless to say, the energy derived from hydrolyzing ATP provides the driving force for various functions of the cells, including protein synthesis pathways (333, 334). The storage capacity of PCr-CK system in skeletal muscles is quite small, however this capacity can be increased by oral ingestion of CR supplement (331).

In conclusion, the results of our study showed that CR supplementation in a 3×Tg-AD mouse model significantly increased intramuscular CR concentration in fast twitch skeletal muscle fibers, resulting in increases in: muscle cell size, the capacity of the muscle fibers for protein synthesis, muscle total protein and AA contents, and improved energy status of the skeletal muscle cells. Emerging research such as the study by Monteiro-Cardoso *et al.* (20) shows that the pathology of AD significantly contributes to skeletal muscle dysfunction in AD. They showed that muscle dysfunction occurs at early stages of AD and results in loss of muscle mass, strength and function. In the present study, we showed that CR supplements could be beneficial to those with AD to favourably enhance muscle growth parameters. CR has been shown to enhance cognitive function in healthy humans and animals and has been tested as a putative therapy in other neurodegenerative disorders (375-377). The advantageous effect of CR on muscle, including protein and AA levels, couple with its reported cognitive enhancing capabilities, suggest it could be a promising treatment for AD. Nevertheless, the co-impact of

exercise and physical stimulus and the effect of CR supplementation on muscle mass, strength and function in AD associated muscle dysfunction are areas requiring further investigations.

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CHAPTER VII. OVERALL CONCLUSION, LIMITATIONS AND FUTURE DIRECTIONS

7.1 SUMMARY AND IMPLICATIONS

The research on AD is mainly focused on cognitive dysfunction and memory loss; however, AD is a systemic disease with broader manifestations. Muscle dysfunction even occurs at early stages of AD and results in loss of muscle mass, strength and function (14). Since muscle dysfunction is a feature of AD it is important to investigate these muscle abnormalities as they may have a huge impact on AD patients. Skeletal muscle tissues, which comprise 40-50% of the body's weight (40), are responsible for mobility and some metabolic functions. Hence, abnormality and dysfunction of skeletal muscles may lead AD patients to become more dependent on others for performing activities of daily living and consequently results in greater burden, including health care costs. Therefore, strategies aimed at attenuating or reversing derangements in skeletal muscles are critical for AD patients. One of these strategies is CR supplementation, which may affect the protein anabolic signalling pathway of the cells. Since it has been shown that CR can also be beneficial for cognitive function of AD patients, the advantageous effect of CR on muscle protein and AA levels as well as protein synthetic pathways would be promising in the treatment of AD.

In conclusion, the results of our study showed that CR supplementation in a 3×Tg-AD mouse model significantly increased intramuscular CR concentration in fast twitch skeletal muscle fibers, resulting in increases in: muscle cell size, the capacity of the muscle fibers for protein synthesis, muscle total protein and AA contents, and improved energy status of the skeletal muscle cells as well as activation of p70S6K which is involved in protein synthesis pathway.

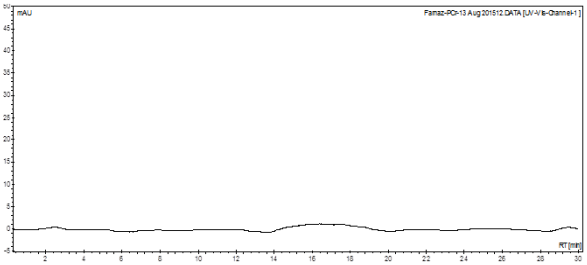
7.2 LIMITATIONS

This study had a number of limitations:

1) We could not include any exercise protocol in our study. This study was done in collaboration with other researchers from Albrechtsen Research Centre and Richardson Centre for Functional Foods and Nutraceuticals whose primary interest was to investigate the effect of CR supplement on cognitive function in 3xTg-AD mice. The assessment of CR supplementation on skeletal muscle was a secondary investigation. Therefore, it was not feasible to incorporate any exercise or functional tests in the study.
2) Assessment of body composition particularly muscle mass is one of the objectives we could not test due to limitation in accessing machinery such as dual-energy X-ray absorptiometry (DXA) at the time of the study.
3) One of our primary objectives was to measure both CR and PCr inside the muscles. This could have been very helpful in understanding the cellular energy status and spatial energy pool after CR supplementation. However, due to a number of technical problems that occurred during the course of the study, we could not get reliable results from the PCr assay, therefore we were not able to include the data of this assay in our result section. The assay method we attempted to use for this study was an HPLC based assay that was a modification of previously reported methods of Zemtsov *et al.* (378) and Sellevold *et al.* (379). Briefly, muscle tissue samples were homogenized in ice-cold 6% perchloric acid. After centrifugation of the homogenates, the pH of the supernatants was raised to 5-7 using 3.75 M potassium carbonate. Filtered supernatant were analyzed by HPLC system (Varian 920LC with ultraviolet

detector, Agilent Technologies, Santa Clara, CA, USA). Unfortunately, we had problems with reproducibility with the standards (**Figure 7.1**). In addition, we were unable to detect PCr in some of our samples, which might be a consequence of a long period of time samples stayed in the freezer or any delay in freezing the samples at the time of tissue collection.

1-PCr standard, 0.04 mg/ml, 206nm



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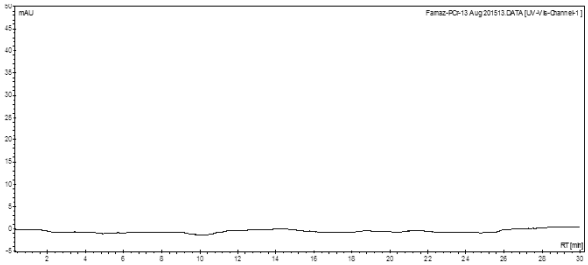

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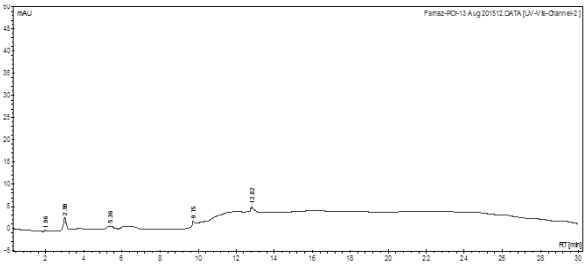

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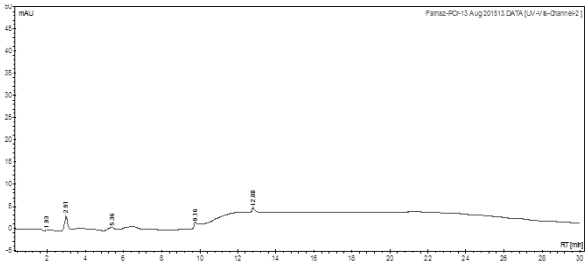

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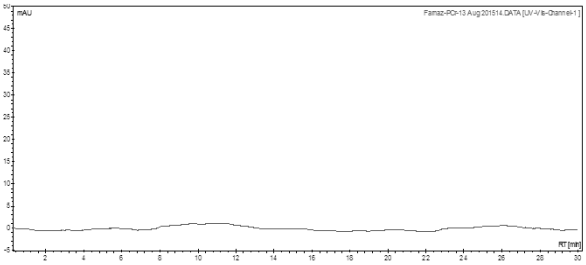
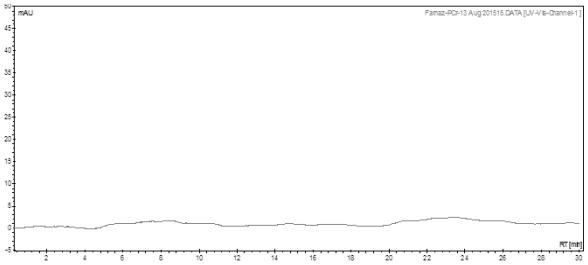
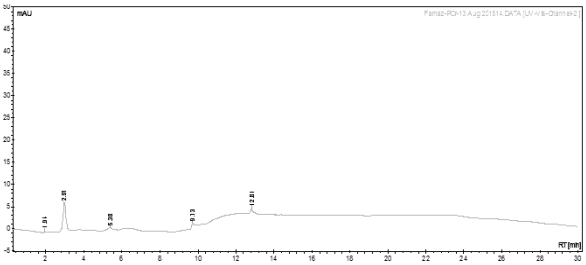


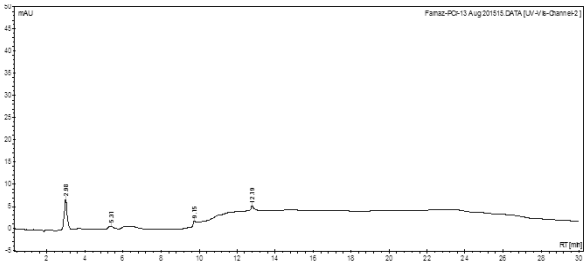
2D-PCr standard, 0.1 mg/ml, 206nm



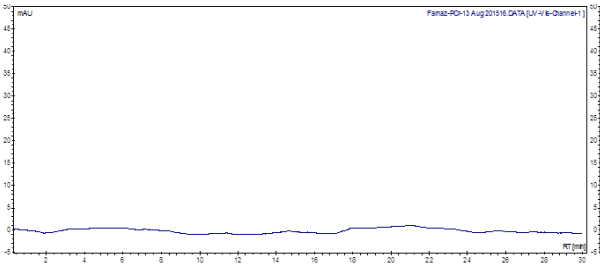
2-PCr standard, 0.1 mg/ml, 259nm



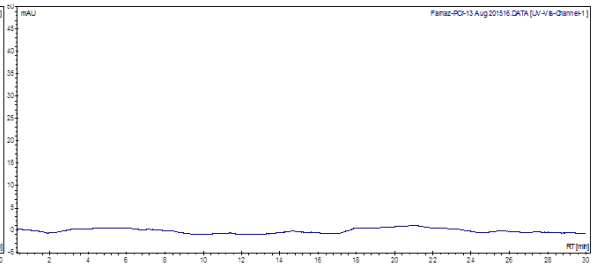
2D-PCr standard, 0.1 mg/ml, 259nm



3-PCr standard, 0.2 mg/ml, 206nm



3D-PCr standard, 0.2 mg/ml, 206nm



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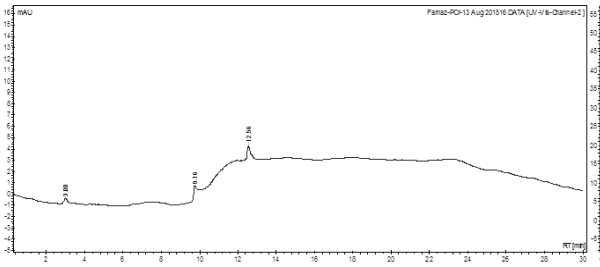

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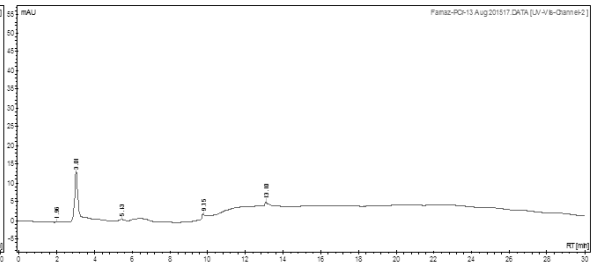
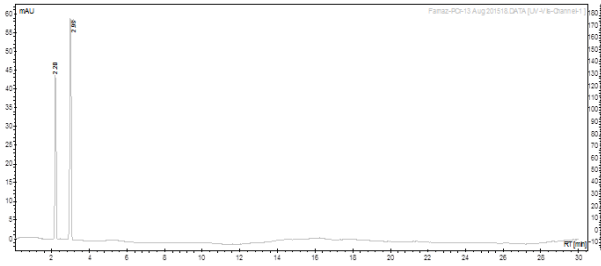
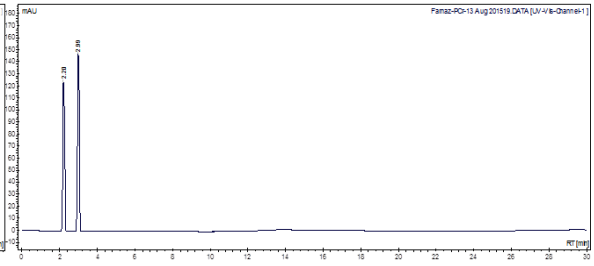


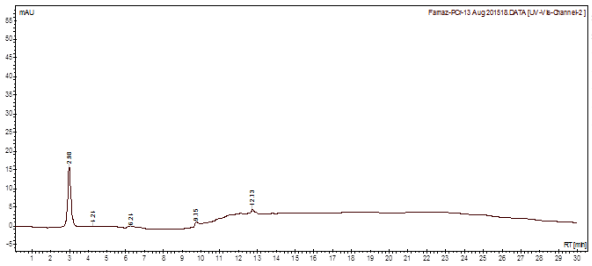
4-PCr standard, 0.4 mg/ml, 206nm



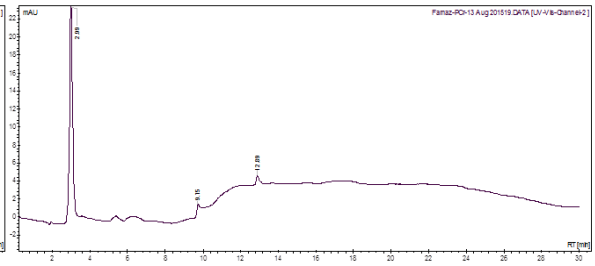
4D-PCr standard, 0.4 mg/ml, 206nm



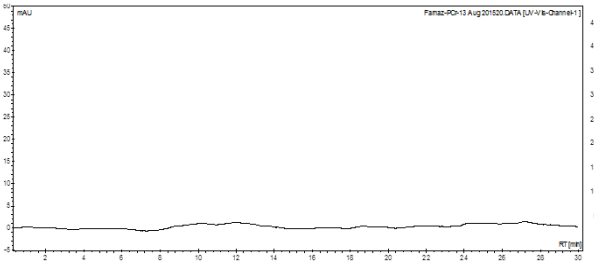
4-PCr standard, 0.4 mg/ml, 259nm



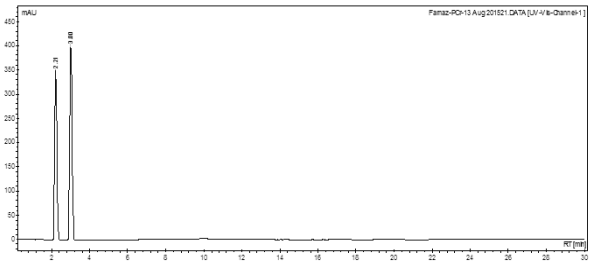
4D-PCr standard, 0.4 mg/ml, 259nm



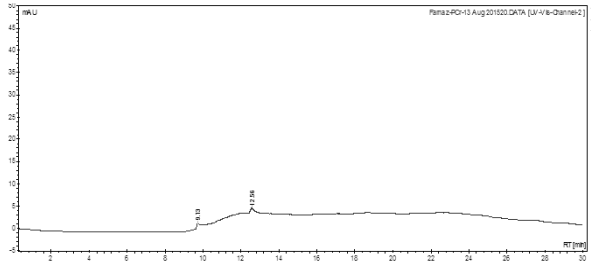
5-PCr standard, 0.8 mg/ml, 206nm



5D-PCr standard, 0.8 mg/ml, 206nm



5-PCr standard, 0.8 mg/ml, 259nm



5D-PCr standard, 0.8 mg/ml, 259nm

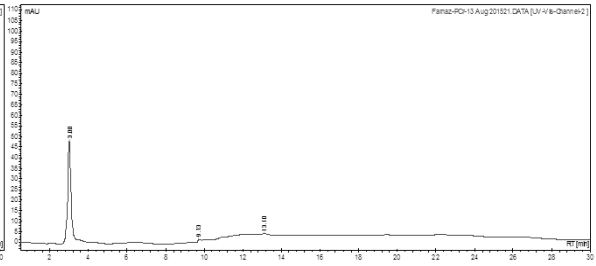


Figure 7.1. Chromatograms of PCr standard solutions with gradient concentrations.

Five standard concentration were used to establish standard curve: 0.04, 0.1, 0.2, 0.4 and 0.8 mg/ml. Light absorbance were read at 206 and 259 nm.

4) This study was conducted on an animal model for AD. Although this animal model mimics some of the more recognized signs, symptoms and pathogenesis of AD, there are definitely lots of differences with AD in humans (380).
5) Assessing the phosphorylation/activation level of elements of intracellular signalling pathways involved in the regulation of MPS was one of our specific objectives in this study. Although we were able to check this level for p70S6K, similar results could not be achieved for mTOR, since no P-mTOR was detected for most samples in western blotting. This could be as a result of general dephosphorylative state due to inappropriate sample freezing. Also, reactivity of the antibodies varies between species and use of an antibody with more reactivity with mouse tissue could possibly resolve the problem.

7.3 FUTURE DIRECTIONS

1) Future studies evaluating the effect of CR supplementation on muscle metabolism in AD with inclusion of exercise training is necessary to assess the combined impact of CR and exercise. Performing muscular functional tests in future investigations would allow researchers to examine any change in skeletal muscles functional capacity in response to CR ingestion.

2) Integration of lean body mass evaluation should be an essential part of future studies in order to assess if metabolic changes observed at the cell level is translatable to any change in body mass and function.
3) As like other animal researches, the results of this study should be confirmed in human subjects.
4) Our primary objective in this study was to evaluate muscle metabolism. However, we were also enthusiastic to see whether CR supplementation increased in other tissues such as kidney, liver and heart. CR concentration enhanced significantly in liver (CR, F: 0.20±0.04, M: 0.14±0.07 and CON, F: 0.004±0.0006, M: 0.02±0.01 nmol/µl, $p<0.05$) and heart (CR, F: 5.08±0.19, M: 5.21±0.18 and CON, F: 4.57±0.19, M: 4.61±0.26 nmol/µl, $p<0.05$) in animals supplemented with CR vs. CON. Nevertheless, the kidney's CR content in CR did not reveal any difference compared to CON ($p=0.9$). Future research should look into creatine metabolism in other tissues in order to compare it with skeletal muscle and to investigate whether there is any beneficial or detrimental effect of creatine on these tissues.

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