# PEANUT PROTEIN SUPPLEMENATION TO AUGMENT MUSCLE GROWTH AND IMPROVE MARKERS OF MUSCLE QUALITY AND HEALTH IN OLDER INDIVIDUALS

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## ABSTRACT

It is generally accepted that resistance training (RT) in conjunction with protein supplementation has positive effects on strength and muscle mass in older individuals. However, to date, no study has examined the effects of a RT program in combination with a high-protein, defatted peanut powder (PP) supplement on these markers. Herein, 39 older, untrained individuals (n=17 female, n=22 male; age=58.6±8.0 years; body mass index =28.7±5.8) completed a 6-week (n=22) or 10-week (n=17) RT program, where full-body training was implemented twice weekly. Participants were randomly assigned to consume either a PP supplement shake once per day (35 g protein, 315 kcal; n=20) or no supplement (CTL; n=19). Right leg vastus lateralis (VL) muscle biopsies were obtained prior to and 24 hours following the first training bout in all participants to assess changes in myofibrillar protein synthetic rates (MyoPS) as measured via the deuterium-oxide ($D_2O$) tracer method. All participants also completed PRE- and POST-intervention testing including dual energy x-ray absorptiometry (DXA), VL ultrasound, mid-thigh peripheral quantitative computed tomography (pQCT) scan, and right leg strength assessment using an isokinetic dynamometer. There was a significant group-by-time interaction for protein consumption when cohorts were pooled ($p=0.008$), and a trend toward significance when cohorts were examined individually as a 10-week ($p=0.086$) and 6-week ($p=0.072$) cohort. MyoPS rates were not significantly different between supplement groups following the first workout bout. Regarding chronic changes, there were no significant group-by-time interactions ($p<0.05$) in DXA-derived fat mass, lean soft tissue mass (LSTM) or percent body fat between supplementation groups. There was, however, a significant increase in VL thickness for PP versus CTL participants when the 6- and 10-week cohorts were pooled (interaction $p=0.041$). There was also a significant increase in knee flexion torque in the 10-week PP group versus the

CTL group (interaction p=0.032). In conclusion, a high-protein, defatted peanut powder supplement in combination with RT may have beneficial effects on select indices of muscle hypertrophy and strength in an untrained, older adult population.

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## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| RT | Resistance training |
| PCS | Protein-containing supplement |
| PP | Peanut-derived protein |
| MyoPS | Myofibrillar protein synthesis |
| SARS-CoV-2 | Severe acute respiratory syndrome coronavirus-2 |
| SkM | Skeletal muscle |
| MyoPB | Myofibrillar protein breakdown |
| 10W | Ten-week |
| 6W | Six-week |
| V1 | Visit one |
| V2 | Visit two |
| PRE | Visit two |
| V3 | Visit three |
| CTL | Wait-list control |
| V4 | Visit four |
| V5 | Visit five |
| V23 | Visit twenty-three |
| V15 | Visit fifteen |
| V24 | Visit twenty-four |
| POST | Visit twenty-four |
| V16 | Visit sixteen |
| POST | Visit sixteen |

# CHAPTER 1: INTRODUCTION

## Aging Defined

Aging has been defined previously as a time-dependent functional decline that affects most living organisms at the cellular, tissue, and organ level (Fedarko, 2011; López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013). This definition presents aging as a construct whereby an organism will experience irreversible deterioration of function over an unspecified amount of time. As such, this definition depicts aging as a wholly negative process. However, this is not the case. Indeed, there are periods of rapid growth and development seen early in life that represent components of aging critical to both health and longevity. Therefore, perhaps a better definition of aging is that it is the totality of changes that occur during an organisms' lifespan (Kirkwood, 2005). This view also has shortcomings in that it fails to take into account the fact that the rate at which changes occurs in each individual varies (da Costa et al., 2016). While these changes can occur rapidly or slowly, these changes can additionally stem from both internal and external factors. For example, puberty and the onset of sexual maturation is a hallmark of the aging process that is predominantly driven by a cascade of endocrine changes. Puberty has been deemed physiological when its onset begins between the ages of eight and twelve years in females, and between the ages of nine and fourteen years in males (Rosenfield, Lipton, & Drum, 2009). However, studies conducted in the United States and Europe have shown a trending decrease in the average age of the onset of puberty in females in the last century (Wyshak & Frisch, 1982). Improvements in health, such as better nutrition and increased access to healthcare, have been posited to be at least partially responsible for the reduced age of sexual maturation (Euling et al., 2008). Regardless the cause, it is clear that external forces, for example food choice and security, can play a pivotal role in the aging process.

Further supporting evidence as to the role that external forces play on the aging process have been observed in studies in identical twins raised separately after birth. These studies have indicated that lifestyle choices and habits can exert significant effects on overall health and the aging process (Antell & Taczanowski, 1999). In a retrospective case study on monozygotic twins separated five months after birth and raised apart, Segal and colleagues observed marked differences in self-reported health (Segal et al., 2015). Although some health outcomes were shared (each had a penicillin allergy), some health outcomes were not (only one developed non-Hodgkin's lymphoma). Despite being genetically identical, this suggests that genetic influences (internal forces) on aging may be overrated and the effects of lifestyle (external forces) are perhaps more important (Antell & Taczanowski, 1999). Because outside forces have been observed to affect the aging process, it can thus be argued that aging is not simply a time-dependent functional decline. Therefore, I propose a more apt definition of aging: it is the result of natural physiological processes interacting with external forces and insults in a variable and disproportionate fashion that culminate in a time-dependent functional decline unique to each individual. Based on this definition of aging, there is no singular aging phenotype. Rather, there are consistent and predictable themes of the aging process. These themes have been the target of many interventions aimed at hindering the aging process.

Because aging is associated with negative health status and a diminished quality of life, for millennia there has been an interest in interventions aimed at delaying the aging process (Cornelissen & Otsuka, 2017). From the works of Herodotus in the 5$^{th}$ century BCE, to Alexander the Great in the 3$^{rd}$ century CE and the travels of Ponce de León in the 16$^{th}$ century CE, there has indeed been a fascination in the search for treatments that reverse the deleterious effects of age. Despite these early historians, conquerors, and travelers being unsuccessful in

their search, the desire to avoid or counteract the process of aging has nonetheless persisted. In 1797, physician Christopher Hufeland published the groundbreaking *Makrobiotik oder Die Kunst, das menschliche Leben zu verlängern* (*Macrobiotics or The 'Art of Prolonging Human Life'*). Already well ahead of his time, Hufeland recognized the importance of both diet and diurnal rhythms on the human lifespan In his manuscript, Hufeland identified two components key to prolonging human life: diet composition and circadian rhythms, which he referred to as "the unity of our natural chronobiology". However, it is not enough to simply increase the human lifespan by delaying the aging process if health also deteriorates. An increased lifespan without a concomitant maintenance of health creates an untenable situation of an ever-decreasing quality of life. In this regard, a focus on successful aging should be the pursuit of any intervention aimed at delaying the aging process. Both the Berlin ageing study (Baltes, Mayer, Helmchen, & Steinhagen-Thiessen, 2008) and the US MacArthur study of ageing (Tabbarah, Crimmins, & Seeman, 2002) have shown that increased longevity in the US and Europe have resulted in fewer years of disability. This indicates that health is preserved longer and later in life for the current generations as compared to previous decades. This is likely due to a greater percentage of these populations having access to better nutrition and health care.

Successful aging, however, cannot be defined simply based on disability. Health encompasses both psychosocial and biomedical constructs. As such, any intervention aimed at improving the aging process must address both constructs as well. From a biomedical approach, successful aging can be loosely defined as "an optimization of life expectancy while concomitantly reducing physical and mental deterioration" (Bowling & Dieppe, 2005). Although the focus of this approach is the absence and/or reduced risk of disease, the importance of independent physical and mental functioning is not overlooked. Conversely, whereas a

biomedical approach views aging through the lens of disease reduction, physical reduction, and mental function reduction, the psychosocial approach emphasizes life satisfaction, social participation and function, and psychosocial resources as measures of successful aging (Bowling & Dieppe, 2005). Under the psychosocial approach, a life free of disease is no healthier than that in which life satisfaction is nonexistent. As such, any intervention that utilizes an overlap of the two approaches, whereby both biomedical and psychosocial constructs are high, is a better assessment of successful aging than simply "diseased" or "not-diseased".

## The Foundations of Aging

Aging is a multifactorial and complicated process. As such, its cause cannot be determined from any single outcome metric. Rather, aging and its associated origins must be viewed as a combination of insults coupled with normal physiologic processes affected by external forces that manifest in a unique and specific phenotype for each individual. Therefore, there is no singular cause of aging. There are, however, hallmarks that contribute to the aging process and its associated negative outcomes on health and longevity. These hallmarks can act individually, collectively, and synergistically to accelerate the aging process. With better understanding of these hallmarks we may be better equipped to treat them through targeted interventions.

### *Genomic Instability*

In 1944, Avery, McCarty, and Macleod first postulated that deoxyribonucleic acid (DNA) was the primary means of transferring genetic information between bacteria strains (Avery, Macleod, & McCarty, 1944) via 4 monomeric nucleotides comprising base pairings. This initial theory laid the groundwork for subsequent researchers to expand our understanding of DNA as a genetic storage device. Later, in 1951, Chargaff and colleagues followed the work of Avery,

McCarty, and Macleod by proposing the basic tenet of DNA in which the double-stranded molecule must contain an equivalent paring of A and T and G and C nucleotide bases (Chargaff, Lipshitz, Green, & Hodes, 1951). This led to Watson and Crick's groundbreaking paper '*Molecular structure of nucleic acids; a structure for deoxy ribose nucleic acid*' (Watson & Crick, 1974) in which they posited the basic structure of the anti-parallel double-helix DNA molecule. These insights collectively serve as our fundamental basis for genetics and provide an understanding as to how four individual bases can be sequenced into a genetic blueprint for human life that is conserved across individuals within the genome of a cell's nucleus. The genome serves to direct all aspects of life. As such, even the slightest perturbations can have far-reaching effects.

Despite the relative simplicity, the stability and integrity of DNA is under a constant barrage of internal and external insults and disruptions (Hoeijmakers, 2009), such as replication errors and ultraviolet radiation exposure (Chatterjee & Walker, 2017). Fortunately, humans have evolved a series of DNA repair mechanisms (Lord & Ashworth, 2012), such as alkyltransferase enzymes and base excision repair during the initial stages of cellular division (Chatterjee & Walker, 2017), that function to negate, counteract, or remove harmful disruptions to DNA. Despite these repair mechanisms, given the length of DNA strands (roughly three billion bases per strand) and the ongoing continuous replication of cells, genetic lesions do still occur (López-Otín et al., 2013). These lesions can manifest as mutations and deletions in the specific DNA sequence, both nuclear and mitochondrial (Park & Larsson, 2011), and as defects and disruptions of the nuclear architecture (Dechat et al., 2008; Worman, 2012), each of which can alter the unique genetic blueprint and exacerbate the normal aging process. Although the vast majority of DNA is made-up of non-coding sequences (i.e. sequences that do not encode for proteins and is

believed to have minimal biologic purpose), lesions occurring on specific gene sequences can have serious negative health consequences. For example, sickle cell anemia results from a single base pair lesion and significantly reduces the ability of blood to deliver oxygen to tissues. In addition, because muscle cells are multinucleated, they are thus exponentially more likely to suffer from lesions and derangements of the genome. As such, the accumulation of genetic damage and the resultant genomic instability across the lifespan is a common denominator of the aging process (Moskalev et al., 2013).

### *Telomere Attrition*

Telomeres are repetitive TTAGGG sequences of DNA that make up the terminal ends of chromosomes (Chilton, O'Brien, & Charchar, 2017; Shay, 2016). Chromosomes house genetic information in the cell's nucleus in the form of genes and must be replicated during cellular reproduction if genetic information is to be conserved. Telomeres serve as a protective endcap to prevent the enzymatic degradation of the encoding genetic DNA sequence during cellular replication (Chilton et al., 2017). Although replicative DNA polymerases (enzymes that synthesize DNA from nucleotide bases) can replicate the interior gene-encoding DNA sequences, these polymerases lack the ability to fully replicate the terminal telomere endcap (López-Otín et al., 2013). This process is unique and reserved for the telomerase enzyme. Because most mammalian somatic cells do not express the telomerase enzyme (López-Otín et al., 2013), this results in an ever-shortening DNA strand with each successful replication. Once enough replications have occurred to exhaust the protective DNA endcap (i.e. telomeres), this exposes genomic DNA to enzymatic degradation (Chilton et al., 2017). This subsequently results in lesions, deletions, and general instability of the genetic sequence. Again, this can have profoundly negative effects on health and longevity. Telomere attrition and eventual exhaustion

also helps to explain the limited replicative capacity of cells. In addition, because of its protective role, when the telomere has been shortened enough DNA is susceptible to degradation and lesion. This is believed to strongly contribute to the biological aging of organisms (Chilton et al., 2017) through a severely reduced regenerative capacity across the lifespan.

### *Epigenetic Alterations*

Unlike the genomic instability of alterations to DNA strands themselves, epigenetic alterations result from changes within the chromosome that do not directly affect the DNA sequence (Gonzalez-Suarez et al., 2009). Rather, these alterations manifest from changes in DNA methylation patterns, post-translational modification of histones, and chromatin remodeling (López-Otín et al., 2013). Collectively, epigenetic perturbations have been shown to affect the aging process in a multitude of ways. For instance, nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylases and adenosine diphosphate (ADP)-ribosyltransferases, both members of a family of proteins collectively referred to as sirtuins (SIRT), are known to alter DNA through histone (i.e. the protein DNA coils around) modifications and side-chain additions. Because DNA strands must coil tightly to conserve precious space and function, any histone modification or side-chain addition would thus affect DNAs ability to coil. Depending on where the modification or addition occurs this may result in disturbed or altered gene expression. As such, SIRT proteins and their effects on DNA gene expression have been studied as potential anti-aging factors in flies (Rogina & Helfand, 2004), worms (Tissenbaum & Guarente, 2001) and yeast (Kaeberlein, McVey, & Guarente, 1999). In mammals, studies have also shown that several members of the SIRT family can delay various aspects of aging (Houtkooper, Pirinen, & Auwerx, 2012; Sebastián, Satterstrom, Haigis, & Mostoslavsky, 2012). Of particular interest are the mechanisms of SIRT1, SIRT3 and SIRT6. The mechanisms underlying the beneficial effects

of these SIRT proteins are believed to involve improved genomic stability (Oberdoerffer et al., 2008; R. H. Wang et al., 2008), enhanced metabolic efficiency (Nogueiras et al., 2012), inflammatory cytokine signaling (Kawahara et al., 2009), glucose homeostasis (Zhong et al., 2010), and the mediation of the benefits of caloric restriction (CR) on mitochondrial proteins (Someya et al., 2010). Collectively, alterations above the DNA sequence can impact the processes of aging.

### *Loss of Proteostasis*

Proteostasis refers to a tightly controlled process of the initial production and proper folding of proteins, maintenance of its conformational shape, control of its subcellular localization and abundance, and, lastly, its disposal by degradation (Klaips, Jayaraj, & Hartl, 2018). Disruption of any one of these processes can negatively affect the aging process. For example, the chronic expression of misfolded proteins has been shown to contribute to the age-related pathologies Alzheimer's disease and Parkinson's disease (Powers, Morimoto, Dillin, Kelly, & Balch, 2009). Because human cells express upwards of 10,000 different proteins (Kulak, Geyer, & Mann, 2017), there is an immeasurable number of potential conformational shapes these proteins can take (Klaips et al., 2018). As such, folding errors are guaranteed to occur (Bartlett & Radford, 2009; Dobson, Šali, & Karplus, 1998). Unless these misfolded proteins are degraded, the resultant aberrant interactions within the cell can have detrimental effects. Because there is a reduced capacity to clear the cell of misfolded proteins with age (Walther et al., 2015), this predisposes the cell to the formation of insoluble protein aggregates that can disrupt cellular communication, function and bioenergetics. Collectively, these perturbations contribute the aging process.

### *Deregulated Nutrient-sensing*

Life is only compatible with nutrient provision. Therefore, how the body responds to nutrient signals is critical to health and longevity. For instance, reduced insulin sensitivity of both hepatic and extra-hepatic tissues can result in elevated blood glucose levels that can eventually lead to complications such as peripheral vascular diseases, kidney disease, and diabetes (Davies et al., 2018). Nutrient-sensing pathways act in response to either the provision or the absence of nutrients. The entirety of the intricate processes of homeodynamics, reproduction, and tissue maintenance are regulated by the signaling cascade stemming from nutrient sensing pathways (Templeman & Murphy, 2018). Whereas insulin and insulin-like growth factor (IGF-1) respond to nutrient provision and anabolic signals, adenosine monophosphate kinase (AMPK) and sirtuins respond to nutrient scarcity and catabolic signals (López-Otín et al., 2013). Interestingly, research has shown positive effects on health and increased longevity in models that utilized genetic manipulation and attenuation of the insulin and IGF-1 signaling pathways in worms and mice (Fontana, Partridge, & Longo, 2010). Additionally, CR-mediated increases in AMPK and SIRT activity has also been shown to exert favorable effects on health and longevity in non-human primates (Mattison et al., 2012). Although the breadth of current data appears to indicate that anabolic signaling and increased nutrient provision increases the deleterious effects of aging, while catabolic signaling and reduced nutrient provision decreases these effects (Fontana et al., 2010), it is crucial to keep in mind that extremely decreased anabolic signaling has been observed to have negative effects on health, such as insulin resistance and testicular degeneration in mice (Wilkinson et al., 2012). In addition, complete ablation of anabolic signaling, observed in a mouse model with an Akt-null mutation, is incompatible with life (Renner & Carnero, 2009). Given these conflicting reports, it is important to understand that there is crosstalk between the anabolic and catabolic pathways

and some overlap of these downstream targets will occur (Templeman & Murphy, 2018). Because there is an observed resistance to anabolic signals with age (Haran, Rivas, & Fielding, 2012), the catabolic signals may not necessarily be amplified. Rather, their signals may be more pronounced because of the decreased anabolic signaling from an increased anabolic resistance.

### *Mitochondrial Dysfunction*

Reactive oxygen species (ROS) are free radical-containing derivatives of molecules with an unpaired outer-shell electron that has reacted with oxygen (Liguori et al., 2018). Given the immeasurable number of reactions occurring at every moment within cells of the human body, ROS formation is a natural physiological process of everyday life. Because of this, the human body utilizes several different antioxidant enzymes and systems to reduce these free radicals to a stable, less harmful state. The prevalent antioxidant enzymes include superoxide dismutase, catalase and glutathione peroxidase (Liguori et al., 2018). Because a majority of cellular adenosine triphosphate (ATP) production occurs in the mitochondria in a process that involves the reduction of oxygen to water, the mitochondria is a prime source for ROS production.

In 1956, Denham Harman first proposed the free radical theory of aging in which he posited that progressive mitochondrial dysfunction associated with aging results in the overproduction of ROS, which subsequently causes further mitochondrial deterioration and global cellular damage (Harman, 1956). Since this initial theory, many have sought to evaluate the relationship between mitochondrial ROS production and aging. Indeed, there has been an observed diminished efficacy of the mitochondrial respiratory chain with aging. This subsequently results in reduced ATP generation, increased electron leakage across the mitochondrial membrane, and the promotion of additional ROS formation (Green, Galluzzi, & Kroemer, 2011). However, conflicting results in both yeast and roundworms have shown that

increased ROS production within the cell may actually prolong the lifespan (Doonan et al., 2008; Mesquita et al., 2010; Van Raamsdonk & Hekimi, 2009). Additionally, studies in mice utilizing genetic manipulations have observed that increased mitochondrial ROS does not exacerbate the aging process (Van Remmen et al., 2003; D. Y. Zhang, Wang, & Tan, 2011). Further, similar studies in mice that impair mitochondrial function without also increasing ROS production do not accelerate signs of aging (Edgar et al., 2009; Kujoth et al., 2005; Trifunovic et al., 2004; Vermulst et al., 2008). This is interesting as it directly contradicts Harman's theory of aging. Collectively, these studies appear to indicate that increased ROS formation is simply a symptom of the larger problem that is mitochondrial dysfunction. Because the body is well-equipped to handle ROS formation, but is considerably less able to handle the cellular and metabolic derangements of impaired mitochondrial function, it thus stands to reason that mitochondrial function rather than total ROS production is the ultimate determinant of aging at the mitochondrial level. Sena and Chandel furthered this idea when they showed evidence that ROS accumulation in the cell triggers both proliferative and survival signals (Sena & Chandel, 2012). In essence, it appears that, as long as the ROS formation does not exceed the cells capacity to neutralize these free radicals, the derangements appear to be minimal. Thus, given the totality of evidence, it may be more accurate to posit that, as mitochondrial derangements increase with age, the associated increased ROS production promotes further mitochondrial dysfunction and downstream deleterious effects through disrupted homeodynamics.

### *Cellular Senescence*

It has been argued that the primary purpose of cellular senescence, or an arrest of the cell cycle, is a beneficial function to prevent the propagation of damage cells (López-Otín et al., 2013), such as potentially oncogenic cells (Collado, Blasco, & Serrano, 2007; Kuilman,

Michaloglou, Mooi, & Peeper, 2010). Cellular senescence can therefore be viewed as a protective process. Hayflick and Moorhead originally described the phenomenon of cellular senescence through their observations that human cells derived from embryonic tissues could only divide a finite number of times in a culture medium (Hayflick & Moorhead, 1961). As such, cellular senescence thus limits the human lifespan to a finite amount of time. Following their work, others have observed that senescent cells accumulate with age. For instance, when measuring senescence-associated β-galactosidase activity, Wang and colleagues observed a 17% yield in older mice versus an 8% yield in their younger counterparts (C. Wang et al., 2009). This observation lends direct evidence to an increased senescence with age. It is worth noting that they observed a difference in total senescent cells in the skin, lungs and spleen, but not SkM, heart or kidney. This illustrates that senescence may not be uniformly distributed across tissues, and some tissues may be more affected than others.

### *Stem Cell Exhaustion*

Stem cells are undifferentiated or partially differentiated cells that are essential for organism tissue maintenance and survival (Ren, Ocampo, Liu, & Izpisua Belmonte, 2017). Due to their ability to divide an indefinite number of times into a variety of cell types, stem cells are therefore key players in organismal homeodynamics through tissue repair and regeneration (Goodell & Rando, 2015). However, a functional attrition of stem cells has been identified in a wide array of murine tissues, including brain (Molofsky et al., 2006), bone (Gruber et al., 2006) and muscle (Conboy & Rando, 2012). This has also been observed in humans via slowed wound-healing, osteoporosis and muscle mass attrition with age. Additionally, studies of stem cell activity in old versus young mice have revealed a decreased cell cycle activity of aged mice (Rossi et al., 2007; Ruzankina et al., 2009). As with cellular senescence, this indicates that stem

cell exhaustion and a reduced regenerative capacity is not limited to specific tissues, but rather impacts the entire organism. Again, this is problematic as a reduced stem cell proliferative capacity directly leads to a reduced tissue maintenance and homeodynamics, each of which is observed with age.

### *Altered Intercellular Communication*

Alterations of communication at the cellular level have also been posited to play a role in aging. Endocrine, neuronal and neuroendocrine changes in communication have been shown to be deregulated in aging (Laplante & Sabatini, 2012; Rando & Chang, 2012; Russell & Kahn, 2007; G. Zhang et al., 2013). It has also been speculated that the aging-related changes in one tissue can impact and contribute to aging-associated deterioration of other tissues through perturbations of intercellular signaling (López-Otín et al., 2013).

## The Consequences of Aging

The causes of aging are numerous and affect individuals in a myriad of ways. Derangements at the molecular, cellular and organismal level produce unique and variable consequences. Indeed, there is no single aging phenotype. Because of this, I will focus on a specific effect of aging central to this dissertation: the diminished muscle mass associated with aging, commonly referred to as sarcopenia. Because sarcopenia has numerous causes, this topic will be the focus of the remainder of this dissertation. Briefly, sarcopenia is regarded as a reduction in muscle mass. Considering muscle's many roles in the body, a reduction is thus detrimental. Sarcopenia contributes to the increased frailty and fragility often seen with age and predisposes the individual to more serious health consequences (Choi, 2016; Siparsky, Kirkendall, & Garrett, 2014; Volpi, Nazemi, & Fujita, 2004). Further compounding the problems of diminished muscle mass are the associated physiological alterations that come with age.

Reduced gastrointestinal function and absorption increase the need for many nutrients as individuals age (Rémond et al., 2015). It has been argued that protein needs increase with age to offset the reduced absorption and increase need (Baum, Kim, & Wolfe, 2016; Burd, McKenna, Salvador, Paulussen, & Moore, 2019). Given this reduced absorption and increased need, alternatives, commonly in the form of protein-containing supplements (PCS), may serve as a convenient way to meet these increased protein demands (Walrand et al., 2016). Because the negative effects of diminishing muscle mass often lead to more substantial deleterious health outcomes, including physical disability, depression, decreased quality of life and even death (Beaudart, Zaaria, Pasleau, Reginster, & Bruyere, 2017), strategies to offset or delay this loss of muscle mass across the lifespan have garnered much attention (Morton et al., 2018). Resistance training (RT), PCS or a combination of the two have consistently been shown to augment muscle mass and strength in many different populations and among people of all ages (Morton et al., 2018; Schoenfeld, Ogborn, & Krieger, 2016; Stokes, Hector, Morton, McGlory, & Phillips, 2018). While the most robust effects of RT and PCS have been observed in younger populations, there is evidence that RT and protein feeding can have similar positive effects on muscle mass in the elderly (Moro et al., 2018).

# CHAPTER 2: LITERATURE REVIEW

## Skeletal Muscle Background

A detailed review of evolutionary myogenesis is well beyond the scope of this review. However, in order to better understand the consequences of sarcopenia it is important to provide a brief background on muscle physiology, type, and function so as to better understand how reductions in muscle can manifest in health derangements. Generally, muscle can be divided into two subgroups: 1) striated muscle, comprising both skeletal muscle (SkM) and cardiac muscle, and 2) smooth muscle, such as that of the gastrointestinal tract and vasculature. Because SkM is central to this dissertation, I will forgo any further discussion of cardiac or smooth muscle and focus solely on SkM.

SkM makes up the largest tissue mass of the human body and is essential for motion and support (Chal & Pourquié, 2017). Roughly 40% of total body weight is accounted for by SkM, which contains up to 75% of all body proteins and accounts for upwards of 50% of protein turnover within the body (Frontera & Ochala, 2015). By volume, SkM is largely water (roughly 75%), with the rest comprised of protein (roughly 20%), inorganic salts, minerals, glycogen, and fat (Frontera & Ochala, 2015; Cody T. Haun et al., 2019). Based on work from Haus and colleagues (Haus, Carrithers, Carroll, Tesch, & Trappe, 2007), Haun and colleagues (Cody T. Haun et al., 2019) posited that, of the roughly 20% of SkM comprised of protein, ~60-70% of SkM protein is comprised of myofibrillar protein related to contraction (of note, ~50% of which is made up of the thick filament myosin and ~20% is made up of the thin filament actin), ~20-30% is comprised of sarcoplasmic proteins responsible for metabolism of the cell, and ~10% is comprised of mitochondrial proteins related to mitochondrial function and reactions of the electron transport chain. These proteins constitute the structural, contractile, and regulatory

properties of SkM. SkM mass depends on a balance between protein synthesis and protein degradation. These counter-processes are regulated by a host of factors such as nutrient intake, hormonal state, activity, injury, disease, and age. Because SkM is subject to voluntary control, its contraction is coordinated via both voltage- and calcium-dependent processes (Kuo & Ehrlich, 2015). Mature skeletal myofibers are composed of aligned myofibrils (Chal & Pourquié, 2017), giving SkM its striated appearance. These myofibers are formed by fusion of myoblasts to produce the multinucleated myotubes. These myotubes subsequently mature into myofibers (Abmayr & Pavlath, 2012), which are then classified according to fiber type: in particular Type I slow-twitch and Type II fast-twitch. Importantly, these fiber types each display characteristic movement rates, responses to neural inputs, and metabolic styles (Talbot & Maves, 2016). As such, each fiber type constitutes a percentage of SkM unique to the individual. Adult myofiber type percentage is thus the culmination of myofiber development history, innervation, and the physiological demands placed on the fiber (Schiaffino & Reggiani, 2011). The first determinant of myofiber type occurs during embryonic development via expression of myosin heavy chain (MyoHC)-specific isoforms whereby Type I slow-twitch fibers are first to develop, followed by Type II fast-twitch fibers (Chal & Pourquié, 2017). The reason for the delay in Type II fast-twitch fiber appearance is due to the delayed expression of transcription factors that promote Type II fast-twitch diversity (Grifone et al., 2004; Richard et al., 2011). In addition, fiber type is also driven by neuronal inputs during the fetal stage of development, as evidenced in a rat and mouse model (Hurren, Collins, Duxson, & Deries, 2015). Specifically, it was observed that myogenic differentiating factors and nerve innervation coincide within embryonic tissue, indicating that neuronal inputs also contribute to fiber type and development. Lastly, demands and stresses placed upon a myofiber can influence its fiber type (Schiaffino & Reggiani, 2011).

This is commonly observed in individuals training for a specific sporting endeavor. For instance, it has previously been reviewed that endurance, strength, and power training can significantly impact the fiber type of SkM (Jacob M. Wilson et al., 2012).

In addition to body posture and movement, SkM also serves as the major glucose disposal site of the body and contributes greatly to overall metabolism and homeodynamics. Indeed, it has been observed that SkM insulin resistance is a major contributing factor to the progenesis of disease such as Type II diabetes mellitus (Petersen & Shulman, 2018). As such, maintaining muscle mass throughout the lifespan is crucial for physical exertion, metabolic health, and longevity. With aging, however, there is an appreciable decrease in SkM and will be the focus of the remainder of this dissertation.

## Sarcopenia: A Working Definition and Diagnostic Criteria

Both the size and number of muscle fibers remain relatively stable until the fifth decade of life, at which point an appreciable decrease in muscle fibers and size occurs that culminates in a nearly 40% decrease of total SkM mass by the eighth decade of life (Deschenes, 2004; Faulkner, Davis, Mendias, & Brooks, 2008; I. Janssen, S. B. Heymsfield, Z. Wang, & R. Ross, 2000; Lexell, 1995). In 1989, Irwin H. Rosenberg first defined this progressive loss of muscle mass with age as sarcopenia, from the Greek *sarx*, “flesh”, and *penia*, “poverty” (I. H. Rosenberg, 1997). This original definition focused solely on muscle mass with no regard to muscle strength, quality, or function. This is problematic as longitudinal studies have shown that the age-related reductions in muscle strength are not proportionately related to the reductions in muscle mass (Clark & Manini, 2008). Rather, reductions in strength appear to outpace reductions in mass as much as 5-fold (Delmonico et al., 2009; Cameron J. Mitchell et al., 2013). Additionally, research conducted in older populations has indicated that low muscle strength is

more strongly associated with poor health status and all-cause mortality than low muscle mass (Artero et al., 2011; Katzmarzyk & Craig, 2002; Newman et al., 2006; Ruiz et al., 2008). Worse yet, these negative outcomes are even more prevalent in elderly with chronic medical conditions (Giacomantonio, Bredin, Foulds, & Warburton, 2013; Warburton & Bredin, 2016) indicating that reduced strength has more deleterious effects on elderly with comorbidities than reduced muscle mass. These results confound the original definition of sarcopenia. As such, the definition of sarcopenia has been expanded several times in an attempt to account for the associated reductions in muscle strength, quality, and function (Fielding et al., 2011; Morley et al., 2011; Muscaritoli et al., 2010).

In 2010, the European Working Group on Sarcopenia in Older People (EWGSOP) sought to provide a practical clinical definition, as well as establish consensus diagnostic criteria, for age-related sarcopenia (Cruz-Jentoft et al., 2010). The EWGSOP proposed a working definition of sarcopenia as "a progressive and generalized loss of muscle mass and strength with a risk of adverse outcomes such as physical disability, poor quality of life, and death" (Cruz-Jentoft et al., 2010). The committee also concluded that a diagnosis of sarcopenia should require 1) low muscle mass, as well as 2) low muscle strength and/or low physical performance (Cruz-Jentoft et al., 2010). Given the aforementioned reductions in muscle strength having a more profound effect on mortality and other negative consequences, it is conceivable that muscle mass reductions may be minimal while muscle strength losses are substantial. Based on the diagnostic criteria of the EWGSOP at the time (2010), this would not result in a diagnosis of sarcopenia. This is problematic for several reasons. First, although muscle mass would be relatively maintained, people with reduced strength may nonetheless suffer increased adverse health outcomes through increased frailty, fragility, and risk of falls. Second, any intervention to treat

sarcopenia would potentially be treating the incorrect symptoms (i.e. reduced muscle mass instead of reduced muscle strength). An intervention that increased muscle mass without also increasing strength may therefore not have significant effects on overall health and longevity. These problems are highlighted by the results of two systematic reviews that pooled sarcopenia diagnoses and subsequently observed severe discrepancies in the reported prevalence of sarcopenia. While Shafiee and colleagues reported a diagnosis of sarcopenia in 10% of the population from the reviewed literature (Shafiee et al., 2017), Cruz-Jentoft and colleagues reported a diagnosis of sarcopenia in 1-29% of the community-dwelling population, 14-33% in the long-term care population, and 10% in the lone acute hospital-care population of the reviewed literature (Cruz-Jentoft et al., 2014). Despite utilizing similar diagnostic criteria (i.e. each assessed sarcopenia based on muscle mass, the EWGSOP accepted basis at the time), the two reviews yielded wildly dissimilar results. This is problematic because the lack of a consensus definition and diagnostic criteria for sarcopenia hinders evaluation of intervention efficacy. Because the success or failure of clinical interventions are largely dependent on the constructs surrounding outcome measures, it is thus critical to have well-defined outcome measures. Knowing this, Cruz-Jentoft and colleagues reconvened in 2018 in an attempt to once again define sarcopenia and establish diagnostic parameters. In their revised guidelines, muscle strength replaced muscle mass as the central determinant of sarcopenia (Cruz-Jentoft et al., 2019). This was driven largely by the aforementioned works, as well as additional studies indicating that muscle strength is a better indicator than muscle mass in predicting adverse health outcomes (Dos Santos, Cyrino, Antunes, Santos, & Sardinha, 2017; Ibrahim et al., 2016; Darryl P. Leong et al., 2015; L. Schaap, Koster, & Visser, 2012). Contrary to their 2010 diagnostic criteria, in which reduced muscle mass was required, the EWGSOP stated in 2018 that

"sarcopenia is probable when low muscle strength is detected", and a diagnosis of sarcopenia is confirmed by "the presence of low muscle quantity or quality" (Cruz-Jentoft et al., 2019). This revised definition places muscle strength as the critical focus of sarcopenia. Additionally, the EWGSOP introduced the concept of severe sarcopenia, a condition they characterized by low muscle strength, low muscle quantity and/or quality, and low physical performance (Cruz-Jentoft et al., 2019). Importantly, the EWGSOP recognized the difficulty in diagnosing sarcopenia based on strength. As such, Cruz-Jentoft and colleagues identified both hand grip strength (Ibrahim et al., 2016; D. P. Leong et al., 2015) and the chair stand test as adequate measures of muscle strength (Cruz-Jentoft et al., 2019). In addition, the EWGSOP also recognized many available modalities of assessing muscle quality, including anthropometry estimation techniques, magnetic resonance imaging (MRI), computed tomography (CT), DXA, and bioelectrical impedance analysis (BIA).

## The Causes of Sarcopenia

Much of the difficulty surrounding defining sarcopenia and identifying its diagnostic parameters stem from the complexities of its etiology. Just as there are a multitude of factors that contribute to the aging process, all of which interact in a myriad of ways to produce an aging phenotype unique to each individual, there are likewise many constituents of sarcopenia. Not to be mistaken for other conditions of progressive amyotrophy, such as muscular dystrophy and cancer-related cachexia, our current understanding of sarcopenia is that it is the cumulative interactions of cellular, neuromuscular, and metabolic perturbations (Mankhong et al., 2020). Because sarcopenia is the result of each of these anomalies interacting, it is thus necessary to explore the minutia in order to better understand how each of these aspects affects the development of sarcopenia. In this way, we may better develop interventions to target the

specific etiologies of sarcopenia. Although there is significant overlap between the causes of aging and the causes of sarcopenia, there are also unique aspects of sarcopenia nonetheless, and I will focus on the causes as they affect SkM.

### *Cellular Senescence*

The reductions in muscle size and number throughout life are largely believed to be driven by cellular senescence. Collectively, the mechanisms that promote cellular senescence in SkM are multifactorial, complex, and involve many different signaling pathways and proteins. Although a detailed review is beyond the scope of this review, a short synopsis to highlight key components is presented. SkM houses an array of progenitor cells, including both satellite cell (SC) and mesenchymal progenitor cells (MPC). Satellite stem cells are capable of regenerating muscle fibers, and these progenitor cells are supported by MPCs that contribute to muscle regeneration (Klimczak, Kozlowska, & Kurpisz, 2018). Under normal physiologic circumstances, after injury to muscle tissue regeneration typically occurs rapidly with satellite stem cells (Rosenblatt, Yong, & Parry, 1994). During this regenerative time, myogenic regulatory factors (MFRs), such as MyoD, myf-5, myf-6, and myogenin, work together to stimulate the proliferation and differentiation of SCs (G. R. Hunter, McCarthy, & Bamman, 2004). In addition, anabolic endocrine signals, such as insulin and IGF-1, can also stimulate proliferation and differentiation of SCs unrelated to muscle damage. These signals will be discussed later and in greater detail. Once stimulated by the actions of MRFs following injury, the typically inactive SCs proliferate into myoblasts, and further fuse into myotubes (Owino, Yang, & Goldspink, 2001; Roth et al., 2000).

With aging there is an observed increased senescence of both SCs and MPCs (Blau, Cosgrove, & Ho, 2015) which dampens the response to both muscle tissue injury and anabolic

endocrine signals. This severely limits the regenerative capacity of SCs and MPCs to build, restore, and maintain SkM. Because SkM are multinucleated cells, the ultimate size of the muscle fiber is somewhat dependent on the number of nuclei in the muscle fiber (Favier, Benoit, & Freyssenet, 2008). Although muscle nuclei do not enter apoptosis during times of muscle atrophy or injury as once believed (Schwartz, 2019), the nuclei are unable to replicate nonetheless (Favier et al., 2008). Rather, new nuclei added to existing muscle fibers come from SCs, which, when activated following injury or exercise, migrate to the site of injury, proliferate, and fuse to the existing damaged fiber (Hawke & Garry, 2001). However, there appears to be an age-dependent decline in SC regeneration, termed SC senescence, which can delay the recovery of muscle tissue following injury and reduce nuclei accretion of the myofiber. This can reduce cellular replicative and metabolic process of SkM. Over time this contributes to the progressive loss of muscle mass seen with aging.

Senescence of these progenitor cells may be due to a variety of reasons, including DNA damage, telomere shortening, mitochondrial dysfunction, oxidative stress, activation of oncogenes, and chemotherapeutic agents used in the treatment of certain cancers (van Deursen, 2014). Although these stimuli induce senescence in a variety of ways, the most prominent axes affected are the p53-$p21^{Cip1}$ and $p16^{Ink4a}$ pathways, whose activation and upregulation inhibit both cyclin-dependent kinase (CDK) 2 and CDK416, culminating in the hyperphosphorylation of the retinoblastoma protein and, ultimately, cell-cycle arrest (Nevins, 2001; Sharpless & Sherr, 2015). Studies done previously involving aged mice (Baker et al., 2008) and the elderly (Sousa-Victor et al., 2014) have both observed an increased expression of $p16^{Ink4a}$ and an accumulation of senescent SCs in SkM with increasing age. These works provide a direct association between $p16^{Ink4a}$ expression and SC senescence. Further supporting this association are results observing

that the silencing of $p16^{Ink4a}$ in geriatric SCs restored quiescence and muscle regenerative functions (Sousa-Victor et al., 2014). Other works have also demonstrated that the elimination of $p16^{Ink4a}$ in BubR1 progeroid mice delayed the development of sarcopenia (Baker et al., 2011). Collectively, these works illustrate a clear and consistent link between upregulated $p16^{Ink4a}$ activity and sarcopenia.

Another contributor to cellular senescence is the cysteine-rich protein 61 (Cyr61 or CCN1). As a member of the CCN gene family, CCN1 expression has been observed to be increased at sites of wound repair (Jun & Lau, 2010), which subsequently induces fibroblast senescence through p53 activation and ROS-dependent activation of the $p16^{Ink4a}$ pathway (Jun & Lau, 2010). Although beneficial to prevent uncontrolled tissue fibrosis during the repair process, an uncontrolled or overexpression of CCN1 could severely damage normal tissue regeneration through cellular senescence. Indeed, Du and colleagues have observed an increased CCN1 expression and concomitant increase of $p16^{Ink4a}$ expression in aged mice, but not their younger counterparts (Du et al., 2014). They also observed that CCN1 expression was increased in both a time- and dose-dependent manner when $C_2C_{12}$ myoblasts were transduced with Wnt-3a (Du et al., 2014), which has been observed previously to trigger accelerated cellular senescence in a murine model of accelerated aging through activation of the p53-$p21^{Cip1}$ pathway (Liu, Zhang, Zhao, & Feng, 2017; D. Y. Zhang et al., 2011). These works provide supporting evidence that CCN1 expression is both instrumental in senescence and upregulated with age.

In addition to senescence via the p16 and CCN1 pathways, members of the mitogen activated protein kinase (MAPK) family are known to impact SC senescence through their many roles in the maintenance, proliferation, division, and differentiation of cells (Bernet et al., 2014). Previous studies examining myogenesis utilizing a cell culture model have demonstrated that the

p38 MAPK pathway plays a role in various myogenic stages, from myoblast proliferation to fusion, and also influences SC cycle withdrawal (Segales, Perdiguero, & Munoz-Canoves, 2016). Specifically, the p38αβ MAPKs are believed to be involved in SC senescence through a signaling cascade that involves the p38-stimulated production of proinflammatory cytokines (Zarubin & Han, 2005). In addition, aging has been observed to enhance MAPK activity and subsequent ROS production (H. J. Kim, Jung, Yu, Cho, & Chung, 2002). Although there is strong evidence that pro-inflammatory signaling induces cellular proliferation and differentiation (Yu & Brown, 2015), aberrant overproduction of pro-inflammatory signaling may inhibit cellular proliferation and induce apoptosis (Landén, Li, & Ståhle, 2016). Given that aging increases pro-inflammatory cytokine production, this may have detrimental effects on the regenerative capacity of cells. Though not completely understood yet, it is believed that increased inflammation with age leads to excessive and aberrant p38 MAPK activation, ultimately leading to impaired self-renewal of aging SCs (Bernet et al., 2014).

### *Mitochondrial Dysfunction and Oxidative Stress*

The mitochondria serve as a major source of ATP production within SkM cells through processes involving the reduction of oxygen to water via electron transport across the mitochondrial membrane. Given the permeability of the mitochondrial membrane, electrons can leak at various complexes involved in mitochondrial ATP production. These electrons can interact with stable molecules to produce unstable molecules (i.e. $H_2O$ and various protein, lipid, and carbohydrate species) and ROS, such as superoxide and, subsequently, hydrogen peroxide. Although this is a natural physiological process, over time the accumulation of insults stemming from electron leakage can produce mitochondrial derangements and dysfunction. This mitochondrial dysfunction can result in the over production of ROS as an individual ages. Once

ROS formation exceeds the cells ability to maintain homeodynamics, the resultant oxidative stress further exacerbates mitochondrial dysfunction.

The increased mitochondrial dysfunction seen with age may be in part due to the increased senescence of SCs with age. In theory, the increased number of senescent SCs would decrease the number of active SCs. This would likely result in upregulation of the mitochondrial oxidative metabolism of active cells to meet metabolic demands (Passos et al., 2010). To meet cellular energy demands, there would be an increased flux through ATP production pathways. An increased flux would potentially produce more ROS. If oxidative stress is already present, the potentially increased ROS production would exacerbate the already present oxidative stress. An increase in oxidative stress could further reduce redox homeodynamics and potentially lead to a progressive oxidation of cellular components such as proteins, lipids, and DNA. This, in turn, promotes further mitochondrial dysfunction and increased oxidative stress in a feed-forward loop that increases cellular energy deficiency, cellular damage, and dysfunction (Kadoguchi et al., 2020), all of which contributes to the sarcopenic phenotype.

The increasing mitochondrial dysfunction with age may also be largely dependent on a person's physical activity level. It has been posited that a larger percentage of fast-twitch, low oxidative capacity Type II muscle fibers and a smaller percentage of slow-twitch, high oxidative capacity Type I muscle fibers is related to poorer metabolic health (Fisher et al., 2017) and higher oxidative stress (Quindry et al., 2011). Indeed, it stands to reason that muscle tissue with a higher percentage of low oxidative capacity muscle fibers would more likely suffer detriments of mitochondrial dysfunction. For instance, if certain muscle fiber types (i.e. Type II fast-twitch) already have a reduced threshold to efficiently meet energy demands, any further reductions in this capacity via mitochondrial dysfunction may contribute to and exacerbate oxidative stress and

extend mitochondrial dysfunction. In addition, increased mitochondrial mass has been observed in senescent SCs of the elderly (Correia-Melo et al., 2016) and is thought to be driven by a reduced mitophagy of the elderly (Moreira et al., 2017). This is important in regard to cellular oxidative capacity because mitochondrial number rather than mitochondrial mass determines energy and metabolic homeodynamics. In this regard, an increased mitochondrial mass would not likely increase metabolic efficiency and, worse yet, may contribute to and further exacerbate metabolic derangements. When coupled with an increased mitochondrial dysfunction and reduced energy production, this may further intensify the sarcopenic phenotype. It may therefore stand to reason that a greater percentage of Type II fast-twitch to Type I slow-twitch fibers may exacerbate oxidative stress through mitochondrial dysfunction with age. Although older adults have been observed to have a reduced Type II fast-twitch muscle fiber content as compared to younger adults (Kosek, Kim, Petrella, Cross, & Bamman, 2006) there appears to be only a modest increase in Type I slow-twitch fibers. This is interesting as the reduced Type II fast-twitch content would likely decrease mitochondrial oxidative capacity, and the modest increase in Type I slow-twitch content may not be sufficient to rescue the diminished capacity. Despite limited research reporting the percentage of fiber-type switching with age, it is nonetheless purported that different regulatory pathways affect the degeneration of muscle fiber types differently (Ciciliot, Rossi, Dyar, Blaauw, & Schiaffino, 2013; Miljkovic, Lim, Miljkovic, & Frontera, 2015; Y. Wang & Pessin, 2013). Therefore, it is unlikely that Type II fast-twitch muscle fiber reduction is the sole determinant of mitochondrial dysfunction with age. However, it is nonetheless likely that a reduced oxidative capacity and increased oxidative stress via a reduction in Type II fast-twitch muscle fibers plays a role in the progression of sarcopenia via mitochondrial dysfunction and increased ROS production.

### *Inflammation*

Inflammation, the body's natural response to a harmful stimulus, is an acute process of removing or counteracting the harmful stimuli, initiating the healing process, and limiting injury or infection. As such, inflammation serves a protective biological role. However, chronic, low-grade inflammation is detrimental to health and is implicated in the pathogenesis of many diseases and disorders, including sarcopenia (L. A. Schaap, Pluijm, Deeg, & Visser, 2006). The many inflammatory pathways utilized by the body involve several common inflammatory markers that activate intracellular signaling pathways, which subsequently activate the production of inflammatory mediators (Chen et al., 2017). The primary inflammatory stimuli consist of interleukin 1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), which mediate inflammation through interactions with their respective receptors (Kaminska, 2005). This interaction triggers an intracellular cascade involving the aforementioned MAPK pathway, NF-κB pathway, and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (Hendrayani, Al-Harbi, Al-Ansari, Silva, & Aboussekhra, 2016; Henríquez-Olguín et al., 2015; Kyriakis & Avruch, 2001). These pathways function in a multitude of ways to regulate cell proliferation, differentiation, survival, and apoptosis (Kaminska, 2005), as well as regulate cytokine production, inflammatory cell recruitment (Chen et al., 2017), and gene expression regulation (O'Shea et al., 2015).

During acute inflammation, such as following a minor skin abrasion, these inflammatory pathways all serve a beneficial role to neutralize external pathogens and retard the spread of infection. However, persistent, uncontrolled activation of these pathways is detrimental to health and longevity. For instance, low serum concentrations of TNF-α (~3-100 ng/mL) from chronic, aberrant cytokine production has been observed to increase muscle atrophy (Li et al., 2005;

O'Leary, Wallace, Bennett, Tsintzas, & Jones, 2017; Sishi & Engelbrecht, 2011; D.-T. Wang et al., 2014), as well as stimulate muscle catabolism (Li et al., 2005; T. Phillips & Leeuwenburgh, 2005). In addition, chronic TNF-α signaling has been shown to alter the degradation of proteins by directly activating the ubiquitin proteasome system (UPS) in a rat model (Llovera, García-Martínez, Agell, López-Soriano, & Argilés, 1997), as well as trigger apoptotic signaling pathways in aged SkM (Pistilli, Jackson, & Alway, 2006). Collectively, the evidence surrounding chronic TNF-α signaling and persistent, low circulating levels indicate that it is detrimental to muscle mass retention. This is especially problematic as there is growing evidence that the levels of the muscle-specific protein degradation ubiquitin ligases, Atrogin1 (MAFbx) and muscle RING finger 1 (MuRF1), as well as TNF-α, increases with age and sarcopenia (Li et al., 2005; T. Phillips & Leeuwenburgh, 2005). This results in a feed-forward system of ever-increasing inflammation with further muscle degradation. Several studies have observed that TNF-α and other inflammatory mediators are elevated 2-4-fold higher in those with sarcopenia compared to their younger counterparts (Bian et al., 2017; Can et al., 2017; Marzetti et al., 2019; L. A. Schaap et al., 2006). Work by Hunter and colleagues has also found that elderly women with elevated levels of circulating plasma TNF-α concentrations before and after completing a 16-week RT program did not experience similar increases in muscle hypertrophy as compared to their counterparts with lower levels of circulating plasma TNF- α (G. R. Hunter et al., 2004). In addition, work done in rodent models has consistently shown that increased circulating inflammatory mediators increase markers of both proteolysis and muscle atrophy (Baumgartner et al., 1998; Dufour, Hannan, Murabito, Kiel, & McLean, 2013; I. H. Rosenberg, 1997). Conversely, TNF-α inhibition has been shown to reduce muscle proteolysis, while TNF-α infusion increased myofibrillar protein breakdown (MyoPB) in a cell culture model (Gary R.

Hunter, Singh, Carter, Bryan, & Fisher, 2019). Collectively, this evidence indicates that, while the inflammatory response is critical to life, an aberrant, chronic, low-grade inflammatory state has serious detrimental consequences on both health and longevity through increased degradation and reduced accumulation of SkM.

### *Fat Accumulation*

The reduction in muscle mass seen with aging is intimately linked with fat accumulation. SkM is a highly metabolically active tissue and, therefore, accounts for a large percentage of an individual's energy expenditure through their basal metabolic rate (BMR). As such, a higher muscle mass correlates with a higher BMR and greater sedentary energy expenditure, while a lower muscle mass correlates with a lower BMR and reduced sedentary energy expenditure. Because muscle mass declines with age, BMR declines with age as well. Indeed, the loss of muscle mass across the lifespan results in a 2-3% decline in BMR and sedentary energy expenditure before age 50 and a 4% decline after age 50 (Buch et al., 2016). Unless energy intake is concomitantly reduced with the reductions in BMR, this predisposes an individual to an increased risk of fat gain as excess calories are stored as fat. Given that the number of adipose (fat) cells is relatively stable after adolescence (Stenkula & Erlanson-Albertsson, 2018), once these fat storage cells reach capacity, excess fat is thus forced into neighboring tissue such as SkM. Although fat infiltration into muscle is detrimental to health and function, some lipid molecules are more detrimental than others.

Ceramides are members of a family of sphingolipid molecules that are ubiquitously expressed and serve as an essential structural component of cell membranes (Young, Mina, Denny, & Smith, 2012). Whereas the *de novo* ceramide synthesis involves the amino acid L-serine and palmitoyl-CoA, both the salvage pathway and direct hydrolysis of sphingolipids each

relies on pre-existing precursors (Duarte et al., 2020; Jeffries & Krupenko, 2018). These endogenously derived ceramides represent a large percentage of the cell membrane lipids. Regardless the pathway utilized, endogenous ceramide production generates a lipid mediator second messenger involved in many biological processes of SkM, including proliferation, differentiation, survival (Bruni & Donati, 2008; Duarte et al., 2020), growth, arrest, and apoptosis (Saïda Mebarek et al., 2007). In addition, ceramides function as a modulator of cellular stress (Chavez & Summers, 2012). Given their prevalence in cellular membranes and their extensive signaling capacities, ceramides thus represent important players in SkM health. In rat L6 myoblasts, short-chain ceramides have been observed to inhibit SkM myogenesis through reductions in phospholipase D (PLD) and myogenin, both of which serve important roles in membrane trafficking and SkM development, in a dose-dependent manner (S. Mebarek et al., 2007). Additionally, increased ceramide content of cell membranes of aged rats has been associated with increased autophagy and age-dependent sarcoplasmic reticulum stress (Russ, Boyd, McCoy, & McCorkle, 2015; Russ, Wills, Boyd, & Krause, 2014). In humans, ceramide content of SkM has been observed to be increased in obese, insulin-resistant individuals (Adams et al., 2004; Srikanthan, Hevener, & Karlamangla, 2010). The detrimental effects of this increased ceramide content in SkM are thought to be mediated through reductions of the Akt/protein kinase B (PKB) signaling pathway via increases in both inflammatory pathways and increased mitochondrial stress (Chavez & Summers, 2012). Because the Akt/PKB pathway is indirectly involved in muscle protein synthesis and muscle hypertrophy (as will be discussed later) and is downregulated with increased SkM ceramide content, it thus stands to reason that increased fat infiltration into SkM with age would result in further reductions in SkM mass through altered intracellular signaling. Additionally, it has been observed that increased SkM

ceramide content also plays a role in the activation of protein kinase C zeta (PKCζ) (Chavez & Summers, 2012). Because PKCζ functions as a negative regulator of the Akt/PKB pathway (Chavez & Summers, 2012), this would further compound the negative consequences of an increased ceramide content on muscle mass. In addition, studies have shown that ceramides promote SC senescence in cell culture models (Chang et al., 2018; Jadhav, Dungan, & Williamson, 2013), as well as increases oxidative stress and mitochondrial fission (Smith et al., 2013). Collectively, these data indicate that an increased fat mass, and likely increased ceramide content relative to SkM mass, is harmful in regard to both health and longevity and contributes to the sarcopenic phenotype.

### *Neuromuscular Disruptions*

Although the focus thus far has been on the metabolic and cellular derangements of sarcopenia, neuromuscular dysfunction may also play a role. Mature muscle fibers are innervated by a single motor neuron. Because there are many more muscle fibers than motor neurons, individual motor axons branch to innervate multiple muscle fibers, collectively referred to as a motor unit. In addition to utilizing limited motor neurons to connect many muscle fibers, branching of the axons ensures that the contractile force of the motor unit is evenly distributed across the muscle (McCormick & Vasilaki, 2018; PURVES et al., 2001). Studies have observed a reduction in the total number of motor units with aging in both humans (Piasecki, Ireland, Jones, & McPhee, 2016) and rodents (Ling, Conwit, Ferrucci, & Metter, 2009; Sheth et al., 2018). In addition, there is evidence of decreased innervation of motor neurons to muscle fibers with aging (Tomlinson & Irving, 1977). This can result in denervation and motor unit remodeling, in which motor unit axons can denervate and be rennervated by a nearby motor neuron (L. Larsson, 1995). This reinnervation has been implicated in some of the aforementioned

age-related fiber-type switching (Anderson et al., 2003; A. Larsson, Praetorius, & Hjörting-Hansen, 1978) from Type II fast-twitch to Type I slow-twitch fibers. It is also believed that Type I slow-twitch motor neurons are more capable of reinnervation (Kadhiresan, Hassett, & Faulkner, 1996), which can also help to explain the reduction of Type II fast-twitch fibers with age. Essentially, when Type II fast-twitch fibers denervate, there is an increased proclivity for motor neurons to reinnervate a Type I slow-twitch fiber. However, a denervated fiber is not always reinnervated. If the denervated fiber is not reinnervated then the muscle fiber will often experience a decrease in mCSA, as well as a decrease in force production (de Oliveira Gonçalves et al., 2016). Worse yet, many denervated fibers often undergo apoptosis and die (Borisov & Carlson, 2000; Borisov, Dedkov, & Carlson, 2001; Vasilaki et al., 2016), further compounding the loss of muscle mass seen with age. This may help to explain the modest increase in Type I slow-twitch fibers following substantial Type II fast-twitch reductions with age.

## Derangements of Muscle Structure and Function with Age

The cumulative effects of the aforementioned cellular, metabolic, and neuromuscular perturbations collectively interact in an immeasurable number of ways to contribute to the individual sarcopenic phenotype. Despite the various ways in which sarcopenia can manifest, there are nonetheless specific outcomes regarding SkM mass, function, and quality that can be expected. These predictable outcomes help to form our construct of the sarcopenic phenotype. As mentioned previously, muscle quantity (i.e. muscle fiber size and number) remains relatively stable throughout the fifth decade of life after which a constant and appreciable decrease occurs (Faulkner et al., 2008; Ian Janssen et al., 2000; Lexell, 1995). Together, these reductions manifest in a reduction of contractile force, strength, and mass (Power et al., 2010; Trappe et al., 2003). Interestingly, research has shown that, when compared to their younger counterparts,

although Type II fast-twitch fibers from older individuals had a smaller fiber cross-sectional area (fCSA) and a reduced total generated force, specific force (i.e. the force normalized to fCSA) was not different (Claflin et al., 2011; Lexell, Taylor, & Sjöström, 1988). This indicates that although there is a fiber-type switching commonly associated with decreased strength and force output, this may not necessarily be the case with the elderly. Rather, perhaps there is a compensatory mechanism of Type I SkM to maintain adequate force production. However, work from the same groups has also revealed that, although there is an increase in fCSA in the elderly for Type I slow-twitch fibers, specific force does not also increase (Claflin et al., 2011; Lexell et al., 1988). These observations reveal that the age-dependent declines in strength are likely facilitated through reductions in Type II fast-twitch, rather than an increase in Type I slow-twitch, muscle fibers. Given the aforementioned discussion regarding the increased mitochondrial dysfunction likely exacerbated by the decrease in Type II fast-twitch fibers seen with age, in theory it stands to reason that increasing or maintaining Type II fast-twitch fibers across the lifespan would have beneficial consequences on health and longevity.

This theory is exemplified well in masters athletes, loosely defined as athletes 35 years and older who train for or take part in athletic competition often specifically designed for older participants (Tayrose, Beutel, Cardone, & Sherman, 2015). Typically, these individuals are trained competitors who continue their athletic endeavors after their careers are over. Faulkner and colleagues have previously observed that, while there was only a 40% reduction in masters athlete marathon runners, there was a greater than 60% reduction in volume lifted by masters athletes weightlifters (Faulkner et al., 2008). Considering that elite endurance athletes tend to have a higher percentage of Type I slow-twitch fibers while elite powerlifters tend to have a higher percentage of Type II fast-twitch fibers (J. M. Wilson et al., 2012), coupled with the

previously-discussed fiber-type switching and Type II fast-twitch reductions with age, it thus makes sense that strength athletes would see a greater reduction in their respective performance with age. This lends further evidence that there is both a shift in fiber type from Type II fast-twitch to Type I slow-twitch and a reduced force production and function of Type II fast-twitch fibers with age, as evidenced by the increased reductions in performance from athletes primarily relying on Type II fast-twitch fibers.

## Molecular Pathways of Muscle Hypertrophy and Atrophy

In order to better understand sarcopenia, it is necessary to understand the delicate balance of muscle hypertrophy and atrophy. Simply put, muscle hypertrophy can be regarded as an increase in muscle size, mass, or volume, while muscle atrophy can be thought of as a decrease in muscle size, mass, or volume. Collectively, muscle hypertrophy, maintenance, and atrophy are governed by two opposing systems: myofibrillar protein synthesis (MyoPS) and MyoPB. The daily totality of these systems results in an individual's net protein balance (NPB). While muscle hypertrophy requires a positive NPB, either by an increase in MyoPS or a decrease in MyoPB, muscle atrophy occurs in a state of negative NPB when either MyoPS is decreased or MyoPB is increased. As such, NPB is not a static construct but rather represents a fluid mechanism that responds to and is altered by both endogenous and exogenous signals. Although a comprehensive review of every effector of hypertrophy and atrophy is beyond the scope of this review, I will focus on two of the most studied effectors of muscle hypertrophy: mechanical stimuli and feeding (in particular, protein feeding). Despite each of these stimuli being quite different, their exerted effects on MyoPS, and therefore NPB, are strikingly similar and will be the focus of the remainder of this review.

### *mTORC1*

In order to proliferate and divide, cells must be able to adequately produce macromolecules through anabolic processes while concomitantly suppressing degradation through catabolic processes. The mechanistic target of rapamycin (mTOR) is a serine/threonine protein kinase complex that serves a critical and central role in the regulation of these processes through protein synthesis, protein turnover, lipid metabolism, and nucleotide metabolism (Saxton & Sabatini, 2017). mTOR complex 1 (mTORC1) is a protein complex that forms one-half (the other half being mTORC2) of mTOR and is believed to play more critical roles in muscle hypertrophy through a series of downstream protein phosphorylation's that enhance activities of protein synthesis and cell growth, as well as suppress activities of protein degradation. For cells to adequately grow and divide, 1) genetic information must be replicated, 2) intracellular components necessary for protein synthesis must be constructed, and 3) macronutrient substrates necessary for mass accumulation must be available. mTORC1 regulates these activities and responds to nutrient provision through p70S6 Kinase 1 (S6K1) phosphorylation. S6K1 serves as a second messenger to increase messenger ribonucleic acid (mRNA) translation and ribosomal biogenesis (Raught et al., 2004), and it's inhibition has been observed to induce cellular autophagy in a cell culture model (Pham et al., 2018; Xu et al., 2017) . Further, S6K1 directly phosphorylates and activates the eukaryotic initiation factor 4E (eIF4) Binding Protein (4EBP), another promoter of mRNA translation (Kang et al., 2013), as well as phosphorylates and inactivates programmed cell death protein 4 (PCDP4), an inhibitor of eI4B (Dorrello et al., 2006). In addition, mTORC1 promotes *de novo* lipid synthesis via phosphorylation and inhibition of the Lipin1 protein (Peterson et al., 2011), an inhibitor of the sterol responsive element binding protein (SREPB) that controls the expression of genes in fatty acid and cholesterol synthesis (Porstmann et al., 2008) required for cell membrane structure and function.

mTORC1 also promotes the synthesis of nucleotides needed during DNA replication and ribosomal biogenesis, promotes cell growth via inhibition of catabolic pathways, and promotes a shift of glucose metabolism that facilitates the incorporation of nutrients into new biomass during periods of growth (Saxton & Sabatini, 2017). In sum, mTORC1 coordinates numerous processes central to growth.

There are several upstream effectors that activate mTORC1. However, an mTORC1-dependent shift to increased anabolism requires the presence of anabolic endocrine signals and sufficient energy and nutrients for biomass synthesis, such as would be provided following feeding (Saxton & Sabatini, 2017). The tuberons sclerosis complex (TSC) is an upstream negative regulator of mTORC1 signaling (Saxton & Sabatini, 2017) that prevents mTORC1 lysosomal relocation and activation. Although several growth factor pathways converge on and inhibit TSC, thus allowing mTORC1 activation, the most prominent of these growth factors is IGF-1, which triggers an Akt-dependent activation of mTORC1 through TSC inhibition (Inoki, Li, Zhu, Wu, & Guan, 2002; Manning, Tee, Logsdon, Blenis, & Cantley, 2002) via activation of the P13K lipid kinase (Memmott & Dennis, 2009). Additionally, changes in amino acid concentrations in the sarcoplasm can also activate mTORC1 through a process that converts the Rags proteins from their inactive to active state, allowing them to bind the Raptor protein and recruit mTORC1 to the lysosomal surface to interact with the Rheb protein (Saxton & Sabatini, 2017). Specifically, the amino acids leucine and arginine have been observed to activate mTORC1 (Bar-Peled et al., 2013), though leucine is believed to be the more potent mTORC1 activator (Mobley et al., 2017; Saxton et al., 2016; Wolfson et al., 2016). Conversely, mTORC1 responds to both endogenous and exogenous stimuli that do not facilitate growth, such as low

ATP levels, hypoxia, and DNA damage (Saxton & Sabatini, 2017). These stimuli all inhibit activation of mTORC1 and subsequently blunt cell growth and proliferation.

### *mTOR in Muscle Hypertrophy and Atrophy*

At the turn of the century, several studies examining mTOR activation in SkM revealed that mTORC1 activation was associated with muscle hypertrophy (Bodine et al., 2001; Ono et al., 2000; Pallafacchina, Calabria, Serrano, Kalhovde, & Schiaffino, 2002) largely through upregulation of protein synthetic pathways and processes involved in cell growth and development. As mentioned previously, both the endocrine hormone IGF-1 and the amino acid leucine have been observed to activate mTORC1. This activation has been observed to stimulate muscle hypertrophy in both a cell culture and mouse model (Anthony, Anthony, Kimball, & Jefferson, 2001; Rommel et al., 2001). In addition to IGF-1- and leucine-mediated activation of mTORC1, Baar and Esser have previously provided evidence that muscle contraction also activates mTORC1 through S6K1 phosphorylation independent of nutrient provision (Baar & Esser, 1999). This may potentially explain the muscle hypertrophy often seen with regular and repeated muscle use, such as following a structured RT program. In addition, work done by Frey and colleagues illustrated that mTORC1 activation via muscle mechanical stimulation was driven via Raptor phosphorylation and substrate recruitment (Frey, Jacobs, Goodman, & Hornberger, 2014). Collectively, these observations provide direct evidence for the associations between mTORC1 activation and muscle hypertrophy. It may therefore seem logical that continuous mTORC1 activation would have even greater effects on muscle hypertrophy. However, although periodic mTORC1 activation has been observed to have positive effects on increases in muscle hypertrophy in a mouse model and human cohort (D'Hulst, Palmer, Masschelein, Bar-Nur, & De Bock, 2019; Song et al., 2017), chronic activation has conversely

been shown to cause severe muscle atrophy, low body mass, and early death in a murine model (Castets et al., 2013). These undesirable effects of over-activation are thought to be due to an inability to induce autophagy and clear tissues of unwanted cellular components (Saxton & Sabatini, 2017) that could be recycled for *de novo* amino acid, protein, and lipid synthesis necessary for cellular homeodynamics (Levine & Kroemer, 2008). In essence, chronic mTORC1 activation does not allow the cell necessary inactive time required to remove and recycle cellular debris. As such, cycling periods of mTORC1 activation and inactivation, a process that would allow for periods of increases in MyoPS pathways and subsequent lulls to allow for recoup and recovery, is likely the optimal approach for muscle hypertrophy. Thus, the totality of evidence indicates that: 1) nutrient provision and muscle contraction each activate mTORC1, 2) mTORC1 activation results in periods of increased MyoPS, 3) a positive NPB can be achieved via increases in MyoPS, 4) regular, cyclic activation of mTORC1 produces positive effects on muscle hypertrophy through increases in MyoPS. However, as previously discussed, aging has profoundly negative consequences on homeodynamics, metabolism, and cellular function. It therefore stands to reason that aging may also cause perturbations in mTOR function.

As mentioned previously, aging is associated with deregulated nutrient-sensing and blunted responses to endocrine signals. Given that certain endocrine signals are the direct result of nutrient provision and can activate mTOR, perhaps this dysregulation of nutrient signals alters or blunts mTORC1 activation. In theory, reduced nutrient sensing would result in a diminished mTORC1 activation, diminished MyoPS and, ultimately, reduced muscle hypertrophy from feeding. This would help to at least partially explain the progressive losses of muscle mass seen with age. Additionally, it has been posited that the anabolic response to mechanical stimuli is severely reduced with age (Wu, Fannin, Rice, Wang, & Blough, 2011). This would also inhibit

the body's ability to accrue new muscle. Lastly, it has been speculated that the exacerbated oxidative stress of aging may stimulate cell-cycle arrest via a CDK inhibition mechanism while concomitantly promoting the over-activation of mTORC1 (Blagosklonny, 2014; Campisi & d'Adda di Fagagna, 2007). mTOR over-activation during cell-cycle arrest has been observed to result in hypertrophic, pro-inflammatory, senescent cells (Blagosklonny, 2014) incapable of adequate tissue maintenance and regeneration. Moreover, these cells lose their ability to restart proliferation (Stallone, Infante, Prisciandaro, & Grandaliano, 2019). This loss of mitotic proficiency is a hallmark of senescence. Considering the accumulation of senescent cells with age, this provides further evidence of mTORC1 dysregulation with age.

Taken together, it is evident that aging causes deleterious effects on SkM at the tissue, cellular, and molecular level. These deleterious effects appear to be largely driven through altered mTOR (de)activation. The resultant aberrant anabolic signaling has profoundly negative effects on MyoPS and muscle hypertrophy. As such, therapies and interventions designed to target mTOR activation indirectly via mechanical contraction, nutrient provision, and endocrine signaling thus represent a promising avenue for the treatment of sarcopenia. RT and protein consumption, both independently and coupled, are easily manipulatable variables for a sarcopenia intervention. As such, they have been studied extensively.

## Sarcopenia Interventions

### *Resistance Training*

Although it is extremely difficult to causally determine the exact effects of inactivity on the development and progression of sarcopenia, it is nonetheless widely accepted that a sedentary lifestyle contributes to the sarcopenic phenotype. This has been well illustrated in models of bed

rest and weightlessness, in which reductions in muscle mass and MyoPS were observed in healthy young men (Ferrando, Lane, Stuart, Davis-Street, & Wolfe, 1996; Ferrando, Stuart, Brunder, & Hillman, 1995). This is likely due to substantial reductions in the contraction-mediated activation of mTOR, as well as reductions in contraction-mediated energy expenditure. Interestingly, RT has been observed to counteract these reductions in muscle mass and MyoPS in a similar bed rest model (Ferrando, Tipton, Bamman, & Wolfe, 1997). Coupled with our current understanding of how RT and the associated mechanical stimuli trigger a cascade of anabolic signals necessary for growth, this lends credence to the theory that RT can be a viable countermeasure to sarcopenia. As such, many researchers have examined the impacts of RT in elderly at risk for sarcopenia.

For instance, Urso and colleagues assessed the efficacy of a 10-week RT program performed 3 times per week on markers of muscle plasticity, muscle nuclei content, and IGF-1 receptor density in the elderly (Urso et al., 2005). The authors concluded that muscle plasticity was maintained in the group assigned to the RT intervention based on Z-band damage (Exercise Z-band damage % change PRE-POST=161±93.7%, Control Z-band damage % change PRE-POST=-40.8±3.5%). Because it has been observed previously that Z-band damage correlates with an increased MyoPS and myofibrillar fractional synthetic rate (FSR; i.e. the rate at which a precursor is incorporated into a product per unit of product mass; in this instance, amino acids into SkM) (Damas et al., 2016), this indicates that an increased Z-band damage correlates with greater rates of muscle hypertrophy at the molecular level. Additionally, central nuclei content increased in the RT group above that of the control group (Exercise central nuclei % change PRE-POST=296±120%, Control central nuclei % change PRE-POST=46.3±18.9%). Because muscle fibers accrue additional nuclei from SCs following tissue damage and regeneration this

indicates that the RT protocol stimulated SC activation and fusion to existing myofibers. Lastly, the RT group showed a robust increase in IGF-1 receptor density compared to the sedentary, control group (Exercise IGF-1 receptor density % change PRE-POST=165.0±54.8%, Control IGF-1 receptor density % change PRE-POST=57.1±18.2%). As previously discussed, IGF-1 is a potent endocrine mediator of mTORC1 activation and subsequent downstream anabolic signaling. Therefore, the results of this study lend evidence to the profound role that RT may play in muscle hypertrophy through its effects on anabolic endocrine signaling and muscle damage.

As previously stated, there has been an observed fiber-type shift that occurs with aging from Type II fast-twitch to Type I slow-twitch, which may subsequently have negative downstream effects. When comparing muscle fiber hypertrophy and myogenic mechanisms after a 16-week triweekly RT program in both young (20-35 years; n=24) and older (60-75 years; n=25) participants, Koseck and colleagues observed that RT attenuated this fiber-type switching (Kosek et al., 2006). Both Type IIa (old, PRE=3980±297 $\mu m^2$ vs. old, POST=4613±319 $\mu m^2$; young, PRE=4726±232 $\mu m^2$; p=ns) and Type IIx (old, PRE=3701±282 $\mu m^2$ vs. old, POST=4552±323 $\mu m^2$; young, PRE=4450±238 $\mu m^2$; p=ns) muscle fCSA in the old cohort increased to baseline levels of the young cohort after 16-weeks of RT. These results indicate that RT can rescue the fiber-type switching seen with age. Importantly, this rescue of fiber-type switching may potentially mediate the associated mitochondrial dysfunction of Type II fast-twitch reductions seen with age. Indeed, RT has been observed to have beneficial effects on both mitochondrial function and biogenesis (Lamb et al., 2020; Tarnopolsky, 2009), and it is posited that these benefits are PGC-1 and SIRT-mediated (Fiuza-Luces, Garatachea, Berger, & Lucia, 2013).

Considering that muscle mass is no longer identified as a better diagnostic criterion for sarcopenia, Reid and colleagues sought to examine whether 12 weeks of triweekly traditional strength training outperformed power strength training in a group of older (65-94 years) adults (Reid et al., 2008). While the strength group was advised to perform concentric, full extension, and eccentric phases for 2, 1, and 2 seconds, respectively, the power group was instructed to perform the same phases as fast as possible, for 1, and 2 seconds, respectively. A control group performing range of motion and flexibility exercises was also assessed. Power output was significantly higher for the power and strength group compared to the control group for knee extension (~2.8-fold, 49% increase and ~2.3-fold, 41% increase for power and strength group, respectively; $p<0.001$). However, bilateral leg press and leg lean mass did not change between groups, and changes in peak power and specific peak power were similar. These results indicate that both RT protocols yielded similar increases in lower-leg strength versus the control group. As such, perhaps it is the act of RT, rather than the protocol itself, that is the ultimate determinant of muscle hypertrophy and strength. Collectively, the results of the aforementioned studies demonstrate that RT has beneficial effects on muscle mass, muscle strength, muscle function and intracellular bioenergetics in the elderly. RT performed 2-3 times weekly thus represents a safe and practical intervention to counteract sarcopenia and its deleterious effects.

### *Protein Consumption*

Skeletal MyoPS is driven and regulated by a diverse host of factors. However, the fundamental prerequisite for MyoPS, muscle mass accretion, and retention is the delivery of diet-derived amino acids from protein-containing foods. Simply put, new product (i.e. SkM) cannot be made without precursor provision (i.e. amino acids from protein-containing foods and supplements). As previously mentioned, leucine is an essential amino acid (EAA) that has been

shown to activate mTORC1 and is therefore purported to be intimately involved in muscle hypertrophy. Because the body cannot endogenously synthesize EAAs, they must be acquired through the diet. As such, several studies have examined the acute and chronic effects of EAA infusion and consumption on markers of muscle anabolism in elderly populations compared to younger cohorts. Volpi and colleagues have previously observed on several occasions that healthy elderly individuals respond to an amino acid stimulus, provided as either a primed, constant infusion or oral ingestion, with increased amino acid delivery, transport, and increased MyoPS (Volpi, Ferrando, Yeckel, Tipton, & Wolfe, 1998; Volpi, Kobayashi, Sheffield-Moore, Mittendorfer, & Wolfe, 2003; Volpi, Mittendorfer, Wolf, & Wolfe, 1999). Importantly, these works observed that the increases in both FSR and NPB were not significantly different in the elderly when compared to the younger cohort. Collectively, these works indicate that the protein-synthetic effect is not diminished with aging, as both FSR and NPB were positively affected in a similar manner between both young and old. Paddon-Jones and colleagues have also provided direct evidence of similar improvements on MyoPS between the elderly and young (Paddon-Jones et al., 2004). Although they observed that the rate at which EAAs increased in arterial circulation was slower in the elderly, the enrichment persisted longer (7.7±0.4% vs 8.8±0.4% average arterial enrichment over the entire infusion period for young vs old, respectively; $p=0.49$). Additionally, work from Symons and colleagues, again examining the difference in MyoPS kinetics between an elderly and young cohort, has shown a similar positive effect on FSR and NPB in the elderly following consumption of 113 g of lean beef containing roughly 10 g EAAs (Symons et al., 2007). Following lean beef consumption, each group experienced a similar ~51% increase in FSR. However, although peak plasma amino acid concentrations occurred roughly 100 minutes following ingestion of lean beef for each group, there was a

significantly higher peak plasma concentration of EAAs in the elderly versus the young cohort (2185±134 nmol/mL vs 1403±96 nmol/mL, respectively; $p<0.001$). These results indicate that, although the protein-synthetic effect is not diminished, the efficiency of protein synthesis may be lessened with age. This is direct evidence that the elderly may therefore require higher levels of plasma amino acids to maximally stimulate MyoPS to the levels of a younger cohort. If this is the case and elderly do require higher plasma amino acid concentrations to maximally stimulate MyoPS, it thus stands to reason that the elderly would need to consume a larger bolus of protein to achieve these increased peak plasma amino acid concentrations.

This idea has driven much of the discussion surrounding the current protein recommendations for the elderly. Currently, the recommended dietary allowance (RDA) for protein for men and women aged 19 years and older is 0.8 $g \cdot kg^{-1} \cdot d^{-1}$. These recommendations were established by the Institute of Medicine and, importantly, were based on short-term nitrogen balance studies in healthy, young adults (Rand, Pellett, & Young, 2003; Trumbo, Schlicker, Yates, & Poos, 2002). As such, these recommendations may not necessarily be sufficient for the elderly. When coupling the physiological alterations that come with aging, such as reduced gastrointestinal function (Rémond et al., 2015) thus likely increasing nutrient need through decreased absorption, with the current recommendations set at the lowest threshold to achieve the nutritional requirement based on studies in a young population, it can thus be argued that protein recommendations for the elderly should be increased. In fact, several investigators have posited that the current protein recommendations do not promote optimal health, nor do they protect the elderly from sarcopenia and muscle loss (Bunker, Lawson, Stansfield, & Clayton, 1987; Houston et al., 2008; Pepersack et al., 2002; Wolfe & Miller, 2008). Additionally, it has been observed that aging skeletal MyoPS responds in a dose-dependent manner to EAA

provision (Katsanos, Kobayashi, Sheffield-Moore, Aarsland, & Wolfe, 2005; Paddon-Jones et al., 2004), whereby provision of lower amounts of EAAs (roughly 7.5 g) produced a diminished MyoPS response in comparison to provision of higher EAAs (10-15 g) that produced a markedly increased MyoPS response. This further illustrates that aging SkM requires a greater EAA stimulus to achieve the same MyoPS response as that of younger SkM. Work from Moore and colleagues has shown a similar trend after regression analysis of previously conducted studies examining the difference in amino acid kinetics between elderly and young revealed that MyoPS reached a plateau after ingestion of 0.40±0.19 and 0.24±0.06 g/kg body mass (elderly vs young, respectively; $p=0.055$) and 0.60±0.29 and 0.25±1.3 g/kg lean body mass (elderly vs young, respectively; $p<0.01$), respectively (Moore et al., 2015). The totality of evidence from these works strongly indicates that, although the elderly can achieve similar MyoPS rates as compared to levels of younger individuals, they nonetheless require a larger protein bolus to do so.

As an example, for an 80 kg elderly individual the RDA would advise consuming 64 g of protein each day to offset daily nitrogen losses. Given the aforementioned discussion regarding the periodic activation of mTORC1 for optimal MyoPS, if this individual were to spread protein consumption across three meals this would equate to roughly 21 g of protein at each meal. Considering that a 21 g serving of most animal or plant-based protein contains roughly 5-8 g of EAAs (Paddon-Jones & Rasmussen, 2009), it is unlikely that this serving would result in sufficient MyoPS to stimulate muscle hypertrophy. Rather, this individual would need to increase the protein content of each meal by roughly 9 g of protein, which should suitably provide the extra 2 g of EAAs needed for adequate MyoPS and subsequently provide an optimal stimulus for muscle hypertrophy. This idea is corroborated by Paddon-Jones and Rasmussen, who have previously proposed that the consumption of three separate 30 g bolus' of protein

spread throughout the day would result in greater hypertrophy due to a more frequent MyoPS stimulation as opposed to three separate bolus' of 10 g, 20 g, and 60 g (Paddon-Jones & Rasmussen, 2009). If we accept that 1) EAAs, specifically leucine, activate mTORC1, 2) mTORC1 activation stimulates MyoPS, 3) periodic and frequent mTORC1 activation results in increased periods of MyoPS and reduced periods of MyoPB, and 4) 10 g of EAAs provided in a 30 g bolus of protein results in ideal MyoPS for the elderly, we can therefore argue that increasing the amount of protein throughout the day would potentially have beneficial effects on diminishing and offsetting the effects of sarcopenia. As such, protein supplementation represents an intriguing avenue to prevent or delay the development of sarcopenia. Although there is less data regarding the efficacy of different protein-containing supplements (PCS) on MyoPS and hypertrophy in the elderly, there is a wealth of data in younger populations. Common PCS examined include dairy-derived whey protein (McAdam et al., 2018; Mobley et al., 2017; West, Abou Sawan, Mazzulla, Williamson, & Moore, 2017), plant-based soy (Haun et al., 2018; Messina, Lynch, Dickinson, & Reed, 2018; Mobley et al., 2017), and other proteins (Joy et al., 2013; Xia et al., 2018). These studies provide direct evidence that incorporating a PCS in the diet has beneficial effects on MyoPS, muscle hypertrophy, muscle quality, muscle function, muscle strength, and/or body composition. Importantly, however, the extent to which these PCSs contribute to the aforementioned metrics remains to be determined.

### *Combination Resistance Exercise Plus Protein Consumption*

Both RT and protein consumption individually have been observed to positively affect muscle protein kinetics and/or muscle mass, composition, and function. Considering that each affects MyoPS and hypertrophy through similar mechanisms, it stands to reason that a combination of the two may produce a synergistic effect greater than either in isolation. Recent

works have illustrated that combination RT and PCS have beneficial effects not only on muscle mass retention and muscle function in the elderly, but also on strength (Deutz et al., 2014; Guimarães-Ferreira et al., 2014; Hidayat et al., 2018; Liao et al., 2018; Liao et al., 2017; Morton et al., 2018; S. M. Phillips, 2015). This is important when considering that the EWGSOP recently redefined sarcopenia to focus on strength rather than muscle mass.

Upon examination of the literature regarding RT and PCS in the elderly, there are vast discrepancies in the amount and/or kind of protein provided, the type of exercise performed, and the duration of the studies. Two studies examining the effects of low-dose protein (i.e. 3-6 g leucine) in combination with a full-body RT program in a sarcopenic urban community reported modest increases in walking speed and steps-per-minute, but no appreciable differences in body composition (H. Kim et al., 2016; H. K. Kim et al., 2012). The most profound effects were observed when comparing the protein plus exercise group to the control group (which received only health information) for leg muscle mass, walking speed, and knee extension strength. However, when comparing the protein plus exercise group to either the exercise alone or protein alone group there were no differences in any outcome variable. While these results indicate that exercise plus protein can have positive effects on muscle mass, function, and strength when compared to a sedentary lifestyle, neither the protein dosage nor exercise intervention alone was sufficient to elicit meaningful results. Similarly, in a combination RT plus PCS study supplying roughly 15 g of soy protein to an elderly population, there was no effect of the supplement on any primary outcome measure, including muscle power, muscle strength, and body composition (Fiatarone et al., 1994). There was, however, a significant increase in muscle strength for both the exercise (~90% increase) and exercise plus supplement (~135% increase) group above that of the control group (~4% increase; $p<0.001$ for exercise vs control and exercise plus supplement

vs control). However, there was no difference between the exercise and the exercise plus supplement group. These results indicate that while RT plus protein consumption can positively impact muscle strength, the protein dosage in this particular study may have been too low to observe an effect. Realizing this, Molnár and colleagues sought to examine the effects of a pharmacologic dose of protein in combination with a RT protocol on measures of sarcopenia. The authors found that the provision of a 40 g whey PCS (consumed 2x/d with meals) in combination with a RT program elicited a significant increase in muscle strength in an elderly population in a long-term care facility (Exercise=22.51±2.35 kg, Exercise plus protein=24.54±2.65 kg) (Molnár et al., 2016). Taken together, these studies not only provide evidence that combination RT and PCS has positive effects on markers of muscle strength, but they also highlight that a higher dose of protein is necessary to elicit a physiological benefit to SkM in response to RT in the elderly.

Concerning the duration of the intervention period, most studies examining the effects of a combination RT and PCS in the elderly have tended to range from 3-6 months (Bonnefoy et al., 2012; Fielding, Travison, Kirn, Koochek, Reid, von Berens, Zhu, Folta, Sacheck, Nelson, Liu, Åberg, et al., 2017; H. Kim et al., 2016; Mariangela Rondanelli et al., 2016; Minoru Yamada et al., 2019). Most studies that have examined the effects of RT and PCS for a period shorter than 3 months have examined elderly with pre-existing conditions, such as elderly with polymyalgia rheumatica (Björkman, Pilvi, Kekkonen, Korpela, & Tilvis, 2011), or those currently receiving treatment for an acute illness in a hospital (Hegerová, Dědková, & Sobotka, 2015). Conversely, a lone study from Bonnefoy and colleagues examined the effects of a RT program and protein supplement in the elderly for 9 months and concluded that the provision of an additional 30 g of protein each day in the form of two ‘nutritional energy drinks’ resulted in a significant increase

in muscle power (p=0.03) at 3 months, but not at 9 months. In addition, the authors observed a nonsignificant increase in fat free mass at 9 months (Bonnefoy et al., 2003). While this study illustrates that a long-term intervention is feasible (63% compliance for the RT group vs 54% compliance for the nonexercised group), given that the most profound results on strength were observed at 3 months, this indicate that elderly SkM may be more responsive to shorter periods of mechanical tension and nutrition. This is corroborated by the literature indicating that profound muscle strength (20.9% vs 3.9% increases) and quality (5.6% vs -1.8%) adaptations are observed earlier in untrained populations (Ahtiainen, Pakarinen, Alen, Kraemer, & Häkkinen, 2003).

Given the aforementioned review of fat infiltration into SkM and the associated fat gain associated with sarcopenia, several researchers have sought to examine the effects of RT and either a PCS or increased protein consumption on weight loss and markers of sarcopenia. Verreigen and colleagues examined the effects of high- (~1.13 g/kg body weight) versus normal (~0.98 g/kg body weight) protein intake in combination with a triweekly RT program and hypocaloric diet (~600 kcal deficit) on markers of body weight, fat mass, and fat free mass (Verreijen et al., 2017). The authors concluded that there was no significant effect of either protein or RT alone on any marker of body composition or strength. There was, however, a significant increase in fat-free mass in the RT and protein group versus the control group (+0.6±1.3 vs +0.0±1.4 kg, respectively; p=0.011). Despite the significant effect of the exercise plus increased protein regimen, the lack of significant results may be due to several reasons, including the body composition assessment metric (air displacement plethysmography) and the relative lack of difference between the high- and normal-protein group (~1.3 g/kg body weight vs ~0.98 g/kg body weight, respectively). Work from Galbreath and colleagues (Galbreath et al.,

2018) has also sought to assess the effects of 14 weeks of RT and protein consumption on markers of strength and body composition in the elderly. When compared to a eucaloric RT group, the high-protein (~1.2 g/kg/day) experienced significant reductions in body weight (-0.87±0.2 vs -3.88±0.6 kg, respectively; $p<0.001$), fat mass (-0.82±0.1 vs -3.36±0.4 kg, respectively; $p<0.001$), and body fat percentage (-0.90±0.2 vs -2.71±0.5%, respectively; $p=0.005$) following the RT intervention. Despite the positive results regarding body composition, the lack of significant results regarding muscle strength may be due to the relatively low increase in protein of the high-protein diet (i.e. advised to increase protein consumption to 1.2 g/kg/day). Given the aforementioned discussion regarding an adequate protein bolus to maximally stimulate MyoPS, it is thus likely that the lack of difference in strength observed between groups is due to a reduced protein consumption in regard to what is optimal. Considering the discrepancies in RT protocol, protein consumption/recommendations, age, health, and gender of the interventions aimed at sarcopenia utilizing a combination RT and protein approach, Sardeli and colleages (Sardeli, Komatsu, Mori, Gáspari, & Chacon-Mikahil, 2018) sought to synthesize the totality of the existing data in a systematic review. After the inclusion of 6 studies, the authors observed that the reductions in body mass and fat body mass were not different between groups performing RT and utilizing CR versus groups performing RT alone. However, the reductions in LBM between the two groups was significant (LBM in RT + CR group was, on average, 93.5% less than the CR alone group; $p<0.001$), and the percentage of muscle quality changes, as defined as force production per unit of muscle tissue, trended toward significance (CR + RT=20.9±23.1 vs CR along=-7.5±9.9%; $p=0.007$). These results indicate that a combination RT plus protein consumption can have positive effects on body composition and muscle function even in a caloric deficit.

The culmination of the aforementioned data indicate that: 1) there is a decrease in muscle mass with age that stems from many reasons, 2) this decrease in muscle mass results in detrimental health consequences, ranging from minor to catastrophic, 3) RT and protein feeding individually can potentially reduce muscle mass losses via augmentation of intracellular pathways associated with anabolic signaling, 4) a combination approach of RT coupled with simultaneous protein feeding appears to have a greater positive impact on SkM than either RT or protein feeding in isolation, and, finally, 5) reversal of muscle mass losses with age appears to have positive effects on both health and longevity.

## Statement of Purpose

To date, no study has examined the effects of a protein supplement derived from peanut protein in combination with a RT program in an untrained, elderly population. Considering the prevalence of diminished muscle mass in the elderly (Choi, 2016; Siparsky et al., 2014; Volpi et al., 2004), the increasing average age and lifespan of the population (Kontis et al., 2017), and the positive effects that RT (Schoenfeld et al., 2016) and PCS (Pasiakos, Lieberman, & McLellan, 2014) can have on muscle retention and overall health, it is worth evaluating if peanut-derived protein (PP) can have the same positive effects. To my knowledge, only one study has sought to examine the effects of peanut consumption on any body composition metric (Barbour, Howe, Buckley, Bryan, & Coates, 2015). These authors observed nonsignificant results in regard to cardio-metabolic risk factors and body composition in older individuals. However, muscle composition, muscle mass, and muscle function were not assessed. As such, no study has assessed peanut supplementation on markers of muscle composition, muscle mass, and muscle function. Therefore, the purpose of this study was to elucidate what effects, if any, a ten-week RT program coupled with or without PP could have on markers of MyoPS, muscle quality, body

composition and strength in an untrained, elderly population. Although our original intent was to recruit two separate ten-week cohorts of participants over the course of one year, due to the unforeseen impacts of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) pandemic, we were forced to consolidate our second cohort of participants into a six-week RT program.

## Significance of the Study

This study will further our understanding of how RT and protein supplementation can impact the health of elderly individuals. Rather than focus on a niche group that is not representative of the general population (i.e. Olympic powerlifters, ultra-marathon enthusiasts, elite track athletes, etc.), I propose to examine how a biweekly, full-body exercise program consisting of five unique sets performed three times each and lasting roughly thirty minutes can improve markers of muscle quality, body composition, and strength. Although there has been an increase in the amount of research targeting the elderly, there are still significant gaps in our understanding of the aging process and ways to diminish its deleterious effects. This study seeks to address some of these gaps and contribute to the growing knowledge of healthy aging.

## Hypotheses

I hypothesize that:

1. Given the aforementioned literature demonstrating that RT alone can elicit improvements in muscle mass and muscle strength, RT in the CTL group will result in improvements in muscle mass and muscle strength.
2. PP supplementation in combination with RT will result in greater improvements in muscle mass and muscle strength given that protein supplementation in combination with RT has been shown to outperform RT alone.

3. PP supplementation in combination with RT will result in greater improvements in body composition given that protein supplementation in combination with RT has been shown to outperform RT alone.

# CHAPTER 3: METHODOLOGY

## Ethical Approval and Participant Screening

Prior to any data collection, this study was approved by the Auburn University Institutional Review Board (IRB) (Protocol # 19-249 MR 1907), conformed to standards set by the latest revision of the Declaration of Helsinki and was registered as a clinical trial (NCT04015479). Men and women aged 50-80 years with minimal RT experience, defined here as not having performed structured RT for at least three months prior, were recruited for this study (n=41).

Participants were contacted via flyer, email inquiry and newspaper advertisement. Interested participants were informed of the study and testing procedures either over the phone or face-to-face at the Auburn University School of Kinesiology. Inclusion criteria included that participants must not be allergic to peanuts, have no known overt cardiovascular/metabolic disease, have had no medically necessary radiation exposure within the last six months other than minor dental x-rays, have normal blood pressure (i.e. <140/90 SBP/DBP) with or without medication and be free of any precluding medical conditions that would contraindicate participating in exercise, giving blood or undergoing a muscle biopsy. Participants deemed eligible provided written and verbal consent to participate. A medical history questionnaire was obtained at the time of consenting and participants were scheduled to return to the Auburn University School of Kinesiology to complete baseline testing.

## Study Design

Our original intent was to recruit two separate ten-week cohorts. Due to the SARS-CoV-2 pandemic, we were forced to end the second cohort after only six weeks. As such, the primary

difference between cohorts was the length of the intervention. The study design is presented in Figure 1.

INSERT FIGURE 1 HERE

Briefly, participants in the 10-week cohort reported to the Auburn University School of Kinesiology on 24 separate occasions, whereas participants in the 6-week cohort reported on 16 separate occasions. Visit one (V1) included screening to determine eligibility, gathering consent and obtaining a health history. Visit two (V2; PRE) occurred at least three days prior to visit 3 (V3) and included a battery of assessments comprised of urine specific gravity (USG) , height and body mass, ultrasound of the right leg vastus lateralis , full body dual energy x-ray absorptiometry (DXA), peripheral quantitative computed tomography (pQCT) scan at the mid-thigh of the right leg and right leg strength assessment using an isokinetic dynamometer. Following the battery of assessments, participants were provided with deuterium oxide (D2O)-enriched water, a 3-day food log, and three separate salivettes to measure D2O enrichment. The food log was returned prior to V3 at each participant's convenience.

V3 included the participant's first muscle tissue sample collection, randomization to either the peanut protein supplement group (PP) or wait-list control (CTL), first resistance exercise bout, and immediate post-exercise PP supplementation or no supplementation. A complete nutritional breakdown for the PP supplement is presented in Table 1. V4 included the participant's second muscle tissue sample collection and salivette return. Visit five (V5) through visit twenty-three (V23) for the 10-week cohort and V5 through fifteen (V15) for the 6-week cohort included a single RE session. During V23 for the 10-week cohort and V15 for the 6-week cohort participants were provided with their second food log. Visit twenty-four (V24; POST) for the 10-week cohort and visit sixteen (V16; POST) for the 6W cohort occurred roughly 72 hours

following V23 and V15 for the respective cohorts and included a repeat of the V2 testing battery including USG assessment, height and body mass assessment, right leg ultrasound, full body DXA, right leg pQCT scan, and second food log collection. Specific methodologies of the testing batteries are detailed below.

INSERT TABLE 1 HERE

## Pre- and Post-intervention Testing Battery

The testing sessions described below occurred during morning hours (05:00–09:00) following an overnight fast for all but 7 participants who reported to the laboratory after working hours at 17:00-18:30 following a ~4-5 hour fast.

### *Body Composition Assessments*

During V2 and V24 (10-week participants) or V16 (6-week participants), participants reported to the Auburn University School of Kinesiology wearing casual sports attire (i.e. athletic shirt and shorts, tennis shoes). Participants submitted a urine sample (~5 mL) to assess USG levels using a handheld refractometer (ATAGO; Bellevue, WA, USA). If the participants USG level was greater than 1.020 the participant was provided with roughly 500 mL of water to drink before any assessments began. Height and body mass were assessed using a digital column scale (Seca 769; Hanover, MD, USA) with mass and height being collected to the nearest 0.1 kg and 0.5 cm, respectively. Thereafter, right leg vastus lateralis images were captured in the transverse plane using real-time B-mode ultrasonography (LOGIQ S7 Expert, GE Healthcare, USA) utilizing a multi-frequency linear-array transducer (3-12 MHz, GE Healthcare, USA) and subsequently analyzed for VL thickness. Participants were instructed to stand and displace bodyweight to the left leg to ensure the right leg was relaxed. Measurements were standardized by placing the transducer at the midway point between the inguinal crease and proximal border

of the patella. All images were captured and analyzed by the same investigator (S.C.O.) with a 24-hr test-retest reliability using intraclass correlation coefficient ($ICC_{3,1}$), standard error of the measure (SEM), and minimal difference (MD) to be considered real of 0.991, 0.06, and 0.16 cm, respectively. Participants then underwent a full body dual-energy x-ray absorptiometry (DXA) scan (Lunar Prodigy; GE Corporation, Fairfield, CT, USA) for determination of total lean soft tissue mass (LSTM) and fat mass (FM). Quality assurance testing and calibration were performed the morning of data-collection days to ensure the scanner was operating to manufacturer specification. Scans were analyzed by the same technician using the manufacturer's standardized software. Test-retest reliability using $ICC_{3,1}$, SEM, and MD were previously determined for LSTM (0.99, 0.36, and 0.99 kg, respectively) and FM (0.99. 0.43, and 1.19 kg). Following the DXA scan, a cross-sectional image of the right thigh at 50% of the femur length was acquired using a pQCT scanner (Stratec XCT 3000, Stratec Medical, Pforzheim, Germany). Scans were acquired using a single 2.4 mm slice thickness, a voxel size of 0.4 mm and scanning speed of 20 mm/sec. All images were analyzed for total muscle cross-sectional area (mCSA, cm2) and density (mg/cm3) using the pQCT BoneJ plugin freely available through ImageJ analysis software (NIH, Bethesda, MD). All scans were performed and analyzed by the same investigator (K.C.Y.). Test-retest reliability using $ICC_{3,1}$, SEM, and MD was previously determined for mCSA (0.99, 0.84, and 2.32 $cm^2$, respectively).

*Right Leg Isokinetic Strength Assessment*

Participants performed maximal isokinetic right leg extensions on an isokinetic dynamometer (System 4 Pro, BioDex Medical Systems, Shirley, NY, USA). Participants were fastened to the dynamometer so that the right knee was aligned with the axis of the dynamometer. Seat height was adjusted to ensure the hip angle was approximately 90°. Prior to

peak torque assessment, each participant performed a warmup consisting of submaximal to maximal isokinetic knee extensions. Participants then completed five maximal voluntary isokinetic knee extension actions at 60°/sec and 120°/sec. Sets were separated by 60 sec of rest. Participants were provided verbal encouragement during each set. The isokinetic extension resulting in the greatest peak torque value was used for analyses. Right leg extensor peak torque testing occurred ~1-3 days prior to the muscle biopsy at the PRE (V2) time point in both the 10W and 6W cohorts, whereas this test occurred approximately 10 minutes following the biopsy at the POST time point for the 10W cohort only (V24). This difference in methodology between time points was due to logistical constraints. However, we have unpublished data suggesting peak torque values are not affected by muscle biopsies when isokinetic testing occurs within a 10-minute post-biopsy window (Haun et al., 2017).

*Supplement Randomization and Resistance Training*

During V3, immediately following collection of the first muscle sample, participants were randomized to either consume PP during the intervention (n=20) or after the intervention (n=19). The PP supplement (PBfit; BetterBody Foods, Lindon, UT, USA) provided the following per daily serving: 315 kcal, 35 g protein, 10.7 g essential amino acids (where 2.44 g was L-leucine), 9.0 g fat and 22.5 g carbohydrate (with 14.8 g fiber and 7.7 g sugars). Randomization was stratified by gender in blocks of four, hence the slight differences in allocation to study arms. Afterwards, participants were escorted to the Auburn University School of Kinesiology Fitness and Performance Optimization Laboratory for their first resistance exercise session. Participants were provided detailed instructions on proper posture, technique, range-of-motion, body positioning and breathing to ensure safety. Participants completed supervised RT twice weekly for either ten weeks or six weeks. All RT sessions were separated

by at least 48 hours to allow for a period of recovery. Each RT session consisted of five exercises including seated leg press, leg extensions, lying leg curls, barbell bench press and cable pull-downs. Upper body exercises were included because changes in appendicular body composition was a secondary aim. For each exercise, participants performed 3 sets of 10-12 repetitions with 1 minute of rest between sets. At the end of each set, participants were asked to rate the level of difficulty where 0 = easy, 5 = moderate difficulty and 10 = hard. If values were below 7, weight was modestly added to increase exertion. If values were 10, or the participant could not complete the set, weight was removed or the number of reps completed was reduced. Participants were encouraged to be as truthful as possible when assessing difficulty and were provided verbal encouragement and feedback during and following each set. The intent of this training method was to consistently challenge participants so that perceived exertion after each set was at a 7-9 rating. Training data for each participant were logged. This allowed us to ensure that training effort was maximized within each training session, and that the participants were successfully implementing progressive overload in an individualized fashion.

Notably, study personnel supervised all training throughout the study. Additionally, participants in the PP group were instructed to consume one daily serving of the PP supplement. On workout days, PP supplements were provided to participants in the PP group immediately following exercise, and supplementation compliance was supervised. On non-workout days, participants were instructed to consume one serving between meals. Product bottles were returned to the study coordinator to ensure compliance to the supplementation protocol.

### *Muscle Sample Collection and Integrated Myofibrillar Protein Synthesis Rate Determination using Deuterium Oxide*

MyoPS rates were determined after the first RT bout with or without PP supplementation using the integrated $D_2O$ technique (C. G. Brook, 1971). Briefly, participants consumed a total 4.5 $mL \cdot kg^{-1}$ of lean body mass (LBM) of $D_2O$-enriched water (70 atom percent; Sigma-Aldrich, St. Louis, MO) to label the body water pool to ~0.2% atom percent excess (APE) over the course of four separate days beginning 2 days prior to V2 through V3. Participants were provided with six individual servings of $D_2O$. Three of these servings contained 1 $mL \cdot kg^{-1}$ LBM $D_2O$ and were to be consumed in a single day, and three of these servings contained 0.5 $mL \cdot kg^{-1}$ LBM $D_2O$ and were to be consumed over the next three consecutive days. Participants were instructed to consume them in the following manner: a) approximately 48 hours prior to V3 participants were instructed to consume the three servings containing 1.0 mL/kg $D_2O$ over the course of a single day to saturate the body water pool; one serving during the morning, one serving during the afternoon and one serving in the evening, b) approximately 24 hours prior to V3 participants were instructed to consume one serving of 0.5 $mL \cdot kg^{-1}$ LBM $D_2O$ to maintain body water concentrations, c) following an overnight fast, on the morning of V3, before reporting to the Auburn University School of Kinesiology for their first muscle biopsy sample donation and first RT session, participants were instructed to consume one serving of 0.5 $mL \cdot kg^{-1}$ LBM $D_2O$ to maintain whole-body $D_2O$ concentrations, d) on the morning of V4, following an overnight fast and before reporting to the Auburn University School of Kinesiology for their second muscle biopsy sample donation, participants were instructed to consume one serving of 0.5 $mL \cdot kg^{-1}$ LBM $D_2O$ to maintain whole-body $D_2O$ concentrations.

Skeletal muscle biopsies at V3 and V4 were obtained from the right thigh (i.e., the vastus lateralis) midway between the patella and iliac crest using a 5-gauge needle with suction and sterile laboratory procedures. Briefly, upon arrival to the laboratory, participants were instructed

to lie in a supine position on an athletic training table. Roughly 5 minutes afterwards, 1.5 mL of 1% lidocaine was injected subcutaneously above the skeletal muscle fascia a small pilot incision was made for needle insertion using a sterile Surgical Blade No. 11 (AD Surgical; Sunnyvale, CA, USA). After 5 minutes of allowing the anesthetic to take effect, the biopsy needle was inserted into the pilot incision just beyond the fascia and approximately 50-100 mg of skeletal muscle was removed using a double chop method and applied suction (Evans, Phinney, & Young, 1982). Following biopsies, tissue was rapidly teased of blood and connective tissue and subsequently stored at -80°C until shipment to Metabolic Solutions (Nashua, NH, USA) for tracer analyses. All biopsies were performed by the same investigator (M.D.R.).

MyoPS rates over the 24-hour period following the first training bout were calculated similar to Bell et al. (Bell et al., 2019) (see equation below).

$$FSR\ (\%day^{-1}) = \left[\frac{(E_{Ala2} - E_{Ala1})}{E_{BW} \times t}\right] \times 3.7 \times 100$$

In the equation above, $E_{Ala1}$ and $E_{Ala2}$ represent $^2H$ enrichment in the first and second muscle biopsies, respectively (in atom percent excess). $E_{BW}$ is the average $^2H$ enrichment (in atom percent excess) of total body water from the second and third salivettes after subtracting background values from the baseline salivette. *t* is time in the number of days $D_2O$ was ingested (which equals 1 herein). The 3.7 coefficient adjusts for average $^2H$ atoms that can be bound to alanine, and final values were expressed as % synthesis per day by multiplying values by 100.

*Dietary recall analysis*

Participants were instructed to self-report their habitual food intake for three consecutive days and return these food logs during V3 and V24 (10-week cohort) or V16 (6-week cohort). Participants were asked not to change their diet in any way with the exception of PP participants who were instructed to consume the supplement as described above. Study staff entered each day

of the food log into the Automated Self-Administered 24-Hour Dietary Assessment tool (ASA24), which uses the United States Department of Agriculture Food and Nutrient Database for Dietary Studies to provide values for 195 nutrients, nutrient ratios and other food components (Subar et al., 2012).

### *Statistical analysis*

All statistical analyses were performed using SPSS v26.0 (IBM Corp, Armonk, NY, USA). For MyoPS and training volume comparisons between the PP and CTL groups independent samples t-tests were used. For all dependent variables over time, repeated measures two-way (group × time; GxT) ANOVAs were performed. When a significant interaction occurred, LSD post hocs were performed between and within groups to determine the level of significance. With the exception of MyoPS data, all data in figures are presented to show the 6-week cohort individually, 10-week cohort individually, and pooled cohorts collectively. Group, time and GxT data are provided for each cohort individually and when pooled. Statistical significance was established as $p<0.05$, and relevant p-values are depicted in-text for tables or within figures.

## Research Aims

Primary Aim 1: To determine the acute effects of PP consumption on SkM myofibrillar protein synthesis (MPS) rates using the $D_2O$ tracer method.

Primary Aim 2: To determine the effects of PP supplementation during resistance training on SkM growth and quality as measured by pQCT.

Secondary Aims: To determine the effects of PP during resistance training on:

1) Whole body and appendicular body composition as measured by DXA

2) Type I and II muscle fiber cross sectional area (fCSA)*

*We were unable to perform the associated analyses given the SARS-CoV-2 circumstances*

## CHAPTER 4: JOURNAL MANUSCRIPT

**Effects of resistance training with or without peanut protein supplementation on skeletal muscle and strength adaptations in older, untrained individuals**

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ABSTRACT

It is generally accepted that resistance training (RT) in conjunction with protein supplementation has positive effects on strength and muscle mass in older individuals. However, to date, no study has examined the effects of a RT program in combination with a high-protein, defatted peanut powder (PP) supplement on these markers. Herein, 39 older, untrained individuals (n=17 female, n=22 male; age=58.6±8.0 years; body mass index =28.7±5.8) completed a 6-week (n=22) or 10-week (n=17) RT program, where full-body training was implemented twice weekly. Participants were randomly assigned to consume either a PP supplement shake once per day (35 g protein, 315 kcal; n=20) or no supplement (CTL; n=19). Right leg vastus lateralis (VL) muscle biopsies were obtained prior to and 24 hours following the first training bout in all participants to assess changes in myofibrillar protein synthetic rates (MyoPS) as measured via the deuterium-oxide ($D_2O$) tracer method. All participants also completed PRE- and POST-intervention testing including dual energy x-ray absorptiometry (DXA), VL ultrasound, mid-thigh peripheral quantitative computed tomography (pQCT) scan, and right leg strength assessment using an isokinetic dynamometer. There was a significant group-by-time interaction for protein consumption when cohorts were pooled ($p=0.008$), and a trend toward significance when cohorts were examined individually as a 10-week ($p=0.086$) and 6-week ($p=0.072$) cohort. MyoPS rates were not significantly different between supplement groups following the first workout bout. Regarding chronic changes, there were no significant group-by-time interactions ($p<0.05$) in DXA-derived fat mass, lean soft tissue mass (LSTM) or percent body fat between supplementation groups. There was, however, a significant increase in VL thickness for PP versus CTL participants when the 6- and 10-week cohorts were pooled (interaction $p=0.041$). There was also a significant increase in knee flexion torque in the 10-week PP group versus the

CTL group (interaction p=0.032). In conclusion, a high-protein, defatted peanut powder supplement in combination with RT may have beneficial effects on select indices of muscle hypertrophy and strength in an untrained, older adult population.

Keywords: muscle, resistance training, aging, peanut protein supplementation

## INTRODUCTION

The size and number of muscle fibers remain relatively stable until the fifth decade of life, at which point an appreciable decrease in both total muscle fibers and size occurs [1, 2]. The gradual age-related decrease in muscle mass and strength, termed sarcopenia, culminates in a reduction of nearly 40% of an individual's total muscle mass by the eighth decade of life [3-5]. Sarcopenia coincides with cellular, neuromuscular and metabolic perturbations [6], and the most recent definition and diagnostic criteria established by the European Working Group on Sarcopenia in Older People (EWGSOP) focuses on reductions in muscle strength, quantity and quality [7]. Not only does sarcopenia directly contribute to increased frailty and fragility through diminished muscle mass, muscle quality and strength [8, 9], but it indirectly contributes to more serious health consequences such as decreased quality of life and premature death [10]. Therefore, interventions targeting the retention of muscle mass with aging have garnered much attention.

Numerous studies have demonstrated that 8-16 weeks of resistance training (RT) can increase muscle mass and strength in older individuals (reviewed in [11, 12]). Given that protein feeding stimulates an anabolic response in skeletal muscle [13], it stands to reason that combining protein supplementation with RT likely optimizes increases in muscle mass [14]. Animal-based protein sources possess the full complement of essential amino acids needed to stimulate the muscle-building process at the molecular level (i.e., increases in post-meal myofibrillar protein synthesis or MyoPS rates) [15]. Moreover, it has been well-documented that dairy-derived protein supplements (e.g., milk or whey protein concentrates or isolates) can enhance increases in muscle mass with RT relative to other protein sources [16]. However, there has been a growing interest in the health benefits of plant-based foods, as well as concerns related to the

sustainability issues regarding the procurement of animal-based proteins [17]. In this regard, data from the National Health and Nutrition Examination Survey indicate that intakes of plant proteins increased significantly from 1999 to 2010 [18], and there is sentiment that consumers will continue to increase plant protein intake for the foreseeable future [19].

Protein isolates from several plant-based foods (i.e. soy, pea, rice, and hemp) are currently sold to consumers with the intent of supporting the rigorous demands of exercise training. There has also been a recent growth in the popularity and availability of peanut flour and defatted peanut powder. With the exception of containing low methionine and threonine levels, peanut protein possesses a full complement of essential and non-essential amino acids [20]. Relative to other plant-based proteins (e.g., wheat or legumes), peanut protein possesses a relatively high protein digestibility corrected amino acid score (0.70/1.00) [20]. Furthermore, it has been posited that peanut protein can be used as an ingredient for protein fortification in low-protein food sources [21]. Despite these positive statistics surrounding peanut protein, no study to date has examined if a peanut protein supplement combined with RT can enhance training adaptations. Therefore, the purpose of this study was two-fold. First, we sought to determine if post-exercise PP supplementation could enhance the MyoPS response to one RT bout in minimally trained, older participants. Second, we sought to determine if PP supplementation with 10 weeks of RT could enhance muscle quality, strength and body composition in these same participants. We hypothesized that RT alone would elicit improvements in muscle mass, muscle strength and body composition, and the addition of PP supplementation in combination with RT would produce substantial increases in muscle mass, muscle strength and body composition above that of RT alone.

METHODS

*Ethical Approval and Participant Screening*

Prior to any data collection, this study was approved by the Auburn University Institutional Review Board (IRB) (Protocol # 19-249 MR 1907), conformed to standards set by the latest revision of the Declaration of Helsinki and was registered as a clinical trial (NCT04015479). Men and women aged 50-80 years with minimal RT experience, defined here as not having performed structured RT for at least three months prior, were recruited for this study (n=41).

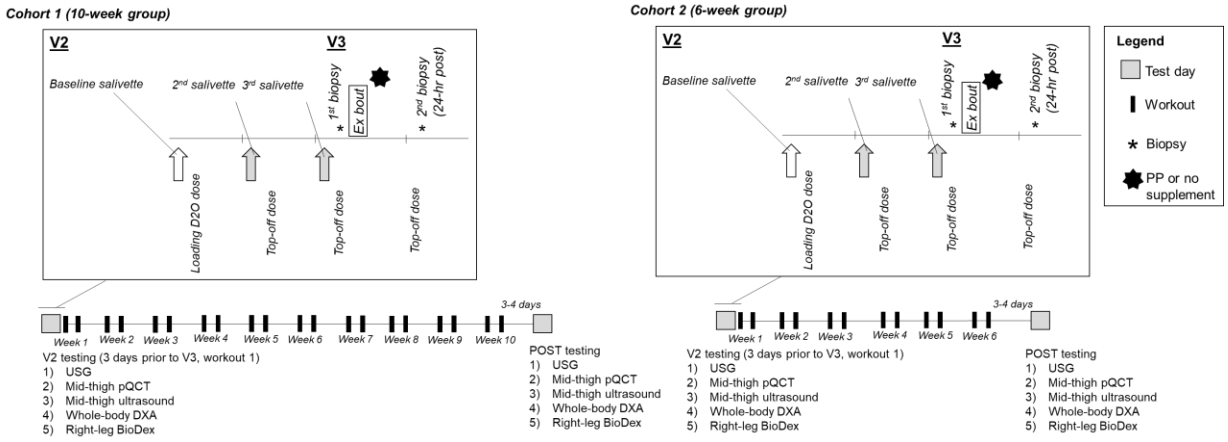
Participants were recruited via flyer, email inquiry and newspaper advertisement. Interested participants were informed of the study and testing procedures either over the phone or face-to-face at the Auburn University School of Kinesiology. Eligibility criteria included that participants should: 1) be between the ages of 50-80 years old; 2) not be actively participating in structured RT for at least 3 months prior; 3) be free of metal implants; 4) have an average blood pressure within normal ranges, with or without medication (i.e. <140/90 SBP/DBP). Exclusion criteria included that participants should: 1) have no known peanut allergy; 2) have a body mass index (BMI) $\geq$ 35 kg/m$^2$; 3) have had no exposure to medically-necessary radiation in the last 6 months; 4) have no medical condition contradicting participation in a RT program, giving blood or donating a skeletal muscle biopsy (i.e. blood clotting disorders or taking blood thinning medications). Participants deemed eligible provided written and verbal consent to participate. A medical history questionnaire was obtained at the time of consenting and participants were scheduled to return to the Auburn University School of Kinesiology to complete baseline testing.

*Study Design*

Our original intent was to recruit two separate ten-week cohorts. However, due to the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) pandemic, we voluntarily decided to

end the second cohort after only six weeks. As such, the primary difference between cohorts was the length of the intervention. The study design is presented in Figure 1 below.

Figure 1. Study Design




Legend: The figure above outlines the study design for the 10- and 6-week cohorts.

Briefly, participants in the 10-week cohort reported to the Auburn University School of Kinesiology on 24 separate occasions, whereas participants in the 6-week cohort reported on 16 separate occasions. Visit one (V1) included screening to determine eligibility, gathering consent and obtaining a health history. Visit two (V2; PRE) occurred at least three days prior to visit 3 (V3) and included a battery of assessments comprised of urine specific gravity (USG) , height and body mass assessment, ultrasound of the right leg vastus lateralis (VL), full body DXA, pQCT scan at the mid-thigh of the right leg, and right leg strength assessment using an isokinetic dynamometer. Following the battery of assessments, participants were provided with $D_2O$, a 3-day food log, and three separate salivettes to measure D2O enrichment. The food log was returned prior to V3 at each participant's convenience.

V3 included the participant's first muscle tissue sample collection, randomization to either the peanut protein supplement group (PP) or wait-list control (CTL), first RT bout, and immediate post-exercise randomization to either PP supplementation or no supplementation. Visit 4 (V4) included the participant's second muscle tissue sample collection and salivette return. Visit five (V5) through visit twenty-three (V23) and visit fifteen (V15) for the 10- and 6-week cohort, respectively, included a single RT session. During V23 for the 10-week cohort and V15 for the 6-week cohort participants were provided with their second set of food logs. Visit twenty-four (V24; POST) and visit sixteen (V16; POST) for the 10- and 6-week cohort, respectively, occurred roughly 72 hours following V23 and V15, respectively, and included a repeat of the V2 testing battery. Specific methodologies of the testing batteries are detailed below.

*PRE- and POST-intervention Testing Battery*

The testing sessions described below occurred during morning hours (05:00–09:00) following an overnight fast for all but 7 participants who reported to the laboratory after working hours at 17:00-18:30 following a ~4-5 hour fast.

*Body Composition Assessments*. During V2 and V24 (10-week participants) or V16 (6-week participants), participants reported to the Auburn University School of Kinesiology wearing casual sports attire (i.e. athletic shirt and shorts, tennis shoes). Participants submitted a urine sample (~5 mL) to assess USG levels for adequate hydration status using a handheld refractometer (ATAGO; Bellevue, WA, USA). If the participants USG level was greater than 1.020 the participant was provided with roughly 500 mL of water to drink before any assessments began, as recommended by Sawka and colleagues [22]. Height and body mass were assessed using a digital column scale (Seca 769; Hanover, MD, USA) with mass and height being collected to the nearest 0.1 kg and 0.5 cm, respectively. Thereafter, right leg VL images

were captured in the transverse plane using real-time B-mode ultrasonography (LOGIQ S7 Expert, GE Healthcare, USA) utilizing a multi-frequency linear-array transducer (3-12 MHz, GE Healthcare, USA) and subsequently analyzed for VL thickness. Participants were instructed to stand and displace bodyweight to the left leg to ensure the right leg was relaxed. Measurements were standardized by placing the transducer at the midway point between the inguinal crease and proximal border of the patella. All images were captured and analyzed by the same investigator (S.C.O.) with a 24-hr test-retest reliability using intraclass correlation coefficient ($ICC_{3,1}$), standard error of the measure (SEM), and minimal difference (MD) to be considered real of 0.991, 0.06, and 0.16 cm, respectively. Participants then underwent a full body DXA scan (Lunar Prodigy; GE Corporation, Fairfield, CT, USA) for determination of total LSTM and fat mass (FM). Quality assurance testing and calibration were performed the morning of data-collection days to ensure the scanner was operating to manufacturer specification. Scans were analyzed by the same technician using the manufacturer's standardized software. Test-retest reliability using $ICC_{3,1}$, SEM, and MD were previously determined for LSTM (0.99, 0.36, and 0.99 kg, respectively) and FM (0.99. 0.43, and 1.19 kg). Following the DXA scan, a cross-sectional image of the right thigh at 50% of the femur length was acquired using a pQCT scanner (Stratec XCT 3000, Stratec Medical, Pforzheim, Germany). Scans were acquired using a single 2.4 mm slice thickness, a voxel size of 0.4 mm and scanning speed of 20 mm/sec. All images were analyzed for total muscle cross-sectional area (mCSA, $cm^2$) and density ($mg/cm^3$) using the pQCT BoneJ plugin freely available through ImageJ analysis software (NIH, Bethesda, MD). All scans were performed and analyzed by the same investigator (K.C.Y.). Test-retest reliability using $ICC_{3,1}$, SEM, and MD was previously determined for mCSA (0.99, 0.84, and 2.32 $cm^2$, respectively).

*Right Leg Isokinetic Strength Assessment.* Participants performed maximal isokinetic right leg extensions on an isokinetic dynamometer (System 4 Pro, BioDex Medical Systems, Shirley, NY, USA). Participants were fastened to the dynamometer so that the right knee was aligned with the axis of the dynamometer. Seat height was adjusted to ensure the hip angle was approximately 90°. Prior to peak torque assessment, each participant performed a warmup consisting of submaximal to maximal isokinetic knee extensions. Participants then completed five maximal voluntary isokinetic knee extension actions at 60°/sec and 120°/sec. Sets were separated by 60 seconds of rest. Participants were provided verbal encouragement during each set. The isokinetic extension resulting in the greatest peak torque value was used for analyses. Right leg extensor peak torque testing occurred ~1-3 days prior to the muscle biopsy at the PRE (V2) time point in both the 10-week and 6-week cohorts, whereas this test occurred approximately 10 minutes following the biopsy at the POST time point for the 10W cohort only (V24). This difference in methodology between time points was due to logistical constraints. However, we have unpublished data suggesting peak torque values are not affected by muscle biopsies when isokinetic testing occurs within a 10-minute post-biopsy window [23].

*Supplement Randomization and Resistance Training*

During V3, immediately following collection of the first muscle sample, participants were randomized to either consume PP during the intervention (n=20) or after the intervention (n=19). The PP supplement (PBfit; BetterBody Foods, Lindon, UT, USA) provided the following per daily serving: 315 kcal, 35 g protein, 10.7 g essential amino acids (where 2.44 g was L-leucine), 9.0 g fat and 22.5 g carbohydrate (with 14.8 g fiber and 7.7 g sugars).

Randomization was stratified by gender in blocks of four, hence the slight differences in allocation to study arms. Afterwards, participants were escorted to the Auburn University School

of Kinesiology Fitness and Performance Optimization Laboratory for their first RT session. Participants were provided detailed instructions on proper posture, technique, range-of-motion, body positioning and breathing to ensure safety. Participants completed supervised RT twice weekly for either ten weeks or six weeks. All RT sessions were separated by at least 48 hours to allow for a period of recovery. Each RT session consisted of five exercises including seated leg press, leg extensions, lying leg curls, barbell bench press and pronated-grip cable pull-downs. Upper body exercises were included because changes in appendicular body composition was a secondary aim. For each exercise, participants performed 3 sets of 10-12 repetitions with 1 minute of rest between sets. At the end of each set, participants were asked to rate the level of difficulty using the modified Borg scale, where 0 = easy, 5 = moderate difficulty and 10 = hard [24]. If values were below 7, weight was modestly added to increase exertion on the subsequent set. If values were 10, or the participant could not complete the set, weight was removed, or the number of reps completed was reduced. Participants were encouraged to be as truthful as possible when assessing difficulty and were provided verbal encouragement and feedback during and following each set. The intent of this training method was to consistently challenge participants so that perceived exertion after each set was at a 7-9 rating. Training data for each participant were logged, allowing us to ensure that training effort was maximized within each training session, and participants were successfully implementing progressive overload in an individualized fashion.

Notably, study personnel supervised all training throughout the study. Participants in the PP group were instructed to consume one daily serving of the PP supplement. On workout days, PP supplements were provided to participants in the PP group immediately following exercise, and supplementation compliance was supervised. On non-workout days, participants were instructed

to consume one serving between meals. Product bottles were returned to the study coordinator to ensure compliance to the supplementation protocol.

*Muscle Sample Collection and Integrated MyoPS Rate Determination using Deuterium Oxide*

MyoPS rates were determined after the first RT bout with or without PP supplementation using the integrated $D_2O$ technique [25]. Briefly, participants consumed a total 4.5 $mL \cdot kg^{-1}$ of lean body mass (LBM) of $D_2O$-enriched water (70 atom percent; Sigma-Aldrich, St. Louis, MO) to label the body water pool to ~0.2% atom percent excess (APE) over the course of four separate days beginning 2 days prior to V2 through V3. Participants were provided with six individual servings of $D_2O$. Three of these servings contained 1 $mL \cdot kg^{-1}$ LBM $D_2O$ and were to be consumed in a single day, and three of these servings contained 0.5 $mL \cdot kg^{-1}$ LBM $D_2O$ and were to be consumed over the next three consecutive days. Participants were instructed to consume these servings in the following manner: a) approximately 48 hours prior to V3 participants were to consume the three servings containing 1.0 mL/kg $D_2O$ over the course of a single day to saturate the body water pool; one serving during the morning, one serving during the afternoon and one serving in the evening, b) approximately 24 hours prior to V3 participants were to consume one serving of 0.5 $mL \cdot kg^{-1}$ LBM $D_2O$ to maintain whole-body $D_2O$ concentrations, c) following an overnight fast, on the morning of V3, before reporting to the Auburn University School of Kinesiology for their first muscle biopsy sample donation and first RT session, participants were instructed to consume one serving of 0.5 $mL \cdot kg^{-1}$ LBM $D_2O$ to maintain whole-body $D_2O$ concentrations, d) on the morning of V4, following an overnight fast and before reporting to the Auburn University School of Kinesiology for their second muscle biopsy sample donation, participants were instructed to consume one serving of 0.5 $mL \cdot kg^{-1}$ LBM $D_2O$ to maintain whole-body $D_2O$ concentrations.

Skeletal muscle biopsies at V3 and V4 were obtained from the right thigh (i.e VL; consistent with the ultrasound and pQCT assessments) midway between the patella and iliac crest using a 5-gauge needle with suction and sterile laboratory procedures as previously described by Mobley and colleagues [26]. Briefly, upon arrival to the laboratory, participants were instructed to lie in a supine position on an athletic training table for roughly 5 minutes to allow for normalization of body-fluid distribution. Afterwards, 1.5 mL of 1% lidocaine was injected subcutaneously above the skeletal muscle fascia. A small pilot incision was made for needle insertion using a sterile Surgical Blade No. 11 (AD Surgical; Sunnyvale, CA, USA). After 5 minutes of allowing the anesthetic to take effect, the biopsy needle was inserted into the pilot incision just beyond the fascia and approximately 50-100 mg of skeletal muscle was removed using a double chop method and applied suction [27]. Following biopsies, tissue was rapidly teased of blood and connective tissue and subsequently stored at -80°C until shipment to Metabolic Solutions (Nashua, NH, USA) for tracer analyses. All biopsies were performed by the same investigator (M.D.R.).

MyoPS rates over the 24-hour period following the first RT bout were calculated similar to Bell and colleagues [28] (see equation below).

$$FSR\ (\%day^{-1}) = \left[\frac{(E_{Ala2} - E_{Ala1})}{E_{BW} \times t}\right] \times 3.7 \times 100$$

In the equation above, $E_{Ala1}$ and $E_{Ala2}$ represent $^{2}H$ enrichment in the first and second muscle biopsies, respectively (in atom percent excess). $E_{BW}$ is the average $^{2}H$ enrichment (in atom percent excess) of total body water from the second and third salivettes after subtracting background values from the baseline salivette. *t* is time in the number of days $D_2O$ was ingested (which equals 1

herein). The 3.7 coefficient adjusts for average $^{2}H$ atoms that can be bound to alanine, and final values were expressed as % synthesis per day by multiplying values by 100.

*Food log analysis*

Participants were instructed to self-report their habitual food intake for three consecutive days and return these food logs at V3 and V24 or V16 (10- and 6-week cohort, respectively). Participants were asked not to change their diet in any way, with the exception of PP participants who were instructed to consume the supplement as described above. Study staff entered each food log into the Automated Self-Administered 24-Hour Dietary Assessment tool (ASA24), which uses the United States Department of Agriculture Food and Nutrient Database for Dietary Studies to provide values for 195 nutrients, nutrient ratios and other food components [29].

*Statistical analysis*

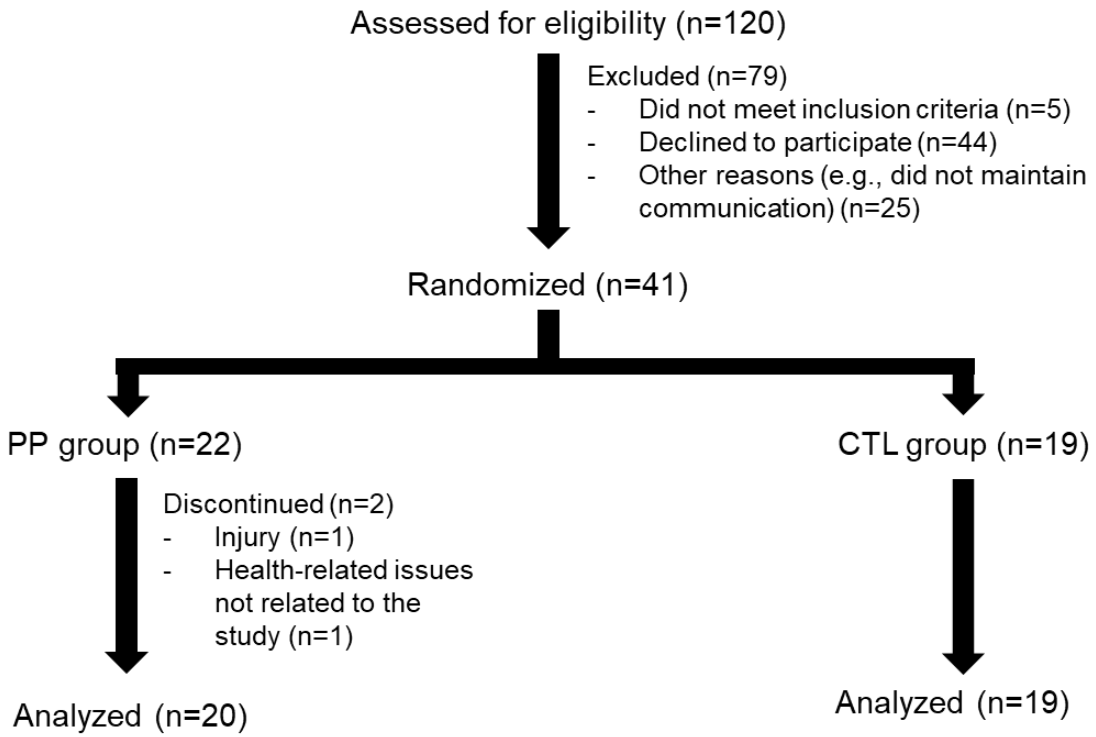
All statistical analyses were performed using SPSS v26.0 (IBM Corp, Armonk, NY, USA). For MyoPS and training volume comparisons between the PP and CTL groups independent samples t-tests were used. For all dependent variables over time, repeated measures two-way (group × time; GxT) ANOVAs were performed. When a significant interaction occurred, LSD *post-hoc* analysis was performed between and within groups to determine the level of significance. With the exception of MyoPS data, all data in figures are presented to show the 6-week cohort individually, 10-week cohort individually, and pooled cohorts collectively. Group, time and GxT data are provided for each cohort individually and when pooled. Statistical significance was established as $p<0.05$, and relevant p-values are depicted in-text for tables or within figures. All data herein are presented as mean±standard deviation (SD).

RESULTS

*Study CONSORT Diagram*

Figure 2 provides a detailed CONSORT diagram of the study. Briefly, 120 potential participants contacted the study coordinator. Of these, 41 were eligible and agreed to participate in the study, and n=22 were randomized to the PP group whereas n=19 were randomized to the CTL group. Two participants in the PP group had to discontinue the study due to injury from weightlifting (n=1) or health reasons outside of the study (n=1), whereas none of the CTL participants discontinued the study. Thus, n=20 PP participants and n=19 CTL participants were included in most analyses unless stated otherwise in the results, figures, or tables.

Figure 2. CONSORT Diagram




Legend: The diagram indicates how many individuals were screened and completed the intervention.

*Baseline Participant Characteristics*

Baseline participant characteristics between the PP and CTL cohorts are presented in Table 1. Notably, there were no differences between cohorts regarding age or body composition metrics.

Table 1. Baseline Participant Characteristics

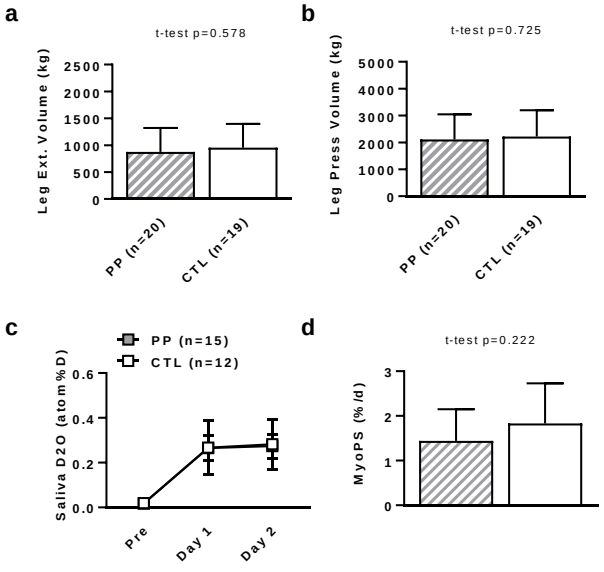
| Variable (units) | Mean ± SD | | p-value |
|---|---|---|---|
| Gender | PP<br>CTL | 12 M / 8 F<br>10 M / 9 F | N/A |
| Age (years) | PP<br>CTL | 60±9<br>58±7 | p=0.61 |
| Height (cm) | PP<br>CTL | 171.7±8.3<br>172.0±9.3 | p=0.41 |
| Weight (kg) | PP<br>CTL | 84.9±17.6<br>88.8±20.5 | p=0.52 |
| BMI ($kg/m^2$) | PP<br>CTL | 27.8±5.5<br>29.7±6.2 | p=0.23 |
| DXA % body fat (%) | PP<br>CTL | 36.0±7.1<br>36.2±7.6 | p=0.92 |

Legend: Baseline participant characteristics are presented as means ± standard deviation values. Abbreviations: PP, peanut protein supplemented participants (n=20); CTL, non-supplemented participants (n=19); DXA, dual x-ray absorptiometry; BMI, body mass index; cm, centimeters; kg, kilograms; $kg/m^2$, kilograms per meter squared.

*MyoPS response to the first bout of training with or without PP supplementation*

There were no significant differences in total leg extension or leg press volumes for the first bout of training between supplementation groups (Figure 3a and 3b). Twenty-seven participants (n=15 PP, N=12 CTL) yielded salivettes with viable enrichment values for tracer analysis (Figure 3c). There was an enrichment effect over time from V2 to V3 ($p<0.001$), but no further differences in enrichment from V3 to V4. There was no difference in the 24-hour MyoPS rates between supplement groups following the first bout of RT (Figure 3d).

Figure 3. MyoPS rates following the first bout of training with or without PP supplementation




Legend: No differences between conditions existed for the leg extensor (panel a) or leg press (panel b) training volume during the first training bout. Saliva $D_2O$ enrichment increased from V2 to V3 ($p<0.001$) regardless of supplementation (panel c), though no further enrichment effect was observed. MyoPS rates 24 hours following the first exercise bout did not differ between PP and CTL group participants (panel d). All data are presented as mean ± standard deviation values. Abbreviations: PP, peanut protein supplemented participants; CTL, non-supplemented participants.

*Food log data over the duration of the study*

Data from the PRE- and POST-training food logs between the PP and CTL cohorts are presented in Table 2. Thirty-one participants (n=16 PP, n=15 CTL) returned completed food logs suitable for analyses, and data from the PP group includes one serving of PP per day. Notably, there was a significant interaction for protein consumption in the pooled participants (p=0.008). While protein consumption decreased slightly in the CTL group (PRE=71±30 vs POST=68±23 g; p=ns), protein consumption significantly increased in the PP group (PRE=95±24 vs POST=119±22 g; p=0.007). There was also a nonsignificant trend in protein consumption for the 10- (p=0.086) and 6-week (p=0.072) cohorts. Additionally, there was a significant interaction for fiber consumption in both the 10-week (p=0.013) and pooled cohorts (p=0.003).

Table 2. Pre- and post-intervention food recall data

| Variable | Pooled CTL (n=15) | | Pooled PP (n=16) | |
|---|---|---|---|---|
| | PRE | POST | PRE | POST |
| Energy (kcal) | 1799±647 | 1683±569 | 2106±383 | 2181±412 |
| Pro (g) | 71±30 | 68±23 | 95±24 | 119±22*# |
| Fat (g) | 74±31 | 66±27 | 89±18 | 91±21 |
| CHO (g) | 210±82 | 206±71 | 216±75 | 209±49 |
| Sugar (g) | 105±54 | 92±54 | 124±63 | 98±44 |
| Fiber (g) | 14±4 | 15±6 | 19±10 | 27±6*# |
| | 10-week CTL (n=7) | | 10-week PP (n=6) | |
| Energy (kcal) | 1682±737 | 1523±578 | 2130±336 | 2150±456 |
| Pro (g) | 72±36 | 62±19 | 92±20 | 111±14# |

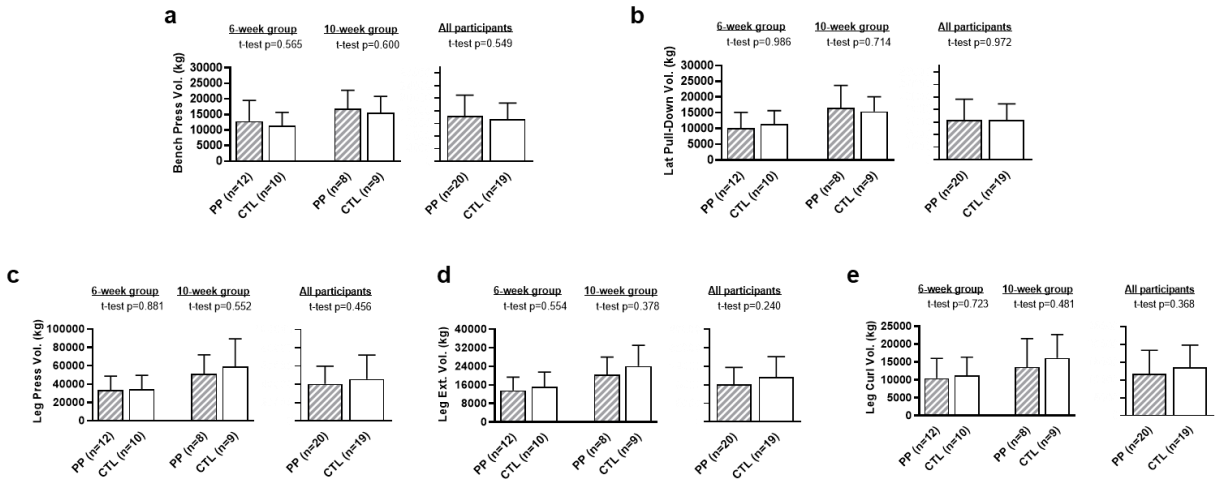
| | | | | |
|---|---|---|---|---|
| Fat (g) | 74±37 | 60±25 | 83±16 | 85±22 |
| CHO (g) | 178±70 | 180±79 | 214±75 | 208±49 |
| Sugar (g) | 83±32 | 85±67 | 90±55 | 87±29 |
| Fiber (g) | 13±4 | 12±7 | 19±15 | 26±9*# |
| | 6-week CTL (n=8) | | 6-week PP (n=10) | |
| Energy (kcal) | 1901±588 | 1823±560 | 2092±426 | 2200±408 |
| Pro (g) | 71±27 | 73±27 | 96±27 | 123±26 |
| Fat (g) | 74±29 | 71±29 | 93±19 | 94±21 |
| CHO (g) | 239±84 | 229±58 | 218±79 | 209±51 |
| Sugar (g) | 124±63 | 98±44 | 87±39 | 79±31 |
| Fiber (g) | 15±5 | 17±3 | 19±6 | 28±5 |

Legend: These data are from the 3-day food recalls averaged to intakes per one day. All data are presented as means ± standard deviation values. Abbreviations: PP, peanut protein supplemented participants; CTL, non-supplemented participants; kcal, kilocalorie; g, gram; Pro, protein; CHO, carbohydrate. Symbols: *, significant increase within PP from Pre to Post ($p<0.05$); #, PP > CTL at Post ($p<0.05$).

*Differences in Resistance Training Volumes after 6- or 10-weeks of Training*

There were no between-group differences in total volume lifted in the 6-week cohort, 10-week cohort, and pooled cohorts for bench press, pronated-grip cable pull-down, leg press, leg extension or leg curl exercises (Figure 4a-e).

Figure 4. Differences in Exercise Volumes over the Duration of Training



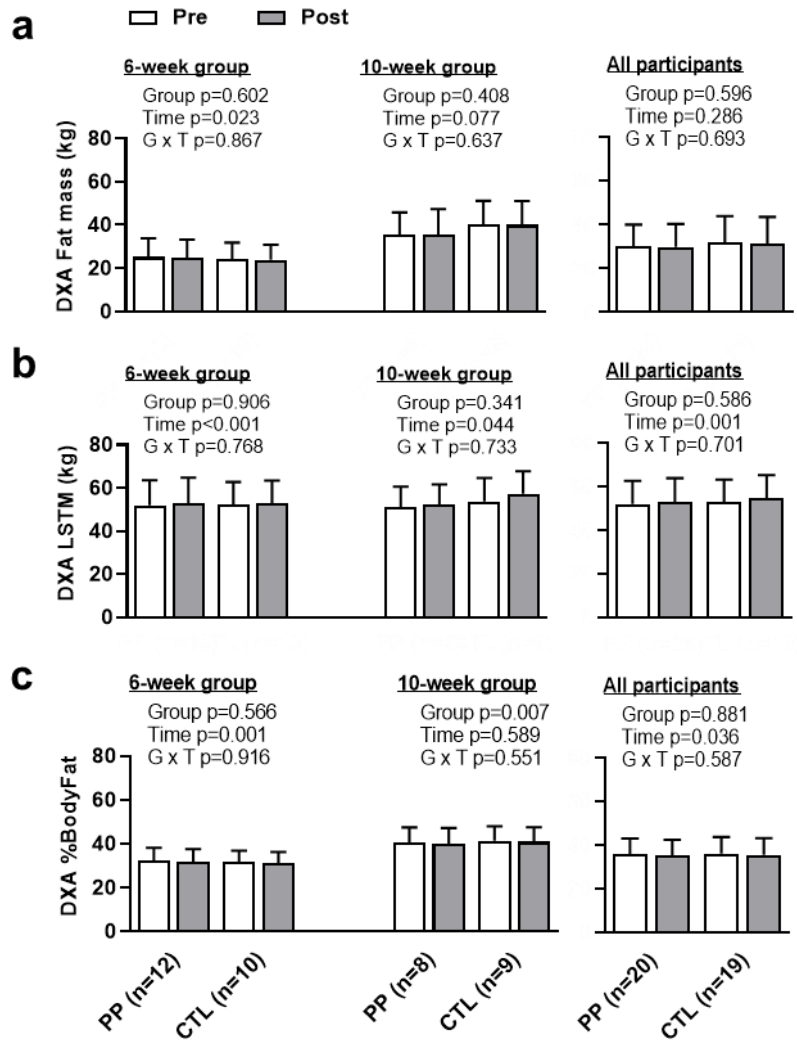

Legend: Bench press volume (panel a), pronated-grip cable pull-down volume (panel b), leg press volume (panel c), leg extension volume (panel d), and leg curl volume (panel e) did not differ between supplementation groups in the 6-week, 10-week or pooled cohorts. All data are presented as mean ± standard deviation values. Abbreviations: PP, peanut protein supplemented participants; CTL, non-supplemented participants.

*Changes in DXA Fat Mass, LSTM and Percent Body Fat*

There was no significant interaction for FM in any of the cohorts. Similarly, there was no main effect of group in any of the cohorts. However, there was a significant main effect of time in the 6-week cohort ($p=0.023$, Figure 5a). While there was no interaction or main effect of group observed for DXA measured LSTM in any cohort (Figure 5b), there was a significant main effect of time for LSTM in the 6-week ($p<0.001$), 10-week ($p=0.044$) and pooled cohorts ($p=0.001$). Lastly, while there was no interaction for body fat percentage in any cohort (Figure 5c), there was a significant main effect of time in body fat percentage in both the 6-week ($p=0.001$) and pooled ($p=0.036$) cohorts, but not the 10-week cohort. Additionally, there was a significant main

effect of group for body fat percentage in the 10-week cohort (p=0.007), but not the 6-week or pooled cohorts.

Figure 5. Changes in DXA Fat Mass, LSTM and Percent Body Fat

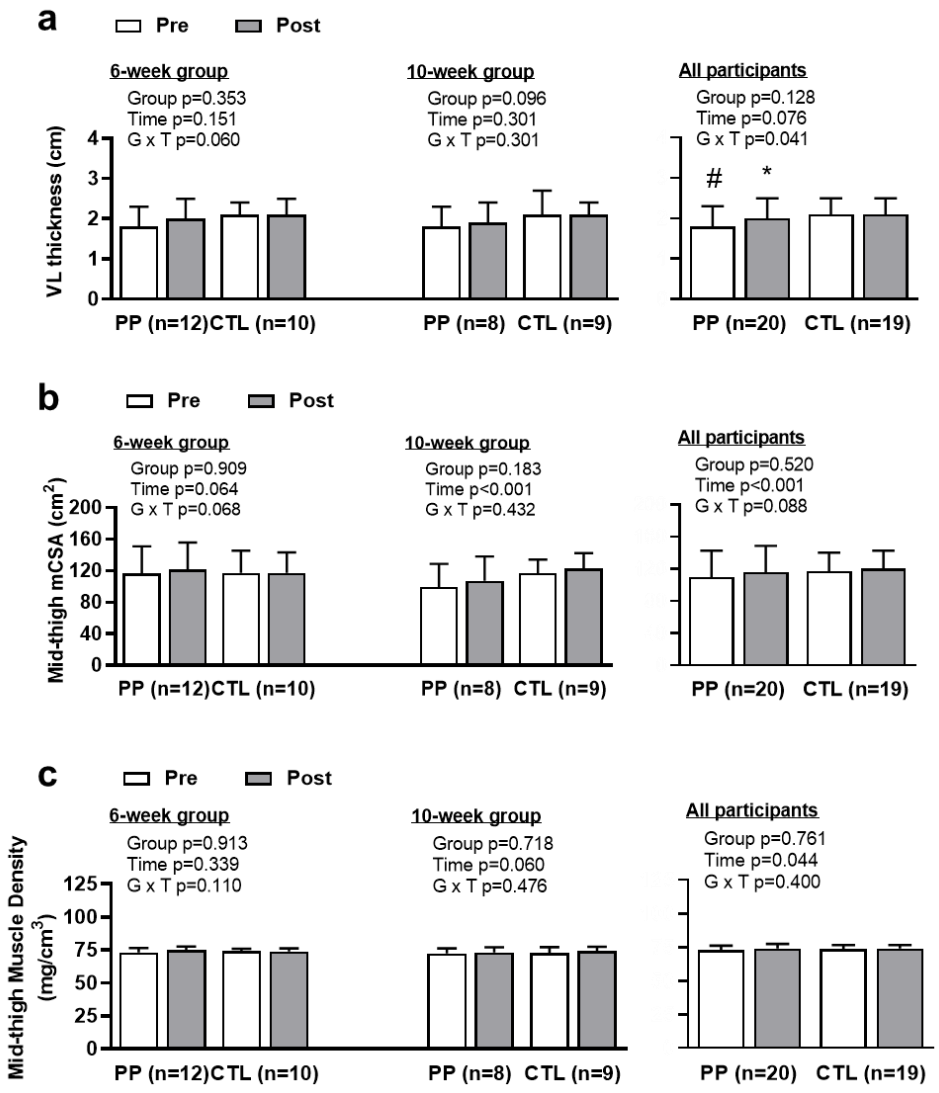



Legend: Changes in DXA-derived FM (panel a), DXA-derived lean soft tissue mass (panel b), or DXA-derived percent body fat (panel c) did not differ between supplementation groups. All data are presented as mean ± standard deviation values. Abbreviations: PP, peanut protein supplemented participants; CTL, non-supplemented participants.

*Changes in Mid-thigh VL Thickness, mCSA and Muscle Density*

There was no interaction for VL thickness in the 6- or 10-week cohorts. However, when pooled, there was a significant GxT interaction for VL thickness (p=0.041). *Post-hoc* analyses revealed that VL thickness significantly increased in the PP group (PRE = 1.8±0.4 cm, POST = 2.0±0.4 cm; p<0.001) (Figure 6a) but did not change in the CTL group (PRE = 2.1±0.5 cm, POST = 2.1±0.5 cm). Importantly, the change in VL thickness observed in the PP group was greater than the minimal difference to be considered real (0.16 cm). There was no interaction for mCSA in either cohort. However, there was a trend toward significance in both the 6-week (p=0.068) and pooled cohorts (p=0.088). Due to the trend toward significance, a forced *post-hoc* analysis was performed revealing mid-thigh mCSA significantly increased in the PP group (PRE= 109.6±32.8 vs POST= 115.8±32.8 $cm^2$; p=0.01) but not the CTL group (PRE=117.1±22.9 vs POST=120.0±22.5 $cm^2$; p=0.07). There was a significant main effect of time for mCSA in the 10-week (p=0.001) and pooled (p=0.001) cohorts, but not the 6-week (p=0.064), and there was no main effect of group for any cohort. Importantly, the change in mCSA observed in the PP group exceeded the minimal difference to be considered real (2.32 $cm^2$). Lastly, there were no significant interactions or main effects for group in the 6-week, 10-week or pooled cohorts for muscle density. However, there was a significant main effect of time when cohorts were pooled (p=0.04, Figure 6c).

Figure 6. Changes in Mid-thigh Muscle Hypertrophy Measurements

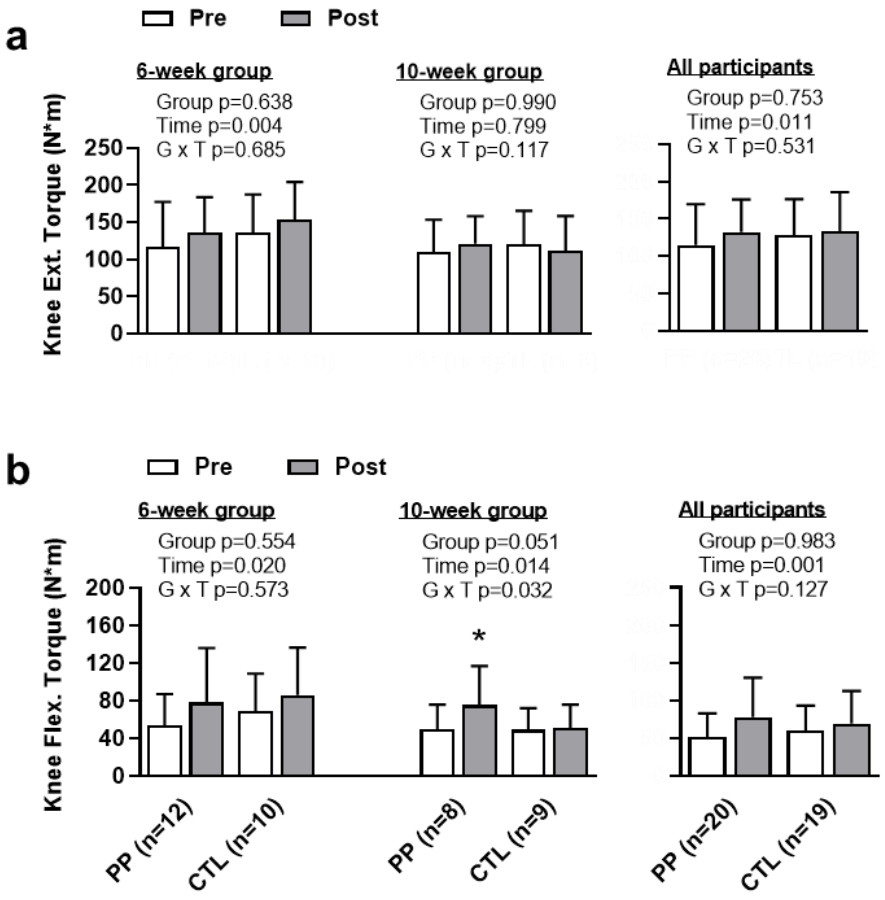



Legend: There was an interaction of VL muscle thickness in the PP group participants when the 6- and 10-week cohorts were pooled, whereas this was not observed in the CTL group participants (panel a). In addition, an interaction was not observed in pQCT-derived mid-thigh lean muscle cross sectional area values (panel b) or mid-thigh pQCT-derived muscle density (panel c). All data are presented as mean ± standard deviation values. Abbreviations: PP, peanut protein supplemented participants; CTL, non-supplemented participants. Symbols: *, significant increase within PP from Pre to Post ($p<0.05$); #, PP < CTL at Pre ($p<0.05$).

*Right Leg Isokinetic Peak Torque*

There was no difference in knee extensor torque at 60º/sec between groups and no interaction. There was, however, a significant effect of time in the 6-week (p=0.004) and pooled (p=0.011) cohorts (Figure 7a). When testing knee flexor torque at 60º/sec, there was no difference between groups in any cohort. However, there was a significant effect of time in the 6-week (p=0.020), 10-week (p=0.014) and pooled (p=0.001) cohorts. Additionally, while there was no interaction in the 6-week and pooled cohorts, there was a significant interaction in the 10-week cohort (p=0.032). While the PP group significantly increased knee flexor torque (PRE = 49.5±26.5 N×m, POST = 75.0±41.9 N×m; p=0.007), the CTL group did not (Figure 7b).

Figure 7. Right Leg Knee Extensor and Flexion Peak Torque



Legend: Knee extension and flexion peak torque increased with training, regardless of supplementation (panel a and panel b). However, PP supplementation significantly increased knee flexion torque of the 10-week cohort group, whereas this metric did not increase in the same CTL group cohort. All data are presented as mean ± standard deviation values. Abbreviations: PP, peanut protein supplemented participants; CTL, non-supplemented participants. Symbols: *, significant increase within PP from Pre to Post ($p<0.05$).

## DISCUSSION

There is good evidence to suggest that protein consumption with RT enhances various training adaptations (reviewed in [14]). Notwithstanding, the majority of these studies have examined the effects of animal-based or soy protein supplements, and no studies to date have evaluated the efficacy of RT with PP supplementation on measures of muscle mass, function, strength and body composition. Therefore, we sought to examine the effects of a novel PP supplement in conjunction with a structured RT program on markers of muscle mass, strength and body composition in an untrained, elderly cohort.

There are several noteworthy findings herein. First, the PP supplementation group significantly increased knee flexion peak torque in the 10-week cohort relative to the CTL group. Additionally, when the 6- and 10-week cohorts were pooled, PP group participants experienced significant increases in VL thickness compared to CTL group participants. A similar trend was also observed regarding mid-thigh mCSA; specifically, the interaction trended ($p=0.088$), and forced *post-hoc* tests indicated that this metric increased in the PP group participants from PRE- to POST-training ($p<0.05$), whereas there was not a significant change in CTL group participants. In addition, our dynamometry data align with previous studies reporting RT with protein supplementation enhances lower-body leg strength relative to placebo supplementation

[30, 31]. Likewise, our muscle imaging data agree with various studies demonstrating protein supplementation with RT enhances muscle mass relative to placebo supplementation [31-34]. However, there are also data showing that protein supplementation with RT does not affect variables related to muscle hypertrophy or strength in older individuals [35-37]. These discrepancies between studies are likely due to various factors including the type of protein administered, as well as the duration and type of RT. While there is strong evidence to suggest protein needs (specifically, the intake of more essential amino acids) increase with age due to decreases in gastrointestinal function [38, 39]. our PP supplement contained a full complement of amino acids and, therefore, likely provided ample amino acids for muscle hypertrophy. It is also notable that several of the studies cited above have examined the effects of whey protein with RT, and positive findings from these studies are possibly related to the high leucine and essential amino acid content as well as the high protein digestibility corrected amino acid score of whey [40]. In this regard, one serving of PP in the current study (i.e., ~35 g protein) provided approximately 10 g of essential amino acids as well as 2.5 g of leucine. In comparison, 35 g of a whey protein isolate provides roughly 17 g of essential amino acids and ~4 g of leucine [40]. As such, whether PP supplementation would be equally as good as or less effective in enhancing training adaptations relative to whey protein supplementation remains to be determined. Notwithstanding, the current findings with PP supplementation are promising and warrant future studies that are longer in duration and with different study populations.

Contrary to the data above, PP supplementation after one bout of RT did not enhance integrated MyoPS rates up to 24 hours following the first training bout. This finding is difficult to reconcile given that PP supplementation enhanced various training adaptations as described above. Given that protein consumption in each cohort increase for the PP group, we expected MyoPS to

exhibit similar increases. However, this was not the case. It is notable that post-exercise increases in MyoPS rates hours following an exercise bout have been shown to demonstrate poor agreement with long-term hypertrophic outcomes (reviewed in [41]). RT studies examining integrated MyoPS rates using $D_2O$ over days or weeks into training have yielded better associations with hypertrophic outcomes [28, 42, 43]. However, again, these correlations are modest at best. Therefore, we posit that the current MyoPS data continue to suggest that tracer data should be viewed independently of chronic training outcomes. Moreover, had we used an acute tracer infusion protocol with a phenylalanine stable isotope, we may have observed enhanced post-exercise MyoPS rates compared to no supplementation within a more acute time frame (i.e., 3-6 hours). Thus, given the paucity of data in this area, future research is needed to examine how the ingestion of PP acutely affects MyoPS rates relative to a placebo supplement or other protein supplements.

A unique aspect of this study is the implementation of pQCT to ascertain muscle quality. While no interactions existed for the metrics provided, it is interesting that muscle density increased over the duration of training regardless of supplementation. This metric is not commonly reported in the exercise physiology literature given that pQCT and CT scanners are not readily available. However, our data agree with a study by Claassen and colleagues [44] where the authors used a CT scanner to report that six weeks of RT increased Hounsfield units of the mid-thigh by ~4-5% in college-aged men. In explaining their findings, the authors speculated that the observed increase in muscle density was due to either an increase in connective tissue density and/or an increase in contractile protein density. Thus, we interpret our data to suggest that RT increases muscle density through an increase in contractile and/or connective tissue density.

Notably, this is an important finding given that a higher muscle density has been shown to be associated with an increased physical function in overweight/obese older participants [45].

What should finally be noted is the lack of agreement between some of our body composition metrics. Although PP supplementation was found to increase VL hypertrophy, no interactions between supplement groups were observed for whole-body DXA LSTM. Additionally, although a significant interaction was not observed for pQCT-derived mid-thigh mCSA changes, the interaction trended (p=0.088), as discussed above. While these data are difficult to reconcile, we have noted in the past that methods used to assess skeletal muscle hypertrophy poorly agree with one another [46]. Thus, our data re-iterate the notion that various measures of muscle mass determination do not exhibit good agreement, and these findings continue to warrant future research in this area.

*Experimental considerations*

A notable limitation of the current study is the duration of the intervention of the second cohort. Given the unforeseen consequences of the SARS-CoV-2 pandemic, we voluntarily decided to conclude the second cohort 4 weeks early rather than jeopardize the health and safety of our participants. Our data are also limited in that, while males and females completed the intervention, a lack of statistical power precluded the determination of gender interactions with PP supplementation. In spite of this limitation, it is notable that other studies have shown that males and females exhibit similar strength and hypertrophic responses to RT [47], as well as protein supplementation [14]. Nevertheless, future studies are needed to determine if gender plays a role in the response to PP supplementation

*Conclusions*

While preliminary, the results of the current study indicate that PP supplementation with 6-10 weeks of RT enhance certain aspects of muscle hypertrophy and strength, compared to a RT program alone, in the elderly population. Future studies are needed to determine whether PP supplementation would be equally or less efficacious in affecting RT variables relative to other protein sources.

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