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Dietary Regulation of Successful Aging

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Abstract

The current growth of the older population is unprecedented in U.S. history. Chronic disease and functional limitation commonly develop prior to old age, leading to prolonged physical disability and decreased well-being. The development of chronic disease and loss of independence is associated with lean body mass (LBM) loss and fat mass gain beginning in middle age. Therefore, it is important to identify modifiable factors to mitigate deleterious shifts in body composition to promote successful aging (SA). The concept of SA is associated with longevity, the absence of disease and disability, and subjective components of well-being, however, an operational definition has yet to be established. For this thesis, we defined SA as low cardiometabolic risk, preservation of physical function, and a positive state of well-being. Nutrition is a key driver of SA and is a proposed modulator of cardiometabolic risk, physical function, and well-being in adults. Among nutrients, several studies have identified dietary protein and the omega-3 polyunsaturated fatty acids (n-3 PUFAs), eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3), as key supportive nutrients for SA in older adults. Therefore, the overall objective of this dissertation was to determine the effect of nutrition, specifically dietary protein and n-3 PUFAs on SA outcomes of cardiometabolic risk, physical function, and well-being. The central hypothesis of this dissertation was that increased intake of high-quality dietary protein or n-3 PUFAs would improve SA outcomes of cardiometabolic risk, physical function, and well-being in adults. Therefore, one meta-analysis (study 1) and two clinical trials (studies 2 and 3) were designed to test our hypothesis. The objective of the first study was to systematically evaluate the available evidence of randomized control trials assessing the effect of beef and beef's nutrients on well-being in healthy, adults ≥ 50 years of age to increase physical function and well-being to promote SA. The objective of the

second study was to determine and compare the acute effects of a high-protein breakfast containing either animal protein or plant protein on appetite, food intake, energy expenditure, and substrate oxidation in young versus older men to decrease cardiometabolic risk and promote SA. The objective of the third study was to determine the individual and combined effect of protein and n-3 PUFAs on body composition, cardiometabolic risk, indexes of sleep, and mood states in postmenopausal women to decrease cardiometabolic risk and increase physical function, and well-being to promote SA. Collectively, the results suggest high-quality protein and n-3 PUFAs act as potential regulators of SA outcomes. However, additional research is necessary to determine the effectiveness of protein and n-3 PUFA-based nutrition strategies to promote SA.

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Dedication

This dissertation is dedicated to my loving husband, Aaron Hawley and to my family. Thank you for your endless support and love.

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List of Publications

Published:

Chapter 4:

Aubree L Hawley, et al. "The Short-Term Effect of Whey Compared with Pea Protein on Appetite, Food Intake, and Energy Expenditure in Young and Older Men." *Current Developments in Nutrition* 4.Geriatric Nutrition (2020): 1-9.

In review:

Chapter 3:

Aubree L. Hawley[1,2], Xinya Liang[3], Elisabet Børsheim[4,5], Robert R. Wolfe[5], Luti Salisbury[6], Hexirui Wu[1,2], Sam Walker[1,2], Angela Tacinelli[1,2], Jamie I. Baum[1,2]. Beef and Nutrients Found in Beef Positively Impact Well-Being in Healthy Adults ≥ 50 Years of Age: A Meta-Analysis and Systematic Review of Randomized Controlled Trials. *Nutrition Research*

CHAPTER 1. Introduction

The number of Americans ages 65 years and older is estimated to nearly double from ~52 million in 2018 to ~95 million by 2060 [1]. The observed growth of the older population corresponds to the projected increased trends in life expectancy from 78.9 years in 2019 to 85.6 years by 2060 [2]. In contrast to the trend in life expectancy, the healthspan, or period of life spent free from chronic disease and disability [3], has remained stagnant in the United States [4]. As chronic disease and functional limitation commonly develop prior to old age [5], the preservation of independence, quality of life, and health is critical [6]. One of the major threats to living independently is sarcopenia, the loss of muscle mass, strength, and function that progressively occurs with age [7]. As the human body ages, skeletal muscle mass declines annually by ~0.1%–0.5% beginning from age 30 and may result in sarcopenia as early as 50 years of age [8, 9]. The progression of sarcopenia is associated with an increased risk of falls [10], decreased quality of life [11], increased morbidity [12], and early mortality [13]. However, advancing age is not always associated with significant functional regression [14] and some individuals maintain a successful aging (SA) trajectory [15, 16]. Therefore, there is a need to identify modifiable factors to promote the prevalence of SA [17].

SA has yet to be universally defined. However, SA is commonly described as a multidimensional concept with subjective and objective components relating to psychological function, physiological function, and well-being [18]. For this thesis, we defined SA in terms of three components 1) low cardiometabolic risk, 2) preservation of physical function, and 3) a positive state of well-being with nutrition as an integral component. Nutrition is a key driver of SA [19], and inadequate nutrition contributes to the increased prevalence of sarcopenia and chronic disease risk in the older population, reducing the chance for SA [20]. Several studies

have identified dietary protein and the omega-3 polyunsaturated fatty acids (n-3 PUFAs), eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3), as key supportive nutrients for older adults [21-28]. Furthermore, an anabolic additive effect of protein and n-3 PUFAs has been identified in skeletal muscle of middle-aged and older adults [29, 30]. However, it is currently unknown if the observed effects project beyond skeletal muscle anabolic signaling to promote SA.

Low cardiometabolic risk is the first component of SA [16, 31]. Cardiometabolic risk is defined as a series of risk factors of metabolic origin (e.g., insulin resistance, dyslipidemia, elevated systolic/diastolic blood pressure) that increase the risk of the development of chronic diseases such as cardiovascular disease (CVD) and type 2 diabetes (T2D) [32]. Increased cardiometabolic risk in older adults is related to changes in both body weight and composition due to alterations in energy intake and/or total energy expenditure [33, 34]. Basale metabolic rate (BMR) makes up 60-70% of total energy expenditure and progressively decreases with age [35, 36]. Skeletal muscle is a primary determinant of BMR [36-39]. An increase in BMR is associated with improvements in body composition and decreases in cardiometabolic risk factors [40, 41]. In addition, a potentially modifiable component of energy expenditure is the thermic effect of food (TEF), the increase in post-prandial metabolic rate [42]. As TEF is reduced in older adults [43], further research investigating dietary factors that affect TEF may lead to better treatment methods to decrease cardiometabolic risk in older adults [44, 45]. In addition to energy expenditure, energy intake can influence energy balance and affect SA [34]. In this dissertation, study two, measured cardiometabolic risk via post-prandial plasma glucose response and energy balance. Short-term energy balance was tested by measuring post-prandial appetite, energy expenditure, and 24-hour food intake. In study three, cardiometabolic risk was measured by

biomarkers of glucose metabolism (glucose, insulin, HOMA-IR) and blood lipid levels (triglycerides, total cholesterol, and free fatty acids). In addition, body composition was measured by dual energy x-ray absorptiometry (DEXA).

Physical function is the second component of SA [16, 18, 46]. The decline in physical function with age differs between individuals and is commonly measured in terms of mobility, balance, and muscle strength [47]. Skeletal muscle plays a critical role in preserving physical function [48, 49], especially after 50 years of age [50]. Handgrip strength, a commonly used assessment of overall muscle strength, is associated with morbidity and mortality in aging adults [51]. Similar to strength, decreased measures of physical function such as the short physical performance battery (SPPB) score is a strong indicator of all-cause mortality in older adults [52]. Considering the strong association of physical function with all-cause mortality [53], early onset interventional strategies are needed to monitor and mitigate physical function decline to promote SA outcomes [54]. In this dissertation physical function was evaluated in study one and study three. In study one, a meta-analysis and systematic review, measured physical function via multiple outcomes such as handgrip strength, SPPB, gait speed, and one-repetition maximum knee extension. Study three, a randomized clinical controlled trial (RCT), measured physical function via handgrip strength pre- and post- 16-week intervention.

The third component of SA is well-being [18]. Well-being is generally defined by emotional well-being such as the presence of positive affect states, life satisfaction, and the absence of negative affect states; and physical well-being such as sleep quality [55-58]. Positive affect states are associated with better health outcomes, lower mortality risk, and longevity in the older population [59-61]. In addition, several studies have reported a relationship between skeletal muscle mass and depressive symptoms [62-64]. Furthermore, studies in adults have

demonstrated an inverse relationship between sleep disorders and poor sleep quality and outcomes of well-being [65-67] and body composition [68-70]. In this dissertation well-being was evaluated in study one and study three. In study one, a meta-analysis and systematic review, aimed to evaluate measures of emotional and physical well-being. However, emotional well-being outcomes did not meet the inclusion criteria. Therefore, lifestyle factors positively associated with well-being were included in the study. Study three, a RCT, measured well-being via the Profile of Mood States (POMS) questionnaire. In addition, sleep quality was evaluated subjectively by the Pittsburgh Sleep Quality Index (PSQI) questionnaire and objectively by accelerometry [71, 72].

Nutrition, specifically dietary protein and n-3 PUFAs, are suggested modulators of cardiometabolic risk, physical function, and well-being and may promote SA [20, 32, 73-75]. As the older population grows and life expectancy continues to rise, it is important to consider optimal nutritional recommendations that will promote SA in older adults [76].

Protein is a dietary focal point for SA as the constituent amino acids (AA) are the essential building blocks necessary to sustain life [20]. The benefits of dietary protein intake for older adults above the current recommended dietary allowance (RDA) of 0.8g/kg/day is well established [20, 77] and experts generally recommend a dietary protein intake between 1.2 and 2.0 g/kg/day or higher and ~30 g of high-quality protein per meal to promote skeletal muscle mass and physical function in older adults [21, 23, 77-82]. Accordingly, dietary protein consumed in higher amounts may prevent sarcopenia, maintain energy balance, reduce cardiometabolic risk, and improve function and well-being in community dwelling middle-aged and older adults [23].

Similarly, n-3 PUFAs, EPA and DHA are also associated with SA [75]. Although, Dietary Reference Intakes (DRI) have yet to be developed, the 2015–2020 Dietary Guidelines for Americans (DGA) recommends a combined daily intake of 250 mg/day EPA + DHA equating to approximately 8 oz per week of a variety of fish in adults with and without CVD [83]. However, the benefits of EPA and DHA intake beyond the recommendations are well-established [83, 84]. Furthermore, 3-4 g of combined DHA + EPA may mitigate deleterious characteristics of aging via suppression of chronic inflammation, incorporation into cellular membranes, and improved cell signaling [29]. Accordingly, dietary EPA and DHA consumed in higher amounts may prevent sarcopenia, improve energy metabolism, reduce cardiometabolic risk, improve physical function, and well-being in community dwelling older adults [28].

Taken together, high-quality dietary protein and n-3 PUFAs may play an integral role in promoting SA. Therefore, this doctoral dissertation investigates the impact of high-quality protein and n-3 PUFAs on components of SA in middle-aged and older adults. The objectives of this dissertation were:

1. To systematically evaluate the available evidence of RCTs assessing the effect of beef and beef's nutrients on well-being in healthy, adults ≥ 50 years of age to promote SA.

2. To determine and compare the acute effects of a high-protein breakfast containing either animal protein or plant protein on appetite, food intake, energy expenditure, and substrate oxidation in young versus older men to decrease cardiometabolic risk and promote SA .

3. To determine the individual and combined effect of protein and n-3 PUFAs on body composition, cardiometabolic health, indexes of sleep, and mood states in postmenopausal

women to decrease cardiometabolic risk, and increase physical function, and well-being to promote SA.

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CHAPTER 2. Literature Review

Nutrition as the Foundation of Successful Aging: A Focus on Dietary Protein and Omega-3 Polyunsaturated Fatty Acids

Abstract

Skeletal muscle is thought to play a critical role throughout the aging process. First, detectable at ~50 years, the deterioration of skeletal muscle mass and strength and power (sarcopenia) are estimated to decline annually at a rate of ~0.8–1% and ~2–3% respectively. People living with sarcopenia often experience diminished quality of life, which can be attributed to a long period of decline and disability. Therefore, it is important to identify modifiable factors that preserve skeletal muscle and promote successful aging (SA). We defined SA in terms of three components 1) low cardiometabolic risk, 2) preservation of physical function, and 3) a positive state of well-being with nutrition as an integral component. Several studies identify nutrition, specifically high-quality protein (e.g., containing all essential amino acids (EAA)) and long-chain omega-3 polyunsaturated fatty acids (n-3 PUFAs), eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3), as positive regulators of SA. Recently an anabolic additive effect of protein and n-3 PUFAs has been identified in skeletal muscle of middle-aged and older adults. Evidence further suggests the additive effect of high-quality protein and n-3 PUFAs may project beyond skeletal muscle anabolism and promote SA. The key mechanism(s) behind the enhanced effects of concomitant intake of high-quality protein and n-3 PUFAs remains to be fully elucidated. Therefore, the first objective of this review is to evaluate skeletal muscle as a driver of cardiometabolic health, physical function, and well-being to promote SA. The second objective of this review is to examine observational and interventional

(whole food and/or supplementation alone, without physical exercise) evidence of protein and n-3 PUFAs on skeletal muscle to promote SA. The final objective of this review is to propose mechanisms by which combined optimal intake of high-quality protein and n-3 PUFAs likely play a key role in SA.

Introduction

The current growth rate of adults ages 65 and older is recognized as one of the most substantial demographic trends in United States (U.S.) history [1, 2] and life expectancy is projected to increase from 78.5 years in 2017 to 85.6 years by 2060 [3]. Maintaining independence, quality of life, and health is crucial as we age [4]. One of the major threats to living independently is sarcopenia, the loss of muscle mass, strength, and function that progressively occurs with age [5]. As the human body ages, skeletal muscle mass declines annually by ~0.1%–0.5% beginning from age 30 and may result in sarcopenia as early as 50 years of age [6, 7]. The progression of sarcopenia is associated with an increased risk of falls [8], decreased quality of life [9], increased morbidity [10] and early mortality [11]. However, advancing age is not always associated with significant functional regression [12] and some individuals maintain a successful aging trajectory [13, 14].

Successful aging (SA) is used in the gerontological literature to cover the multifactorial processes of aging throughout the lifespan [15]. SA has recently been identified as a multidimensional construct with subjective and objective components such as positive and negative affect states, sleep health, and measures of physical and cognitive function [16]. However, a universal definition or standardized criteria of SA has yet to be established. Nevertheless, investigators have generally based their definitions of SA on the absence of

physical disability and maintenance of physical performance and to a lesser extent cognitive function and well-being with increased age [17]. Due to the variability among SA definitions, approximately 14-42% of older adults (aged ≥60 years) are classified as successful agers [16-20]. Moreover, there is a need to identify a SA construct which can be quantified with objective and subjective components in order to promote the development of SA. Therefore, in this review we defined SA as low cardiometabolic risk, preservation of physical function, and a positive state of well-being with nutrition as an integral component. Furthermore, evidence suggests the SA components are influenced by a common physiological factor, skeletal muscle mass [21-23], and are further supported by adequate nutrition [24].

The first SA component is defined by low cardiometabolic risk [14, 25]. Cardiometabolic risk is defined as a series of risk factors of metabolic origin (e.g., insulin resistance, dyslipidemia, elevated systolic/diastolic blood pressure) that increase the risk of the development of chronic diseases such as cardiovascular disease (CVD) and type 2 diabetes (T2D) [26]. Increased cardiometabolic risk in older adults is related to shifts in body weight and composition due to alterations in energy intake and/or total energy expenditure (TEE) [27, 28]. Basale metabolic rate (BMR) makes up 60-70% of TEE and progressively decreases with age [29, 30]. Skeletal muscle is a primary determinant of BMR [30-33]. An increase in BMR is associated with improvements in body composition and decreases in cardiometabolic risk factors [34, 35]. Furthermore, the thermic effect of food (TEF), the increase in post-prandial metabolic rate [36], is reduced in older adults [37]. Additional research investigating dietary factors that affect TEF and TEE may lead to better treatment methods to decrease cardiometabolic risk in older adults [38, 39].

The second SA component includes physical function [14, 16, 19]. The decline in physical function with age differs between individuals and is commonly measured in terms of mobility, balance, and muscle strength [40]. Muscle strength, a key component of physical function, is defined by the force-producing capacity of skeletal muscle [41]. Handgrip strength, a commonly used assessment of overall muscle strength, is associated with morbidity and mortality in aging adults [42]. Physical function is also defined by whole-body function, involving skeletal muscle and the peripheral nervous system (e.g., balance), and is related to the ability to move from one place to another [43]. Considering the strong association of physical function with all-cause mortality, early onset interventional strategies are needed to monitor and mitigate muscle strength and performance decline to ensure SA [44].

The third component of SA is well-being [16]. Well-being is generally defined by emotional well-being such as the presence of positive affect states, life satisfaction, and the absence of negative affect states; and physical well-being such as sleep quality [45-48]. Positive affect states are associated with better health outcomes, lower mortality risk, and longevity in the older population [49-51]. In addition, several studies have reported a relationship between skeletal muscle mass and depressive symptoms [52-54]. Furthermore, studies in adults have demonstrated an inverse relationship between sleep disorders and poor sleep quality and outcomes of well-being [55-57] and body composition [58-60]. Therefore, further research is needed to examine possible modulators of well-being and potential factors influencing the relationship between well-being and body composition in older adults.

Nutrition plays an essential role in the health, function, and well-being of older adults [26, 61]. Nutritional strategies can mitigate the development of sarcopenia [62], cardiometabolic risk [63], physical impairment [61], and poor well-being [64, 65]. Among nutrients, several

studies have identified dietary protein and the omega-3 polyunsaturated fatty acids (n-3 PUFAs), eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3), as key supportive nutrients for skeletal muscle health in middle-aged and older adults [26, 66-72]. In addition to the proposed benefits on skeletal muscle, optimal protein and n-3 PUFA intake can help maintain energy balance [73, 74], reduce cardiometabolic risk [26, 68, 75], and promote well-being [72, 76, 77]. Observational studies have proposed dietary intake as an integral factor separating usual aging from SA [78-80]. Conversely, as the SA construct has developed in the gerontological literature, nutrition is rarely viewed as an integral component [16, 81-84]. However, this review proposes that nutrition is a foundational factor promoting SA via regulation of skeletal muscle mass with advanced age.

Therefore, the first objective of this review is to evaluate skeletal muscle as a driver of low cardiometabolic risk, high physical function, and positive well-being to promote SA. The second objective of this review is to examine observational and interventional (whole food and/or supplementation alone, without physical exercise) evidence of protein and n-3 PUFAs on skeletal muscle to promote the SA outcomes. The final objective of this review is to propose mechanisms by which combined optimal intake of high-quality protein and n-3 PUFAs likely play a key role in SA.

The Role of Skeletal Muscle in Successful Aging

Skeletal Muscle in Cardiometabolic Risk. Advancing age is the greatest risk factor for increased cardiometabolic risk [85] and the possibility of achieving SA decreases with increasing age [86, 87]. The age-related reduction in skeletal muscle mass and physical function is associated with increased morbidity and mortality via the development of cardiometabolic-based

chronic disease such as T2D, CVD, and obesity [33, 61]. Age-related skeletal muscle loss and fat mass gain are also associated with a higher prevalence of multiple chronic diseases (MCDs) [88]. Once age-related decline in muscle strength, mass, and function fall below established cut-off points, older adults (≥ 60 years) are classified as sarcopenic [44]. Sarcopenia, now recognized as an independent geriatric condition and muscle disease [44], is consistently associated with elevated cardiometabolic risk [10], all-cause mortality [89], and is exacerbated by obesity [90, 91]. Sarcopenia and obesity act synergistically, which increases the risk of chronic disease, premature disability, and decreased quality of life [92].

The prevalence of obesity has doubled since 1980. Obesity rates continue to rise with obesity rates in middle-aged and older adults estimated at 44.8% and 42.8%, respectively [93, 94]. Although 9-16% of obese individuals are metabolically healthy [95], midlife obesity is associated with decreased likelihood for achieving SA [96]. Moreover, obesity has been linked to the progression of sarcopenia and is associated with an increased risk for disabilities [97]. One reason that reduced skeletal muscle mass contributes to the accumulation of excess adiposity is due to its role in energy expenditure [98]. Total energy expenditure (TEE) is the net energy utilized by the body to maintain homeostatic function, digest nutrients, and conduct movement [38]. The TEE consists of three basic components: 1) basal metabolic rate (BMR), 2) the thermic effect of food (TEF) and 3) the thermic effect of activity (TEA) [99]. BMR makes up 60-70% of TEE and represents the energy required to maintain the body's homeostatic processes at a rested fasting state. Skeletal muscle is the primary determinant of BMR and variances in BMR contribute to the pathogenesis of obesity [31, 32]. Indeed, BMR declines approximately 1-2% per decade beginning in the third decade of life and is associated with reduced skeletal muscle

mass and increased adiposity [30, 33]. Importantly, skeletal muscle is a modifiable contributor to BMR and can be augmented via lifestyle interventions [100].

In addition to absolute adiposity, relative adiposity, the proportion of muscle mass to fat mass, the lean-to-fat (LTF) ratio, has been linked to an individual's overall cardiometabolic risk [101]. For example, data obtained from the Korean Sarcopenic Obesity Study found the lowest tertile of LTF (appendicular lean mass to visceral fat) was associated with a 5.43 times higher odds ratio for metabolic syndrome when compared to the highest LTF in older adults [102]. This is further supported by a large cross-sectional analysis which identified a lower risk of CVD and all-cause mortality in adults in the highest LTF quartile (appendicular lean mass to trunk fat mass) [103].

Skeletal muscle is proposed to have a bi-directional relationship with cardiometabolic health [104]. Skeletal muscle accounts for approximately 40% of total body mass and is inversely associate blood glucose levels [33]. Furthermore, skeletal muscle is the primary site of blood glucose disposal, accounts for approximately 80% of postprandial glucose uptake [105], and is inversely associated with T2D [106, 107]. Indeed, middle-aged men in an early stage of T2D have low-density skeletal muscle area compared to healthy individuals [108]. Therefore, the maintenance of skeletal muscle over the lifespan is critical in regulating blood glucose homeostasis, reducing cardiometabolic risk, and in the prevention of chronic disease to promote SA.

The Role of Skeletal Muscle in Physical Function. Independence is a necessary facet of SA and is highly correlated with skeletal muscle function in older adults [25, 109]. Skeletal muscle plays a critical role in preserving physical performance [110] and muscle strength in older adults [111, 112]. Moreover, older adults experience an annual decline of muscle strength

and power between 1.5% and 3.5%, respectively [113]. Low muscle strength and mass are identified risk factors of all-cause mortality. For example, two longitudinal studies found community-dwelling men with low grip strength and appendicular lean mass (ALM) had higher odds for mortality after approximately 10 years [114, 115]. In addition, decreases in strength are associated with an increased risk of disability in activities of daily living (ADLs). The inability to complete ADLs is associated with increased cardiometabolic risk, cognitive decline, and decreased well-being [116]. Furthermore, older adults without ADL limitation have increased positive outlook and life satisfaction compared to their counterparts with ADL limitations.

The presence of sarcopenia is associated with increased falls, fractures, and muscle mass loss, further emphasizing the importance of skeletal muscle preservation to ensure SA. Moreover, a recent exploratory study investigated a physiological model of SA and identified muscle strength (i.e., HGS and leg extension) as a significant predictor of SA outcomes including self-rated health, walking speed, and decreased dependency risk at baseline and after the 9-year follow up period [25]. In addition, a recent analysis from the Nutrition and Successful Aging Study (NuAge) found muscle mass decline only explained a small part of the variation of muscle strength and function in healthy older adults [117]. Therefore, further research is needed to establish a relationship between skeletal muscle and SA.

Skeletal Muscle and Well-being. Well-being is generally defined by emotional well-being such as the presence of positive affect states, life satisfaction, and the absence of negative affect states; and physical well-being such as sleep quality [45-48]. Well-being and health are closely linked with advanced age [118], albeit a link to skeletal muscle mass is less established. A recent prospective longitudinal study in hospitalized older adults identified low skeletal muscle mass as a risk factor for outcomes of well-being such as depression symptoms and

decreased quality of life [119]. In agreement, longitudinal and cross-sectional analyses have identified an association between skeletal muscle mass and subjective well-being, health, and physiological function in healthy older adults [120, 121]. Sarcopenia is also associated with decreased well-being indicated by quality of life measures (e.g. SF-12, SF-36, SarQol) [122]. However, other cross-sectional analyses have found little to no relationship between sarcopenia and well-being aside from subjective health measures [21, 123]. Therefore, further research is needed to confirm a relationship between skeletal muscle and well-being.

In addition, well-being is commonly correlated with muscle strength and physical performance [118, 124]. For example, a cross-sectional analysis in older men and women found negative affect states (total mood disturbances, anger, and depression) were negatively correlated with physical fitness [125]. In agreement, walking speed is significantly associated with high levels of emotional well-being including decreased depression, anxiety, and fear of falling accompanied by increased feelings of vitality [126]. Furthermore, adults with reduced muscle mass and function are nearly twice as likely to have depression compared to their counterparts [114]. As the associations of skeletal muscle mass, strength, and performance with well-being gain strength, behavioral modulators of well-being and skeletal muscle are of great interest.

Sleep is not only a behavior necessary to sustain life, but a proposed driver of SA. Sleep quality and duration is associated with cardiometabolic risk, physiological function, and well-being with advanced age [127]. The 2020 Sleep in America poll found 55% of Americans attributed daytime drowsiness to disrupted sleep quality as opposed to short sleep duration [128]. Americans further reported daytime drowsiness worsened their mood, irritability, and deterred them from evening socialization and healthy behaviors (e.g., exercise), all which are aspects of well-being [128]. Approximately 50% of older adults have continual sleep problems such as

frequent awakenings and increased sleep latency [129]. Poor sleep duration (<7h or >8h of sleep each night) is associated with low skeletal muscle health [130, 131] and decreased hedonic well-being [132]. A relationship between sleep and low skeletal muscle mass, strength and function is suggested due to the shared positive associations with age, cardiometabolic risk factors, and decreased well-being [130, 133]. For example, a cross-sectional study of Chinese community dwelling older adults found sarcopenia, especially in women, to be associated with poor sleep quality, cognitive decline, malnutrition, and depression [130]. However, more evidence is needed to establish a relationship between physical function, between skeletal muscle, and sleep.

Well-being, at the physiological level, is associated with cortisol [134], energy metabolism [135], inflammation (e.g., IL-6 and CRP) [136], and neurological regulators such as brain-derived neurotrophic factor (BDNF) [137], and orexin-A (OXA) [138]. Although the potential mechanisms that mediate the relationship between skeletal muscle and outcomes of well-being are not fully understood, several mechanisms may contribute to well-being. First, stress, sleep disruption, and advancing age are associated with cortisol levels [139, 140]. Elevated cortisol is associated with an increased risk for sarcopenia, cognitive decline, and decreased cardiometabolic health via insulin resistance, loss of hypothalamic and hippocampal glucocorticoid receptors, and alterations in peripheral gene expression [141, 142]. Furthermore, decreases in cortisol concentrations are reflective of down-regulation of the hypothalamic-pituitary adrenal axis (HPA) and subsequently improvements in cardiometabolic health [142]. Second, BDNF, a member of the neurotrophic family, plays a role in neurite outgrowth, synaptogenesis, and in the prevention of apoptosis [143]. BDNF has been connected to various physiological functions in the brain relevant to cognitive function, sleep, and mood states [144]. Lastly, human OXA and orexin-B (OXB) are excitatory neuropeptide solely synthesized in the

lateral and posterior hypothalamic area and project widely throughout the central nervous system [145]. OXA has been identified as the peptide of greater physiological relevance due to its ability to rapidly cross the blood-brain barrier by simple diffusion and its lower degradation rate in the blood [146]. Furthermore, OXA signals two G protein-coupled receptors, orexin receptor 1 (OXR1) and orexin receptor 2 (OXR2) [147]. OXA is associated with facets of well-being such as arousal, motivation, and regulation of sleep cycles [138, 145]. Hypothalamic expression of OXA decreases with age [148], plasma levels decrease with obesity [149], and OXA expression has been identified in human adipose tissue [150]. However, little is known about the effects of OXA on human skeletal muscle. Contrarily, in avian species, muscle cells secrete and express OXA and ORX1 and ORX2, respectively [151]. Therefore, further investigation of OXA as a promoter of well-being through skeletal muscle is warranted and will be discussed later in this review.

Nutrition and Successful Aging

Nutrition is a key contributor to SA. Poor nutrition can contribute to the development of sarcopenia and obesity and increase the risk for chronic disease [61, 152, 153]. As the older population grows and life expectancy continues to rise, it is important to consider optimal nutritional recommendations that will promote SA in older adults [154]. Several studies identify dietary protein and n-3 PUFAs as key nutrients for older adults [26, 61, 62, 152, 153].

Dietary Protein Recommendations and Current and Optimal Intake for Older Adults

Current Dietary Protein Recommendations. Dietary protein is a focal point for SA as the constituent amino acids (AA) are the essential building blocks necessary to sustain life [61]. The

current dietary protein recommendations have been based on studies that estimate the minimum protein intake necessary to maintain nitrogen balance [155]. However, the drawback with relying on these findings is that they do not address age-related anabolic resistance, measure physiological and behavioral endpoints relevant to skeletal muscle, nor SA outcomes such as cardiometabolic risk, physical function, and well-being. Currently, the Food and Nutrition Board of the Institute of Medicine has set the recommended daily allowance (RDA) for protein at 0.8 g/kg/day, covering the minimum requirements of 97-98% of all healthy adults >18 years of age, including older adults [156]. In addition to the RDA, recommendations for protein intake are also provided in the context of a complete diet within the Acceptable Macronutrient Distribution Range (AMDR) [157]. The AMDR expresses protein intake recommendations as a percentage of total caloric intake (10-35% of daily energy intake from protein) and is more relevant in the context of a complete diet than the RDA [67]. The AMDR upper value of 35% far exceeds the RDA of 0.8 g/kg/day by approximately four times at ~3.0g/kg/day. Surprisingly, the current percent daily value (DV%) for protein is based off of 50 grams of dietary protein in the context of a 2000 kcal/d diet equating to 10% of daily intake, the minimum amount of daily protein according to the AMDR. Contrarily, a moderate or high consumption of 1.2 g/kg/day to 2.0 g/kg/day of dietary protein easily falls within the AMDR and should be considered for older adults to preserve muscle mass, strength, and performance to promote SA [61, 66, 158]. Therefore, the upper range of the AMDR provides an appropriate recommendation, within dietary guidelines, to promote SA

Recommendations for Dietary Protein Intake for Successful Aging. Recent dietary protein and aging research has focused on the optimal daily and per meal intake to promote skeletal muscle mass and function in older adults [159-161]. Optimal protein intake, defined in

terms of skeletal muscle, is the minimal dose of protein intake that stimulates a maximal anabolic response and maintains or improves skeletal muscle mass and function over time [162, 163]. According to the PROT-AGE recommendations, older adults are recommended to consume 25-30 g (e.g., 0.4g/kg/bw per meal) [164] of protein and 2.5-2.8 g of the branched-chain amino acid (BCAA) leucine per meal, equating to 1.0-1.2 g/kg/day of dietary protein intake [165, 166]. In line with these recommendations, a cross-sectional study in healthy older adults found a positive associate between daily protein distribution of >25 g of protein per meal and appendicular skeletal muscle [167]. According to NHANES data, older men and women consume 1.01 ± 0.03 g/kg/day and 0.97 ± 0.04 g/kg/day of dietary protein, respectively [161, 165, 168]. However, 19.21 ± 2.11% and 13.17 ± 1.33% of older men and women respectively fall below the RDA for dietary protein [168]. In addition, protein consumption in older adults follows a highly skewed distribution pattern with a disproportionate amount of daily protein consumed during the evening meal (<60% of daily protein) and far less at breakfast [169]. For example, a cross-sectional analysis found older men and women consumed 11.4 ± 0.4 g and 15.3 ± 0.5 g of protein at breakfast compared to 44.5 ± 1.0 g and 44.8 ± 1.0 g at dinner [170].

One potential benefit of optimal dietary protein intake and distribution is to overcome anabolic resistance, the reduced response to low doses of protein and AA that occurs with age [171, 172]. There appears to be an AA threshold for stimulation of muscle protein synthesis (MPS) (fractional synthesis rate) of ~2.5 g leucine, ~15 g of EAA, or ~30 grams of high-quality protein [173]. For example, Pennings et al. [174], examined ingestion of 10, 20, and 35 g of whey protein isolate in healthy older adult men (73 ± 2 y). AA absorption and subsequent stimulation of MPS (fractional synthesis rate) were limited at post-ingestion of 10 g of protein, but increased from basal levels in a dose dependent manner following 20 g (16 ± 13 %) and 35 g

(44 ± 16 %) of protein. In addition, Symons et al. [163] investigated the effect of 30 g and 90 g of high-quality beef protein and found both quantities were equally effective in stimulating MPS (~50% increase) in healthy young and older men. Although intakes of protein beyond ~30 g may not further increase MPS, research is suggestive of further benefits via suppression of catabolic processes and inflammation as well as promotion of cardiometabolic health and physical function in favor of SA [68, 175]. As older adults may consume dietary protein in a skewed distribution and below levels recommended by aging experts, protein supplementation and/or increases in dietary protein exists as a strategy to mitigate anabolic resistance and improve functional outcomes of SA in older adults.

Dietary Protein and Cardiometabolic Risk. Although most older adults fall within the RDA for dietary protein intake [168], there is increasing evidence that diets with greater levels of high-quality protein, especially at the expense of simple carbohydrates, may decrease cardiometabolic risk [176]. Proposed therapeutic effects a high protein diets include lower energy intake associated with increased thermogenesis [39] and preservation of skeletal muscle [177] translating to decreased waist circumference, systolic and diastolic blood pressure, triglycerides, fasting insulin, and increased HDL [61, 66, 178, 179]. According to observational data, higher protein diets (1.0 to 1.5 g/kg/day) are associated with lower BMI and waist circumference and higher HDL cholesterol levels compared to protein intake at the RDA [180]. Similarly, a cross-sectional analysis of middle-aged men (50.5 ± years) found individuals who consumed BCAAs in the highest quartile (<0.17g/kg/day) had a lower incident of cardiometabolic risk factors [181]. Furthermore, a recent cross-sectional analysis in female twins (18-76 years) found higher BCAA intake was associated with lower insulin resistance, systolic

blood pressure, and adiposity related metabolites [182]. Overall, dietary BCAA intake exists as a possible strategy to restore cardiometabolic health and promote SA [183].

Several randomized, controlled clinical trials (RCTs) have observed cardiometabolic benefits following high-protein diets and/or supplementation when coupled with weight-loss and/or exercise in middle-aged and older adults [184-186]. However, RCTs under caloric maintenance remain limited, especially in older adults. Therefore, weight maintenance trials will be reviewed. Layman et al. [187] investigated the effect of following a high protein diet (high protein: 1.6g/kg/day, ~30% energy) versus the protein RDA (0.8g/kg/day; ~15% energy) after four months of weight loss on long-term weight maintenance. The high-protein diet was more effective for long-term fat loss (38% greater fat loss) and produced sustained reduction in triglycerides and increases in HDL cholesterol [187]. In line with these findings, a meta-analysis of long-term weight maintenance diets, found individuals following higher protein, low carbohydrate diets had a higher prevalence of sustained weight-loss and decreases in fasting triglycerides and insulin levels supporting a cardiometabolic benefit [188]. Contrarily, a RCT examining the effects of a high-protein diet (high protein: 1.4 g/kg/day versus RDA 0.8g/kg/day) in older adults (70 ± 5 years) observed no differences in outcomes of cardiometabolic risk (e.g., HDL, LDL, fasting glucose, blood pressure) nor markers of inflammation including: tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), and c-reactive protein (CRP) [189]. In addition to dietary protein supplementation, EAA mixtures may improve cardiometabolic health in older adults. For example, an RCT implementing two doses of 8g/day of EAAs (10:00 AM and 5:00 PM) observed significant reductions in insulin resistance, TNF- α, and increases in insulin-like growth factor 1 (IGF-1) and lean body mass (LBM) [190]. In agreement, Scognamiglio et al. [191] supplemented 12g/day of EAA for 3 months and observed significant

improvements in myocardial performance with non-significant improvements in systolic and diastolic blood pressure compared to control. Further RCTs are needed to establish the relationship between protein intake and cardiometabolic health in the absence of weight loss in healthy middle-aged and older adults.

The Role of Dietary Protein in Physical Function. As previously reviewed, most middle-aged and older adults consume, at minimum, the RDA for dietary protein. Observational studies indicate that higher protein intakes are associated with increased strength and physical performance, comprehensively reviewed elsewhere [192]. In addition, observational data suggest, the quality of dietary protein (e.g., animal versus plant sources of protein), defined by its ability to deliver all EAA in proportion to individual requirements, may be an important modulator of muscle strength and performance [193, 194]. For example, data from the Framingham Offspring study indicated that animal-protein foods rather than plant-based protein foods were positively associated with physical performance in older adults [195]. However, observational studies have also indicated total protein, regardless of source, is positively associated with muscle strength and performance [196, 197]. In general, these observational data suggest that increased dietary protein intake promotes and preserves muscle strength and performance to promote SA.

However, several studies have reported inconsistent results regarding the impact of protein and AA supplementation on strength and physical performance with some reporting a positive [198, 199] and others reporting no effect [200, 201]. However, heterogeneity is commonly high in regard to the studied population (e.g., healthy, frail, diabetic, or sarcopenic individuals), duration, and supplement form and dose. However, some studies have identified a positive effect of increased dietary protein and EAA with physical function in older adults [202-

205]. For example, Mitchell et al. [206] conducted a well-controlled feeding study of 35 healthy older men to test the effect of protein at the current RDA (0.8 g/kg/day) compared to two times the RDA (1.6 g/kg/day) for 10 weeks and found the higher protein diet led to increased power (e.g., knee extension peak power) and strength preservation (handgrip strength) compared to no change and decreases in the control group respectively. Moreover, in the absence of exercise [207], weight-loss [208], and multi-nutrient supplementation (e.g., vitamin D, E, and B vitamins) [209], few studies have investigated the effect of increased protein supplementation on outcomes of muscle strength and physical performance in healthy middle-aged and older adults. However, evidence does suggest EAAs promote physical function in older adults with facets of metabolic syndrome and chronic disease. For example, Borsheim et al, supplemented 11g of EAAs + arginine two times daily for 16 weeks in older adults with impaired glucose tolerance and found a significant increase in physical performance measured by gait speed, timed 5-step test, and timed floor-transfer test compared to baseline [210]. Furthermore, according to a meta-analysis of 36 studies, the effectiveness of protein in combination with micronutrients supplementation significantly increases in studies with a duration ≥ 6 months and in frail or malnourished study populations [211]. Therefore, more evidence is needed to establish the effect of protein and AA on older adults to promote muscle strength and function prior to the development of frailty and chronic disease to ensure SA.

The Role of Dietary Protein in Well-being. Dietary protein and its constituent AAs are essential for maintaining neuronal function and have been linked to affect states in older adults [69]. The effect of dietary protein and depression in older adults has partially focused on the AA tryptophan. Tryptophan, the precursor to serotonin, cannot be synthesized in adequate amounts in the body, therefore must be obtained from dietary sources [212]. Low plasma tryptophan is a

risk factor for depression as it leads to decreased serotonin levels [213]. Data from NHANES 2001-2002 (n=29,687) found tryptophan intake to be inversely associated with subjective depression and positively associated with subjective sleep duration despite even the lowest levels of usual intake surpassing the EAR for tryptophan [214]. The average intake of tryptophan was 826 ± 3 mg/day which is approximately three times the EAR of 4 mg/kg/day tryptophan (~280 mg/day for a 70-kg adult), albeit adults aged 51-70 and ≥ 71 y consumed 9% and 22% lower levels on average, respectively.

Sleep is an integral facet of well-being and is influenced by diet with equivocal findings in regard to dietary protein [215]. However, substantial observational data suggest dietary protein intake is associated with improvements in sleep [216-218]. For example, Kant et al. [216] conducted a cross-sectional analysis using NHANES data and found short sleepers consumed a lower percentage of protein, higher total sugars, a lower prevalence of breakfast consumption, with a higher frequency of snacks. Furthermore, according to a systematic review and meta-regression of cross-sectional studies and RCTs [217] good sleeper, defined as sleep duration ≥ 7 hours, PSQI global sleeping score ≤ 5, sleep latency ≤ 30 minutes, and sleep efficiency >85%, consumed greater amounts of dietary protein and a lower percentage of energy from dietary carbohydrates and fat than poor sleepers. These studies suggest that consuming greater amount of dietary protein may benefits sleep quality and healthy adults.

Few RCTs have investigated the effect of dietary protein on sleep quality and duration in middle-aged and older adults. One RCT conducted by Zhou et al. [219], assessed the effect of a high protein energy restricted diet for 16-weeks on middle-aged obese adults (56 ± 3 y) and found high protein diets to improve sleep quality. The mechanism of the effect of protein on sleep after acute feeding may be related to tryptophan, tyrosine, and the synthesis of the brain

neurotransmitters serotonin, melatonin, and dopamine [77, 220]. As BCAAs are transported into the brain across the blood brain barrier via the same carrier that transports large neutral amino acids (LNAA), phenylalanine, tyrosine, and tryptophan, the competition between BCAAs and the aromatic amino acids may influence synthesis of some neurotransmitters, including dopamine, norepinephrine, and serotonin [221]. Although higher protein intake results in greater postprandial plasma AA, high protein diets do not translate into constant low tryptophan- or tyrosine-to-LNAAs ratios. For example, Zhou et al. [219], did not find acute changes in the ratio of tryptophan to LNAAs and tyrosine to LNAAs. The relationship between acclimated protein intake and the body's ability to produce or remove brain tryptophan and serotonin is yet to be elucidated. However, in an intervention conducted in rhesus monkeys, a higher protein diet increased plasma and cerebrospinal fluid concentrations of tryptophan and serotonin metabolites indicating a probable beneficial sleep effect [222]. More long-term RCTs are needed to investigate the relationship between tryptophan: and tyrosine: LNAA ratios in older adults consuming high protein diets. Therefore, the beneficial effect of dietary protein on sleep has yet to be established.

Dietary Omega-3 Polyunsaturated Fatty Acid Recommendation, Current, and Optimal Intake

Current Omega-3 Polyunsaturated Fatty Acid Dietary Recommendations. n-3 PUFAs play a crucial role in SA [223]. n-3 PUFAs are a group of polyunsaturated fatty acids characterized by a double bond at the third carbon from the methyl (-CH3) end of the hydrocarbon chain. The human body is able to metabolize and convert alpha-linolenic acid (ALA; 18:3 n-3), the essential plant-based n-3 PUFA, to the more biologically active and

therapeutic longer chain n-3 PUFAs eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) by a series of desaturation and elongation reactions primarily in the liver [224]. The conversion rate to EPA and DHA is inefficient in humans and is further inhibited by age emphasizing the importance of dietary intake [225]. The 2015–2020 Dietary Guidelines for Americans (DGA) acknowledges the benefits of n-3 PUFAs and recommends a combined daily intake of 250 mg/day EPA + DHA equating to approximately 8 oz per week of a variety of fish in adults with and without CVD [166]. Furthermore, The American Heart Association's (AHA) Strategic Impact Goal Through 2020 and Beyond recommends at least two 3.5-oz fish servings per week, with an emphasis on oily fish (e.g., salmon, mackerel, herring), providing ~500 mg/day of EPA and DHA [226]. DRIs have yet to be established for EPA and DHA and DGA and AHA recommendations are derived from findings from prospective cohort studies and RCTs suggesting EPA and DHA rich eating patterns are associated with reduced risk of CVD [166]

The U.S. diet falls short of n-3 PUFA DGA and AHA recommendations. According to NHANES data (2003–2008) adults ≥ 50 years currently consume far below recommended levels of fatty fish (~ 0.19 oz/day) equating to 58 mg/day and 81 mg/day EPA and DHA, respectively [227]. NHANES data further demonstrates that, even after accounting for supplement intake and potential conversion of plant-based n-3 PUFAs, daily EPA and DHA intake from foods and supplements is well below recommendations with ~20% and ~10% of adults ≥ 55 years meeting or exceeding the DGA and AHA recommendations, respectively [228]. The low consumption of n-3 PUFA in the Western diet is of particular importance in the older population. Older adults require higher amounts of n-3 PUFAs due to decreased absorption, n-3 PUFA capacity to cross the blood-brain barrier, and physiological capacity to convert shorter chained fatty acids (ALA)

into longer fatty acids (EPA and DHA) resulting in a lower composition of n-3 PUFAs in body tissue [229, 230].

The Recommended Intake of Omega-3 Polyunsaturated Fatty Acids in Aging. To date, although somewhat conflicting, a growing number of studies indicate that n-3 PUFAs may exert beneficial effects on the aging brain [231, 232] and skeletal muscle [233] which could result in decreased cardiometabolic risk, improved physical function, and increased well-being. The inconsistent findings may be attributed to the inconsistent doses (~200 mg to 5 g) and duration (~4-wks to 6 months) of RCTs, since n-3 PUFA uptake increases in a time- and dose-dependent manner [234, 235]. For example, Yee et al. [234] supplemented four varying daily doses of n-3 PUFA for six months ranging from 0.84 g (0.47 g EPA and 0.37 g DHA) to 7.56 g (4.2 g EPA and 3.36 g DHA). Over the 2- to 6-month period, the two highest doses (5.04 g and 7.56 g) resulted in a significant increase in total serum lipid EPA and DHA concentrations [234]. Importantly, maximum tissue uptake is tissue dependent and has been reported for plasma phospholipids (56 days), erythrocytes (180 days), adipose tissue (indefinite), [236] and more recently, skeletal muscle (≥ 28 days) [237].

Recently, dietary and supplemental n-3 PUFAs have received considerable attention in the context of nutrition, aging, and skeletal muscle [229, 233, 238]. The beneficial impact of n-3 PUFAs on health is often related to the replacement of omega-six polyunsaturated fatty acids (n-6 PUFAs) by n-3 PUFAs in cell membrane phospholipids [239]. It is well recognized that Western diets have considerably higher n-6 PUFAs: n-3 PUFAs ratios than is considered optimal (15:1-20:1 versus 1:1-4:1) [240]. This shift in the n-6 PUFAs: n-3 PUFAs acid ratio in cell membranes has been shown to induce changes in numerous biological processes related to age-related decline including the expression of pro- and anti-inflammatory lipid mediators and

cytokines [239], gene expression [241], and is associated with increased chronic disease risk [242], functional impairment [63, 243], and depression [65]. n-3 PUFA supplementation has recently been observed to increase the relative and absolute EPA and DHA incorporation into skeletal muscle phospholipids [237]. The incorporation of n-3 PUFAs into skeletal muscle phospholipids supports observational findings of a positive and dose dependent relationship between fatty fish consumption and grip strength [244].

RCTs indicate ~3-5 g/day of combined EPA and DHA can promote skeletal muscle health and mass in older adults [26, 236]. The effect of n-3 PUFAs on skeletal muscle mass in adults is strengthened in the presence of an anabolic stimulus such as high-quality protein and/or EAA [245, 246]. For example, Smith et al., [245] supplemented ~ 4g/day of combined EPA and DHA for 8-weeks and found, in the presence of insulin and AAs, MPS rates increased by ~100% in older adults with no changes in basal MPS. Interestingly, inflammatory markers were unaffected throughout 8 weeks, with anabolic signaling proteins mTORC1 and p70s6k upregulated suggesting a possible mechanism by which n-3 PUFAs may have an anabolic effect. Therefore, we hypothesize that n-3 PUFAs may act on skeletal muscle as an anabolic primer, such that AAs/ high quality protein elicits a greater response when there is greater n-3 PUFA presence in skeletal muscle [236].

The Role of *Dietary Omega-3 Polyunsaturated Fatty Acids in Cardiometabolic Risk.* n-3 PUFA supplementation is an opportunity to decrease cardiometabolic risk as U.S. adults consume well below recommended levels [227]. In fact, the most commonly cited health benefit associated with n-3 PUFAs intake is cardiometabolic health [247] via mitigation of inflammation [248]. Seminal research in Greenland Inuit people first suggested high EPA and DHA intake was responsible for low CVD mortality [249]. Moreover, the Zutphen study, conducted in the

Netherlands, followed middle-aged adults over 20 years and showed a positive relationship between fish consumption and CVD prevention [250]. In agreement, U.S. prospective cohort studies have observed an association between higher circulating EPA and DHA and lower total mortality and coronary heart disease risk in older adults [251]. However, recent studies have challenged the efficacy of n-3 PUFA supplementation for the management of CVD risk due to inconsistent findings [252].

In RCTs, EPA and DHA supplementation in older adults on cardiometabolic risk is conflicting. For example, a 12-week RCT supplementing n-3 PUFAs in older women (N=24; EPA: 360mg/day; DHA: 1290mg/day) observed a 29% reduction in triglycerides with no effect on fasted blood glucose or insulin [253]. In agreement, a study supplementing n-3 PUFAs for 3-months (N=74; EPA:540mg/day; DHA:360mg/day) in middle-aged women (51.6 ± 7.8 years) found fish oil alone reduced triglycerides and LDL cholesterol by 5.4% and 8.4% respectively [254]. In contrast, n-3 PUFA supplementation (EPA: 1860mg/day; DHA: 1500mg/day) for 6 months was not effective in lowering blood lipids (e.g., TG, HDL, LDL) in healthy older adults [255]. However, muscle mass and strength were significantly improved, indicating n-3 PUFAs may also reduce overall mortality and cardiometabolic risk apart from blood lipids and via muscle mass and quality. Similarly, in postmenopausal women, n-3 PUFA supplementation did not affect markers of inflammation (e.g., TNF- α, IL-6 and CRP), but improved markers of physical performance (e.g., walking speed) [256]. An extensive 2018 meta-analysis of 79 RCTs found EPA and/or DHA to have little or no effect on mortality or cardiovascular health [257]. but did not consider outcomes of muscle mass or quality. Therefore, due to the strong association between cardiometabolic risk and skeletal muscle, future n-3 PUFA research should

be directed towards simultaneous evaluation of traditional markers of cardiometabolic risk in conjunction with skeletal muscle mass, strength, and function (reviewed in later sections).

Logan et al. [253] investigated the effects of 12-week n-3 PUFA (Total: 3 g/day; EPA and DHA) supplementation on body composition, strength, physical function, inflammatory markers, metabolic rate, and substrate oxidation in community dwelling older women compared to a placebo olive oil control group. The n-3 PUFA group had a 4% increase in muscle mass, a 7% improvement in the "Timed up and go test", a 14% increase in RMR, 19% increase in fat oxidation, and 10% and 27% increases in energy expenditure and fat oxidation during exercise respectively with no differences in inflammatory markers.

The Role of Dietary Omega-3 Polyunsaturated Fatty Acids in Physical Function.

Recently, dietary n-3 PUFAs have received considerable attention in the context of optimizing physical function in older adults. First, observational studies have identified a positive relationship of n-3 PUFAs and strength [244, 258] and physical function [259, 260]. Furthermore RCTs, notably two seminal RCTs in healthy middle-aged and older adults [245, 261], identified a potential muscle anabolic effect of combined EPA and DHA [262]. In addition to the observed anabolic effect of n-3 PUFAs, RCTs have identified a strength and performance effect of n-3 PUFAs in middle-aged and older adults. For example, Smith et al. [255] assessed the effects of 6 months of n-3 PUFA (EPA: 1.86 g/day; DHA: 1.5 g/day) supplementation on muscle mass and function in older adult men and women. n-3 PUFAs significantly increased muscle thigh volume (3.6%), handgrip strength (2.3 kg), and 1-repetition max (4.0%) with a non-statistical increase in isokinetic power (5.6%) when compared to a placebo group. Similarly, Logan et al. [253] investigated the effects of 12-week n-3 PUFA (total: 3 g/day; EPA and DHA) supplementation on body composition, physical function, inflammatory markers, metabolic rate, and substrate

oxidation in community dwelling older women compared to a placebo olive oil control group. The n-3 PUFA group had a 4% increase in muscle mass and a 7% improvement in the "Timed up and go test". In contrast, other RCTs have failed to show benefits of n-3 PUFAs in older adults. For example, Kryzminska-Siemaszko et al. [263] investigated the effect of 12-week n-3 PUFAs supplementation (Total: 1.3 g/day; EPA: 660 mg/day; DHA: 440 mg/day) on body composition and physical function in older adults (74.6 ± 8 years) with decreased muscle mass and found no significant differences in handgrip strength, "Timed up and go test", or gate speed. The lack of significance may be attributed to the low dose of n-3 PUFAs (<4 g/day). The majority of available data suggest diets including n-3 PUFAs increase physical function to promote SA. However, more research is needed to confirm these conclusions [264].

The Role of Dietary Omega-3 Polyunsaturated Fatty Acids in Well-being. One of the primary symptoms of poor well-being in the older population is depression [265]. n-3 PUFAs are a promising strategy to prevent and mitigate depression, partially, due to the incorporation into cerebral tissue as DHA levels fluctuate with diet, age, and sex [225]. Low consumption of n-3 PUFAs results in decreased brain DHA levels [266]. Brain inflammation progressively increases with age, but increased intake of DHA and EPA reduce inflammation by displacing arachidonic acid and cholesterol from the cell membrane [267]. For example, a recent systematic review and meta-analysis of RCTs in older adults (≥ 65 years of age) with depression evaluated the effects of n-3 PUFA supplementation and found an overall positive effect only in individuals with mild to moderate depression [76]. In addition, a longitudinal cohort study in adults 55-85 years (Hunter Community Study) found n-3 PUFA consumption to be inversely associated with depression [268]. Patients with depression have been reported to have low n-3 PUFA levels in RBC membrane (mg/100mg of total phospholipids) and a low dietary intake of DHA and EPA

[269]. Although n-3 PUFAs have been investigated in the context of depression, little is known on the effect of n-3 PUFAs on positive affect states.

In addition to depression, a link between sleep and n-3 PUFAs has been observed in adults. Observational evidence by Dashti et al. [216] found an association between recommended sleep duration and low carbohydrate and increased n-3 PUFA intake in older women (65-85 years). The n-3 PUFA index, calculated by the EPA and DHA content of erythrocyte membranes, expressed as a percentage of total fatty acids, is associated with sleep quality, metabolic health, and mortality [270]. A low n-3 PUFA index has been identified in obstructive sleep apnea patients [271]. In a randomized, double blind pilot study, postmenopausal women with greater baseline DHA RBC content presented less signs of frailty [256] indicating a relationship between frailty, sleep, and n-3 PUFA status. However, RCTs investigating the effect of n-3 PUFAs, DHA and EPA, are scarce in middle-aged and older adults. Hansen et al. investigated the effect of 6-months of fatty fish consumption of 300 g of Atlantic salmon three times per week (4.8g EPA and DHA per serving) in adults 21-60 years of age compared to a control group. The investigators found the fish group had significantly lower sleep latency at the conclusion of the intervention [272]. However, the sleep latency did not change in the intervention group, but worsened in the control group. Therefore, it cannot be concluded that fatty fish consumption is beneficial for sleep in this study. Further RCTs are needed to investigate the role of n-3 PUFAs and sleep in the older population.

Simultaneous Supplementation of Protein and Omega-3 Polyunsaturated Fatty Acids on Successful Aging

Increasing dietary protein and n-3 PUFAs intake is a potential strategy to promote skeletal muscle and SA in middle-age and older adults. However, RCTs examining the effect of dietary protein and n-3 PUFA combined supplementation have solely been conducted in the context of a multi-nutrient supplement or in combination with caloric restriction and/or exercise [273-275]. For instance, Bell et al. [274] investigated the effect of a multi-nutrient supplement that had 30 g of WPI, 2.5 g of creatine, 500 IU of vitamin D, 400 mg of calcium, and 1.5 g of n-3 PUFAs (700 mg EPA; 445 mg DHA), that was consumed twice daily. After six weeks of supplementation, LBM and muscle strength increased in healthy older adults. In addition, Su et al. [275] conducted a caloric restriction intervention in obese women (> 40 years) and found a high-protein meal replacement (25 g) and fish oil (2,130 mg) reduced percent android fat and the prevalence of metabolic syndrome by almost twofold in comparison to caloric restriction alone. As RCTs examining the combined effect of dietary protein and n-3 PUFAs are scarce, the modest effects observed in the described trials warrant further investigation.

Mechanisms of Successful Aging

According to the existing literature, combined doses of n-3 PUFAs of approximately 4 g/day and dietary protein of approximately 25-30 g/meal meets the suggested recommended nutritive doses to activate MPS in middle-aged and older adults [63, 162]. The mechanisms explaining an additive effect of protein and n-3 PUFAs remain to be fully understood. However, we speculate that n-3 PUFAs may promote SA outcomes by increasing neurotransmitter

sensitivity, membrane fluidity, and by enhancing the anabolic effects and neurotransmitter synthesis from EAA [276].

First, research suggests n-3 PUFAs and EAAs from dietary protein can improve the domains of SA via activation of the mammalian/mechanistic target of rapamycin (mTOR) pathway. mTOR is a serine-threonine kinase that serves as a nutrient, growth factor, and energy sensor and exists in two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [277]. The complex mTORC1 is involved in the activation of protein synthesis in skeletal muscle [278]. Leucine, a well-established regulator of protein synthesis [279], activates mTORC1 [280-285] in tissues such as the brain [281] and skeletal muscle [282], through Sestrin 2 [286]. In addition, the mTORC1 pathway is a regulator of MPS [287], muscle regeneration and repair [288], muscle protein breakdown (atrophy) [289], cerebral cellular survival [290], and is activated following n-3 PUFAs supplementation combined with AA infusion [246, 261]. Furthermore, stimulation of mTORC1 is a suggested approach to prevent age-related fiber atrophy, increase LBM, and improve physical function with advanced age [291, 292]. However, the combined effect of n-3 PUFAs and AA dietary intake on mTORC1 activation is unknown.

Second, with respect to the cellular membrane, n-3 PUFAs may increase neurotransmitter uptake and release. Acetylcholine is a neurotransmitter that supports muscle contraction, making synaptic transmission quicker at the neuromuscular junction resulting in a quicker contractility as well as improved cognitive function [276]. Moreover, acetylcholine interacts with additional excitatory transmitters in the brain such as OXA [293]. Fadel et al. [293-295] have identified a relationship between OXA and improved age-related cognitive decline, primarily via hippocampal and hypothalamic regulation via the cholinergic system. However, the effect of nutrients on the relationship between OXA, acetylcholine, and SA is largely unknown. Few

studies, primarily animal studies, have investigated the effect of dietary protein and n-3 PUFAs on OXA. Elliot et al. [296] delineated BCAA supplementation as a potential therapy to restore glutamate density to orexin neurons in mice with traumatic brain injury. Furthermore, in a study evaluating the effects of a high-protein diet in obese Zucker rats found that obese Zucker rats receiving the higher protein diet had higher levels of plasma orexin compared to the other treatments. [297]. Currently little research has investigated the effect of n-3 PUFAs on orexin neurons. A study examining the effect of fish oils and vegetable oils (olive, sunflower, linseed, and palm) found no effect on the presence or distribution of OXA, OXB, and OX2R in the hypothalamus and gastrointestinal system in rainbow trout [298]. Animal studies have shown dietary n-3 PUFAs to protect neurons from apoptosis by reducing oxidative stress [299] and therefore may protect again the loss of orexin neurons with age, albeit further research is needed to support or refute this theory.

In vitro analyses indicate OXA activates mTORC1 via extracellular calcium influx and lysosome pathway involving Rag GTPases and Erk/Akt-independent pathways [300]. It is probable that elevated OXA concentrations and protein and n-3 PUFA supplementation may further stimulate mTORC1 with age, resulting in inhibition of catabolic pathways linked to age-related decline in OXA, skeletal muscle, and well-being. Furthermore, n-3 PUFAs may enhanced OX2R signaling via incorporation into cellular membranes [225]. Given that neuronal function and anabolic signaling decline with advanced age, combined n-3 PUFA and protein supplementation may be a potential interventional strategy to mitigate age-related decline. However, further investigation of the mechanisms underlying the proposed effects are warranted.

Conclusion

Age-related loss of skeletal muscle mass increases the likelihood of cardiometabolic risk, loss of physical function, and poor well-being. These concerns continue to grow as the older population increases in the U.S. On the basis of the reviewed evidence, we propose that increased protein above the RDA and n-3 PUFAs above the DGA recommendations for middle-aged and older adults is required for older people to maintain skeletal muscle mass and to promote SA. Given that sarcopenia is, in part, underpinned by the reduced ability of dietary protein to stimulate MPS, increasing amounts of protein coupled with increased incorporation of n-3 PUFAs into cellular membranes may result in better preservation of muscle mass and neuroregulation. However, more research is needed to establish an additive effect of protein and n-3 PUFA on skeletal muscle mass, cardiometabolic risk, physical function, and well-being as a possible strategy to promote SA. As part of a multimodal intervention, increasing dietary protein and n-3 PUFA intakes may increase the prevalence of SA in middle-aged and older adults, beyond muscle mass maintenance. However, more research is needed.

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CHAPTER 3. Beef and Nutrients Found in Beef Positively Impact Well-Being in Healthy Adults ≥ 50 Years of Age: A Meta-Analysis and Systematic Review of Randomized Controlled Trials

Abstract

Shifts in well-being occur as we age. Nutrients found in beef are associated with outcomes of well-being such as physical and cognitive function, lean body mass, and mood. However, it is unclear how beef and nutrients found in beef impact well-being in healthy adults ≥ 50 years of age. The objective of this meta-analysis and systematic review was to evaluate the available evidence of randomized controlled trials (RCTs) assessing the effect of beef and nutrients found in beef on well-being in healthy adults ≥ 50 years of age. We hypothesized that RCTs using beef, or nutrients found in beef, would improve well-being outcomes in healthy adults ≥ 50 years of age. PubMed, CINAHL, and Web of Science were searched up to September 30, 2019 for eligible RCTs. Nine RCTs with 55 effect sizes were included in the meta-analysis. The random-effects model indicated an overall positive effect of beef and its nutrients on well-being (g = 0.20, 95% CI = [0.05, 0.34], p=0.01), with substantial heterogeneity. An overall positive effect of amino acids (g=1.53, 95% CI: [1.04, 2.03], p<0.01) and protein (g=0.71, 95% CI: [0.52, 0.92], p<0.01) was found on well-being outcomes with no effect of arginine, vitamin B-12, leucine, and zinc. Physical function (g=0.83, 95% CI: [0.49, 1.17], p<0.01) was influenced by beef and nutrients found in beef. This meta-analysis identified a need for further research regarding the effect of beef and nutrients found in beef on defined functional outcomes of well-being in healthy adults ≥ 50 years of age. PROSPERO CRD42020145729

Introduction

The older adult population in the United States is a segment of unprecedented growth [1]. Longer life spans and aging baby boomers will lead to nearly double the population of Americans ≥ 65 years of age over the next thirty years. Aging increases the risk of developing chronic diseases such as heart disease, cancer, and diabetes [2], which are responsible for the majority of health care costs for older Americans [3]. People living with chronic disease often experience diminished quality of life (QoL) due to gradual physiological and psychological decline and disability [3, 4]. Shifts in characteristics of QoL, such as decreased grip strength and cognitive function, begin prior to old age in the sixth decade of life [5-7]. However, advancing age is not always associated with significant functional regression [8], and some individuals maintain a successful aging trajectory [9, 10].

Successful aging is commonly defined as a multidimensional concept characterized by facets of high levels of physiological functioning, active social and emotional engagement, and beneficial extrinsic factors (e.g. improved nutrient intake and increased exercise) [9, 11-13]. Underlying the framework of successful aging is well-being [14]. Although a single definition for well-being has yet to be solidified, well-being is defined by the Center for Disease Control and Prevention (CDC) as positive emotions (presence of positive and absence of negative affect states), life satisfaction and fulfillment, and positive functioning [15]. Results from epidemiological studies demonstrate that well-being is associated with self-perceived health, longevity, healthy behaviors, mental and physical health, social connectedness, and productivity [16, 17]. In addition, data suggest that well-being is inversely associated with poor lifestyle factors such as dysregulated sleep quality, low physical activity, decreased lean body mass

(LBM), and poor diet [18]. However, few clinical trials have investigated the effect of nutrition on outcomes of well-being prior to the development of disability and chronic disease [19].

Adequate nutrition is a key contributor to successful aging [20]. Diets rich in nutrients found in high amounts in beef such as protein, essential amino acids, vitamins B-6, B-12, choline and minerals zinc and iron are associated with improved markers of chronic disease [21-24]. Research has validated the importance of protein intake above the current Recommended Dietary Allowance [25] of 0.8 grams per kilogram of body weight on strength and physical function for older adults [26, 27]. High quality protein sources, such as unprocessed meat, are negatively correlated with frailty [28], chronic disease, and muscle loss [29] in older adults. Beef is high in nutrients relative to calories [30, 31] and is protein dense (i.e. gram of protein per gram of food source) [32] when compared to alternative protein sources such as legumes, eggs, and dairy [21]. For example, a 3-ounce (~84g) serving of lean beef accounts for a fraction of daily calorie requirements (8.2%), ~25g of dietary protein, ~6.0 mg zinc (40 % daily value (%DV)), 2.2 □g B-12 (37 %DV), 0.4 mg B-6 (18 %DV), and 2.7 mg iron (15 %DV) [33].

Cross-sectional analyses have identified positive associations between unprocessed beef/lean red meat, LBM, physical function, and nutrient status [34, 35]. However, randomized controlled trials (RCTs) investigating the effect of beef alone are limited. A recent meta-analysis and systematic review of clinical trials found beef protein to provide similar benefits to commonly used whey protein isolate on LBM and exercise performance in adults [36]. In observational studies, LBM is commonly associated with positive physical and cognitive functioning, increased longevity, and improved QoL [37-39]. Furthermore, diets higher in nutrients found in greater amounts in beef, such as vitamin B-6, vitamin B-12, choline, zinc, and iron, are associated with improved markers of metabolic health [21, 22], but it is unclear how the

combination of protein and these nutrients impact well-being and QoL in aging adults. Nevertheless, studies of lean, red meat have reached contradictory conclusions in terms of health effects, in part because lean meats are often grouped together with processed meats [40-42]. However, recent studies suggest that lean red meat intake, such as beef, is not associated with increased risk of chronic disease [43-45].

RCTs have found positive benefits following beef consumption in adults when coupled with weight-loss and exercise, or in the presence of chronic disease [36, 46, 47]. However, RCTs investigating the effect of beef and nutrients found in beef under caloric maintenance in healthy, older adults remain limited. Lean beef contributes ~18% and ~22% of the dietary reference intakes for protein for males and females ≥ 51 years of age, respectively [48]. A recent meta-analysis revealed that beef consumption can promote LBM and exercise performance when combined with exercise training but did not explore the effect of beef apart from exercise [36]. Other meta-analyses focused on protein [49], vitamin B [50], zinc [51], and iron [52] supplementation focus on older adults with chronic disease, younger populations, multi-nutrient supplements, or do not assess outcomes of QoL or well-being. To our knowledge, a meta-analysis of RCTs has yet to summarize the existing data on the effects of beef and nutrients found in beef on markers of QoL and well-being in older adults.

Therefore, the objective of this meta-analysis and systematic review was to evaluate the available evidence of RCTs assessing the effect of beef and nutrients found in beef on QoL and well-being in healthy adults ≥ 50 years of age to promote successful aging. We hypothesized that RCTs using beef, or nutrients found in beef, would improve the successful aging outcomes, QoL and well-being, in healthy adults ≥ 50 years of age. We searched PubMed, CINAHL, and Web of Science databases and the reference list of the selected articles or related reviews for potential

trials up until September 30, 2019 by using key words such as older adults, beef, dietary protein, essential amino acids (EAA), branched chain amino acids (BCAA), tryptophan, arginine, cysteine, glycine, glutamate, vitamin B6, vitamin B12, choline, zinc, and iron. The QoL and well-being concept included search terms such as “well-being”, “quality of life”, “depression”, “cognitive function”, “mood”, “sleep”, “physical function”, “frailty”, and “strength”.

Materials and Methods

Approach. This systematic review and meta-analysis of randomized controlled trials was performed following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (PRISMA) [53]. The protocol for this meta-analysis was registered in PROSPERO (CRD42020145729).

Search Methods for Study Identification. Using predesigned search strategies, we systematically searched PubMed, CINAHL, and Web of Science databases for all RCTs up to September 30, 2019 investigating the effect of beef and nutrients found in beef on QoL and well-being in healthy older adults (aged $\geq$ 50 years). Three concepts were included in the search: population, nutrients, and QoL and well-being. The population search terms were “aging adults”, “older adults”, and “elderly”. The intervention search terms were “beef”, “red meat”, “animal dietary protein”, “dietary protein”, “essential amino acids”, “branched chain amino acids”, “leucine”, “tryptophan”, “arginine”, “cysteine”, “glycine”, “glutamate,” “vitamin B6”, “pyridoxine”, “vitamin B12”, “cobalamin”, “choline”, “zinc”, and “iron”. The QoL and well-being concept included search terms as “well-being”, “wellbeing”, “quality of life”, “depression”, “cognitive function”, “mood”, “sleep”, “physical function”, “frailty”, and

"strength". We also searched the reference list of the selected articles or related reviews for potential RCTs.

Eligibility Criteria. We included RCTs that examined associations between QoL, well-being, and beef and nutrients found in beef among healthy older adults (aged ≥ 50 years). Detailed inclusion and exclusion criteria can be found in Table 1.

Study Selection. Two reviewers independently reviewed the retrieved articles. All abstracts and titles were screened according to the inclusion and exclusion criteria. Disagreements were resolved by discussion to achieve consensus. Figure 1 depicts the flow of information through the different study selection phases including the studies identified, included, excluded, and the justifications for exclusions.

Study Extraction and Quality Assessment. Two investigators independently retrieved data regarding the study design. Participant characteristics, supplementation regimens, follow-up duration, outcome measures, statistical model, and experimental design. We assessed the quality of the RCTs using the National Heart, Lung, and Blood Institute website quality assessment tool for Quality Assessment of Controlled Intervention Studies (Table 2) [54].

Outcomes Assessed. The primary outcome was well-being as defined by the RCTs. Well-being was grouped into four categories: LBM, cognitive function, physical function, and QoL. Studies were synthesized using effect sizes as the dependent variable and summary measures [55]. An effect size was computed for each outcome of each included RCT. As a result, nine articles yielded a total of 55 effect sizes for the meta-analysis. First, to calculate the effect size Cohen's *d* effect size [56], which quantifies the standardized mean difference between the treatment (T) and baseline (B) groups (control), defined as:

$$d = \frac{\bar{x}_T - \bar{x}_B}{s_{pool}}, \quad (1)$$

where $\bar{x}_T$ and $\bar{x}_B$ are the means of the treatment and baseline groups, respectively, and s_{pool} is the pooled standard deviation computed as a function of sample sizes (n_T, n_B) and standard deviations (s_T, s_B) from both groups: $s_{pool} = \sqrt{\frac{(n_T-1)s_T^2+(n_C-1)s_B^2}{n_T+n_B}}$ was calculated. The Cohen's *d*, however, has been shown to overestimate the effect in small studies. Thus, the Hedges' *g* [57] effect size, a transformation of the Cohen's *d* was used to correct for small sample bias. Hedges' *g* was transformed from Cohen's *d* as follows:

$$g \backsimeq d \times \left(1 - \frac{3}{4(n_T + n_B) - 9}\right). \quad (2)$$

A positive *g* indicates a benefit of the treatment group, and a negative *g* indicates a benefit of the control group.

Heterogeneity Tests. The heterogeneity of effect sizes was evaluated using various statistical measures. To examine the between-study heterogeneity, the Cochran's Q statistic [58] and Higgin's and Thompson's I^2 [59] were used to assess the degree of heterogeneity. A significant Q statistic indicates heterogeneity (effect sizes come from different populations), whereas a non-significant Q statistic indicates homogeneity (effect sizes come from the same population). The I^2 represents the proportion of variability in effect sizes that is not accounted for by sampling errors. The I^2 of 25%, 50% and 75% indicates low, moderate and substantial heterogeneity, respectively [60]. Subgroup analyses and meta-regression analyses were conducted to investigate the possible sources of heterogeneity.

Publication Bias. Begg's funnel plot and Egger's test were used to statistically evaluate asymmetry and potential publication bias [61].

Statistical Analysis

Cohen's *d* effect sizes were computed and then transformed to Hedge's *g.* The overall differences between the control and treatment groups were examined using both the fixed-effects and random-effects models. The two common approaches for modeling effect sizes are 1) the fixed-effects model that assumes a homogeneous population of effect sizes, and 2) the random-effects model that assumes a distribution of true effect sizes [55]. More specifically, in a random-effects model, the variability of the effect sizes comes from both the sampling errors and the variation of true effects across studies [62]. The random-effects model provides wider confidence intervals for the effect estimates. In this study, a random-effects model was used to explore the overall variability of effect sizes and a mixed-effect model (random errors, fixed moderator effects) was used to evaluate each moderator at a time. The analysis of effect sizes was conducted using the computer program R [63], with the R packages meta [64] and dmetar [65]. All statistical tests were 2-sided, and statistical significance was defined as $P < 0.5$.

Results

Study Characteristics. Nine RCTs fulfilled the inclusion criteria and were included in the meta-analysis. The characteristics of the chosen studies are shown in Tables 3 and 4. The RCTs had a total of 864 participants. The RCT sample size ranged from 14 to 249 participants. Six studies recruited both male and female participants, one study recruited only male participants and two studies recruited only female participants. The duration of each RCT ranged from 8 to 104 weeks with one acute response intervention lasting 100 minutes. The average length of RCT was 33 weeks when excluding the acute study. The included RCT nutrients were consumed as a liquid (n=2), pill (n=6) or whole foods containing beef (n=1). The publication years of the nine

RCTs ranged from 2005 to 2018 and were sourced from 7 different journals. Outcomes pertaining to well-being were identified and analyzed separately as physical function (n=17), cognitive function/mood (n=36) and LBM (n=2) (Table 4). RCTs containing outcomes of QoL did not meet the search criteria. Five RCTs included outcomes pertaining to physical function, two RCTs included outcomes pertaining to LBM and physical function and four RCTs included outcomes pertaining to cognitive function. In addition, there is an inverse relationship between effect size and study characteristics of study duration, age, and BMI (Figure 2).

Meta-regression (Table 5) was used to examine study and participant characteristics as continuous moderators including age, BMI, study duration (time in weeks), and publication year. Continuous moderators of age (g=-0.02, p=0.02), BMI (g=-0.30, p=0.01) and study length (g=-0.01, p<0.01) were significant. There was no effect of BMI or publication year. There was a significant effect of heterogeneity on all meta-regression factors for continuous moderators (p<0.01 for all parameters).

Risk of Bias and Quality of Included Studies. The quality assessment score of the RCTs, as measured by the National Heart, Lung, and Blood Institute website quality assessment tool for Quality Assessment of Controlled Intervention Studies ranged from 11 to 13 out of a maximum of 14, indicating that all studies were high quality (Table 2).

Overall Effect of Beef and Nutrients Found in Beef on Well-being. The overall effect of beef and nutrients found in beef were modeled based on the 55 effect sizes computed from the outcomes of 9 RCTs, summarized in Table 6. The fixed-effects model indicated an overall positive effect of beef and nutrients found in beef on well-being in healthy, older adults (g = 0.08, 95% CI = [0.03, 0.13], p<0.01). The significant Q-statistic suggested statistical heterogeneity among effect sizes (Q (62) = 270.97, p<0.01). This was also supported by the high

I^2 of 80.1%, which indicated 80.1% of the variability in effect sizes was beyond the sampling errors. Due to the substantial heterogeneity of effect sizes, a random-effects model was fitted, which estimated a wider confidence interval for each effect. The random-effects model provided a greater overall effect estimate (g = 0.20, 95% CI = [0.05, 0.34], p=0.01) than the fixed-effects model. A forest plot (Figure 3) identifies the largest significant positive effects were found in the article of Scognamiglio et al [66]. An influence analysis [65] was conducted to identify the influential cases (extremely small or large effects). Five effect sizes were identified to have remarkably large effects; all came from Scognamiglio et al [66], consistent with what was observed in the forest plot. These effect sizes largely contributed to the between-study heterogeneity, and if being removed, the mean effect size g would drop to 0.02 (p=0.49).

Effect of Beef and Nutrients Found in Beef on Outcomes of Well-being. The results of the subgroup analysis suggest that physical function (g=0.83, 95% CI: [0.49, 1.17], p<0.01) was significantly influenced by beef and nutrients found in beef. The effect of physical function was positive reflecting an overall improvement in physical function. There was no significant effect of beef and nutrients found in beef on other outcomes of wellbeing including LBM or cognitive function/mood. Sex had a significant impact on effect size. There was a significant positive effect in studies including both men and women (g=0.22, 95% CI: [0.06, 0.38], p=0.01) with no effect in females only or males only. The results of the intervention nutrient subgroup analysis suggest a significant positive effect of amino acids (g=1.53, 95% CI: [1.04, 2.03], p<0.01) and protein (g=0.71, 95% CI: [0.52, 0.92], p<0.01) with no effect of arginine, vitamin B-12, leucine, and zinc. There was a significant negative effect of vitamin B-12 + vitamin B-6+ folic acid (FA) (g=-0.14, 95% CI: [-0.22, -0.064], p<0.01). There was a significant effect of heterogeneity on all subgroup analyses by moderator categories (p<0.05 for all parameters).

Publication Bias. There was evidence of publication bias using Begg's funnel plot and Egger's test of the intercept ($p<0.01$) (Figure 4) [61]. Caution should be taken when interpreting the results on account of the possible publication bias.

Discussion

To our knowledge, this is the first meta-analysis and systematic review to synthesize scientific literature regarding the impact of beef and nutrients found in beef on well-being in healthy adults ≥ 50 years of age. The results suggest that interventions incorporating beef, protein, and amino acids are potentially beneficial for outcomes of physical function in healthy older adults. Surprisingly, only one RCT evaluated the effect of beef as a whole food [67] and only two RCTs [67, 68] examined LBM, physical function, and multiple domains of well-being, within the same trial.

In the present meta-analysis, only two RCTs evaluated the effect of beef and nutrients found in beef on LBM and physical function. One RCT evaluated the effect of 7.5 g/d leucine [68] and the other evaluated the effect of high protein whole foods including beef (1.1 g protein·kilogram body weight^{-1}·d $^{-1}$) [67]. There was no effect of leucine or beef on LBM, which is supported by a previous meta-analysis evaluating the effect of protein supplementation sourced from non-beef protein sources on LBM [69]. In contrast to these findings, a meta-analysis and systematic review summarizing the effects of protein, not sourced from beef, on body composition and physical function in older adults found protein significantly increased LBM compared to the control group [70]. The lack of effect observed in the current meta-analysis, and contradictory findings throughout the literature, are likely attributed to the inconsistencies in methodology. RCTs showing a beneficial effect of protein supplementation on

LBM in healthy, older adults commonly use a supplementation period of at least 12-weeks [71, 72] and supplement with higher amounts of protein [73, 74] than what were used in the RCTs included in this analysis.

Only five RCTs measuring physical function, using 11 different physical function measurements, were analyzed in this study. These include gate speed/distance [66, 67, 75], handgrip strength [66, 67, 76], sit-and-stand [67, 75], and 1 repetition maximum (1RM) knee extensions [67, 68]. The diversity of physical function tests conducted within the five RCTs is reflective of the substantial heterogeneity found in this meta-analysis. Verhoeven et al [68] and Kim et al [67] found no effect of leucine supplementation or protein intake on strength and physical function in healthy, older men. Consumption of beef improved 1RM knee extension (kg) post-dietary intervention, although improvements were only observed when protein was consumed evenly throughout the day [67]. The lack of significance among other physical function tests may be due to the low protein amount or short duration. The largest effect sizes found in this meta-analysis came from Scognamiglio et al. [66]. In this study, 12 weeks of daily amino acid supplementation resulted in significant improvements in ambulatory function and hand-grip strength compared to the control group. The robust effect of amino acids in this RCT may be due to the older age of the participants (74 ± 5.5 years), the sedentary activity level of participants, and/or the longer study duration of 12 weeks.

Observational studies suggest a positive role of protein dense foods on cognitive function [77, 78]. However, in this meta-analysis there was no effect of either beef or nutrients found in beef on cognitive function in healthy older adults. We examined RCTs investigating the effect of nutrients found in beef, including vitamin B-12, B-6, folate, and zinc [79-81], on measures of cognitive function and found no effect of these nutrients on cognitive function in healthy, older

adults. This is supported by two recent meta-analyses which found no effect of B-vitamin intake, individually or in combination with other nutrients, on cognitive function in middle-aged and older adults [50, 82]. Dietary zinc is hypothesized to play a crucial role in regulating neuroplasticity, cognitive function, and positive and negative affect states in older adults [83-85]. The RCT included in this meta-analysis measured the effects of 15 mg/d or 30 mg/d of zinc on positive and negative affect states in healthy, older adults and found no effect compared to a placebo control [86]. In contrast, observational studies report an association of dietary and plasma zinc levels on positive and negative affect states [87, 88] and cognitive function in older adults [87].

Very few RCTs have investigated the effect of beef and nutrients found in beef on well-being in healthy, older adults independent of weight loss and/or exercise interventions. However, in the studies involving weight loss a beneficial effect of lean beef consumption on well-being has been found [89, 90]. For example, a 6-month weight loss trial in obese, older adults ≥ 60 years of age found consumption of 30 g of high-quality protein per meal, predominantly sourced from lean beef, reported an improvement in physical function when compared to a lower protein control group [89]. Similarly, O'Connor et al [90] compared a Mediterranean diet plan with 200 grams or 500 grams of lean red meat (beef and pork) per week and reported positive effects on outcomes of well-being including reduced physical limitations, improved mental health, and reduced fatigue [90].

There are several limitations to this meta-analysis and systematic review. First, there was a small and heterogenous set of studies which met the inclusion criteria for the meta-analysis. Few studies shared the same quantitative estimate on the relationship between beef and nutrients found in beef and a specific well-being outcome. In addition, beef and the nutrients studied in

this meta-analysis were provided in different forms such as whole foods, pills or liquid, which were not directly sourced from beef which may influence outcomes. Studies were conducted in nine different countries with diverse samples of different size, age, and sex. Lastly, we aimed to include a well-being domain of quality of life, albeit available RCTs did not meet our search inclusion criteria. The small heterogenous set of studies included in this meta-analysis emphasize a need for standardized measurements of well-being outcomes in future RCTs in healthy, older adults.

In summary, the results of our meta-analysis suggest that compared with a control group, protein and amino acids found in beef, may positively influence well-being through improved physical function in healthy adults ≥ 50 years of age.

Recommendations for Future Research

There is an evident need for additional well-designed RCTs evaluating the efficacy of beef and nutrients found in beef in healthy adults ≥ 50 years of age to promote well-being. Future research should adopt a population representative sample of healthy older adults, absent of chronic diseases, and examine the effect of lean beef on outcomes of well-being. For example, RCTs should implement lean beef supplementation within a multidimensional approach with homologous defined functional outcomes of LBM, cognitive function, physical function, and QoL to advance research in the field of aging and nutrition. Moreover, future studies should investigate the molecular mechanisms underlying the potential effect of beef consumption, apart from exercise and weight-loss, on well-being in healthy older adults.

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Conflict of Interest

This meta-analysis and systematic review was supported by the Beef Checkoff, and was not involved in the design, implementation, analysis, or interpretation of the data.

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Tables

Table 1. Study Selection Process: Inclusion and Exclusion Criteria

Criteria	Inclusion Criteria	Exclusion Criteria
Beef and Beef's Nutrients	Beef	Other sources of red meat
	Beef sourced protein	Non-beef sourced protein
	Zinc	Multivitamin supplements
	Arginine	
	Vitamin B-6	
	Vitamin B-12	
	Folic Acid	
	Essential amino acids (individually and as a group)	
	Choline	
	Cysteine	
	Glycine	
	Glutamate	
Study Design	Randomized controlled clinical trials	Exercise
	Registered clinical trial	Method of nutrient supplementation (e.g. injection)
		Review article
		Meta-analysis
		Longitudinal or cross-sectional data
		Epidemiological
		Weight-loss
		Non-human model

Table 1. Study Selection Process: Inclusion and Exclusion Criteria (Cont.)

Criteria	Inclusion Criteria	Exclusion Criteria
Outcomes	Well-being and Quality of Life (e.g. SF-36, SF12, EQ-5D, HRQOL)	Mechanistic
	Strength (e.g. handgrip, 1RM)	Appetite
	Physical function (e.g. gait, walking speed, sit-stand test)	Bone Health
	Cognitive function, Mood, Depression (e.g. POMS, MMSE)	Fat mass
	Sleep (e.g. PSQI, Actigraph)	
	Lean body mass	
Journal Characteristics	Peer-reviewed full text	Conference abstracts
	English language	Non-English language
		Statistics cannot be quantified
Participant Characteristics	Humans	Chronic disease
	Healthy	Age not specified
	≥50 years of age	Cognitive disorders/Dementia
		Non-human model

Table 2. Quality Assessment of Controlled Intervention Studies

	Study ID (Reference number)								
Criteria	1	2	3	4	5	6	7	8	9
1. Was the study described as randomized, a randomized trial, a randomized clinical trial, or an RCT?	1	1	1	1	1	1	1	1	1
2. Was the method of randomization adequate (i.e., use of randomly generated assignment)?	X	1	X	X	1	1	X	1	X
3. Was the treatment allocation concealed (so that assignments could not be predicted)?	1	1	1	1	1	1	X	1	1
4. Were study participants and providers blinded to treatment group assignment?	1	1	1	1	X	1	1	1	1
5. Were the people assessing the outcomes blinded to the participants' group assignments?	1	1	1	0	X	1	X	1	1
6. Were the groups similar at baseline on important characteristics that could affect outcomes (e.g., demographics, risk factors, co-morbid conditions)?	1	1	1	1	1	0	1	X	1
7. Was the overall drop-out rate from the study at endpoint 20% or lower of the number allocated to treatment?	1	1	1	1	1	1	1	X	1
8. Was the differential drop-out rate (between treatment groups) at endpoint 15 percentage points or lower?	1	1	1	1	1	1	1	X	1
9. Was there high adherence to the intervention protocols for each treatment group?	1	1	1	1	X	X	1	1	X
10. Were other interventions avoided or similar in the groups (e.g., similar background treatments)?	1	1	1	1	1	1	1	1	1
11. Were outcomes assessed using valid and reliable measures, implemented consistently across all study participants?	1	1	1	1	1	1	1	1	1
12. Did the authors report that the sample size was sufficiently large to be able to detect a difference in the main outcome between groups with at least 80% power?	1	1	1	0	X	1	1	1	0
13. Were outcomes reported or subgroups analyzed prespecified (i.e., identified before analyses were conducted)?	1	0	1	0	1	1	1	1	1
14. Were all randomized participants analyzed in the group to which they were originally assigned, i.e., did they use an intention-to-treat analysis?	1	1	1	1	1	1	1	1	1
Total Score	13	13	13	10	10	12	11	11	11

1 denotes Yes, 0 denotes N0, and X denotes not reported

Table 3. Main characteristics of subjects in randomized controlled trials included for the meta-analysis

Study ID	Primary Author (Year)	Country	Sample Size (Experimental)	Sample Size (Control)	Age (Years)	Age (SD)	Sex	BMI
1	Aguiar, 2015 [73]	Brazil	10	10	71.6	6.1	F	26.6
2	Dangour, 2015 [77]	England	97	100	80.0	3.6	B	27.3
3	Eussen, 2006 [78]	Netherlands	50	53	82.0	5	B	NR
4	Fricke, 2008 [74]	Germany	11	12	53.9	4.3	F	23.5
5	Kim, 2018 [65]	United States	7	7	59.2	6.3	B	27.5
6	McMahon, 2006 [79]	New Zealand	125	124	73.5	5.8	B	26.8
7	Scognamiglio, 2005 [64]	Italy	48	47	74.0	5.5	B	25.3
8	Stewart-Knox, 2011 [84]	Ireland	62	62	68.1	4.1	B	NR
9	Verhoeven, 2009 [66]	Belgium	15	14	71.0	15.3	M	26.1

Int, intervention; M, male; F, female; B, both males and females; NR, not reported

Table 3. Main characteristics of subjects in randomized controlled trials included for the meta-analysis (Cont.)

Study ID	Primary Author (Year)	Int. Duration	Statistical Model	Attrition Rate
1	Aguiar, 2015 [73]	Acute	Repeated measures ANOVA	0%
2	Dangour, 2015 [77]	52	ANCOVA Logistic Regression	5.0%
3	Eussen, 2006 [78]	24	ANOVA	16.9%
4	Fricke, 2008 [74]	26	ANOVA	0%
5	Kim, 2018 [65]	8	ANCOVA	0%
6	McMahon, 2006 [79]	104	Estimating equations w/ exchangeable correlation matrix	8.3%
7	Scognamiglio, 2005 [64]	12	Repeated measures ANOVA	5.0%
8	Stewart-Knox, 2011 [84]	26	Mixed ANOVA	Not Reported
9	Verhoeven, 2009 [66]	12	Repeated Measures ANOVA	3.6%

Int, intervention; M, male; F, female; B, both males and females; NR, not reported

Table 4. Intervention arms, nutrients consumed, well-being measures, and results.

Study ID	Intervention Arms	Nutrient/diet methodology	Well-being parameter	Study Results Post-Intervention	Overall Conclusion
1 [73]	Intervention: L-Arginine 8 g, liquid form Control: Corn starch 8 g, liquid form	Supplements were orally administered in water in a double-blind placebo-controlled randomized design. Physical tests and examinations were initiated 80 min after supplementation	Physical Function	**Tandem gait:* ARG:16.8±1.2 vs. PLA: *18.8±1.3s; (NS) **Sit-stand:* *ARG:4.9±0.1. vs. PLA: 5.1±0.3s; (NS) **Timed up and go* *ARG:7.2± 0.3 vs PLA: 7.4±0.4s; (NS)	Acute arginine supplementation does not significantly effect endothelial function or muscle performance in older women.

[1]All values are means ± SDs unless indicated as * denoting SEMs. Non-significant p-values are denoted by NS; #ofn: number of nouns; significant p-values are denoted by the given p-value, P=0.05

[2] PLA, placebo.

Table 4. Intervention arms, nutrients consumed, well-being measures, and results (Cont.)

Study ID	Intervention Arms	Nutrient/diet methodology	Well-being parameter	Study Results Post-Intervention	Overall Conclusion
2 [77]	Intervention: Vitamin B-12 (cyanocobalamin) 1 mg Control: 1 mg control tablet (nutrient not reported)	Participants had moderate vitamin B-12 deficiency in the absence of anemia and of neurologic and cognitive signs or symptoms. Supplements administered as a daily oral tablet for 12 months in a double-blind placebo-controlled randomized manner.	Cognitive Function/ Mood	*30-item general health questionnaire* *Vit B-12: 2.4±0.5 vs PLA: 2.7±0.5; Adj effect size and 95% CI -0.1(-1.3,1.1);* *California Verbal Learning Test: [A] Total words correct in first 3 trials* *Vit B-12: 23.9*±0.7 vs PLA: 24.6±0.7; Adj effect size and 95% CI -1.4 (-2.9,0.1) *[B] Words recalled at delayed recall* Vit B-12 7.5±0.3 vs PLA:7.7±0.4 Adj effect size and 95% CI -0.4 (-1.0,0.2) *Symbol letter modality, n correct* Vit B-12: 39.6±1.1 vs PLA: 4-.1±1.2; Adj effect size and 95% CI -1.3 (-3.2,0.6) *Simple-reaction time* *Vit B-12: 0.3*±0.01 vs PLA: 0.3±0.01; Adj effect size and 95% CI 0.01 (-0.02,0.04) *Choice-reaction time* *Vit B-12: 0.7*±0.01 vs. PLA: 0.7±0.02; Adj effect size and 95% CI - 0.003 (-0.03,0.02) *Verbal fluency* *Vit B-12: 20.8*±0.5 vs PLA 19.9±0.6; Adj effect size and 95% CI 1.1(-0.01,0.22)	12-months of vitamin B-12 supplementation does not significantly effect neurologic or cognitive function.

[1]All values are means ± SDs unless indicated as * denoting SEMs. Non-significant p-values are denoted by NS; #ofn: number of nouns; significant p-values are denoted by the given p-value, P=0.05

[2] PLA, placebo.

Table 4. Intervention arms, nutrients consumed, well-being measures, and results (Cont.)

Study ID	Intervention Arms	Nutrient/diet methodology	Well-being parameter	Study Results Post-Intervention	Overall Conclusion
3 [78]	Intervention : Vitamin B-12 (cyanocobalamin) 1000 µg, tablet Control: AVICEL PH102, tablet	Participants had moderate vitamin B-12 deficiency in the absence of anemia and of cognitive impairment. Supplements administered as a daily oral tablet for 24 weeks in a double-blind placebo controlled randomized manner.	Cognitive Function/ Mood	*Construction: complex figure of Rey (pts)* *Vit B-12: 30.0*±7.5 vs PLA:29.2±7.0 (NS) *Attention: digit span forward-attention (pts)* Vit B-12: 7.5±1.7 vs PLA: 7.8±1.6 (NS) *Motor planning 2-sensomotor speed (millisecond)* Vit B-12: 647±265 vs PLA: 618±300 (NS) *Finger tapping-sensomotor speed (millisecond)* *Vit B-12: 412*±175 vs. PLA:389±168 (NS) *Trail making test-sensomotor speed [1]* *Vit B-12: 77.5*±52.3 vs PLA: 73.9±43.9 (NS) *15 word learning immediate recall-memory(pts)* Vit B-12: 35.2±12.1 vs PLA: 35.7±11.1 (NS) *15 word learning delayed recall-memory(pts)* VitB-12: 5.5±3.9 vs. PLA: 6.1±3.9 (NS) *15 word learning recognition-memory(pts)* Vit B-12: 26.6±3.7 vs PLA: 27.0±3.6 (P<0.05) *Complex figure of Rey, immediate recall-memory (pts*) Vit B-12: 12.2±7.7 vs PLA: 12.7±7.4 (NS) *Complex figure of Rey delayed recall-memory (pts)* Vit B-12: 11.4±7.0 vs PLA: 11.9±7.3 (NS) *Digit span backward-memory (pts)* Vit B-12: 4.6±1.6 vs PLA: 5.3±1.7 (P<0.05)	Oral supplementation of vitamin B-12 for 24 weeks does not effect cognitive function.

[1]All values are means ± SDs unless indicated as * denoting SEMs. Non-significant p-values are denoted by NS; #ofn: number of nouns; significant p-values are denoted by the given p-value, P=0.05

[2] PLA, placebo.

Table 4. Intervention arms, nutrients consumed, well-being measures, and results (Cont.)

Study ID	Intervention Arms	Nutrient/diet methodology	Well-being parameter	Study Results Post-Intervention	Overall Conclusion
3 [78]	Intervention : Vitamin B-12 (cyanocobalamin) 1000 µg, tablet Control: AVICEL PH102, tablet	Participants had moderate vitamin B-12 deficiency in the absence of anemia and of cognitive impairment. Supplements administered as a daily oral tablet for 24 weeks in a double-blind placebo controlled randomized manner.	Cognitive Function/ Mood	*Motor planning 3-executive function (millisecond)* Vit B-12: 863±376 vs PLA: 990±696 (NS) *Trail making test (part C/part A)-executive function (millisecond)* Vit B-12: 2.8±1.2 vs PLA: 2.8±1.0 (NS) *Stroop test (part 3/part 2)-executive function (millisecond)* Vit B-12: 2.2±0.9 vs PLA: 2.8±1.0 (NS) *Similarities WAIS-executive function (pts)* Vit B-12: 6.1±2.6 vs PLA: 5.4±2.8 (NS) *Raven-executive function (pts)* Vit B-12: 16.6±3.5 vs PLA: 16.5±3.9 (NS) *Word fluency animals-executive function (#onf)* Vit B-12: 17.6±5.5 vs PLA: 16.5±5.9 (NS) Word fluency letter-executive function (#ofn) VitB-12: 15.5±7.9 vs PLA: 17.5±8.8 ($p<0.05$)	Oral supplementation of vitamin B-12 for 24 weeks does not effect cognitive function.

[1]All values are means ± SDs unless indicated as * denoting SEMs. Non-significant p-values are denoted by NS; #ofn: number of nouns; significant p-values are denoted by the given p-value, P=0.05
[2] PLA, placebo.

Table 4. Intervention arms, nutrients consumed, well-being measures, and results (Cont.)

Study ID	Intervention Arms	Nutrient/diet methodology	Well-being parameter	Study Results Post-Intervention	Overall Conclusion
4 [74]	Intervention: L-Arginine hydrochloride 18 g and 14.8 g, tablet L-Arginine/ daily Control: Dextrose, tablet	Supplements administered orally for 26 - weeks in a double-blind placebo controlled randomized manner. Secondary analysis from Baecker et al.,	Physical Function	*Maximal isometric grip force (MIGF) of non-dominant hand via Jamar dynamometer (Newton) *ARG: 0.909*±2.3*: vs PLA: 1.167*±2.368 (NS)	Oral supplementation of L-Arginine for 26 weeks did not significantly influence MIGF in postmenopausal women.

[1]All values are means ± SDs unless indicated as * denoting SEMs. Non-significant p-values are denoted by NS; #ofn: number of nouns; significant p-values are denoted by the given p-value, P=0.05
[2] PLA, placebo.

Table 4. Intervention arms, nutrients consumed, well-being measures, and results (Cont.)

Study ID	Intervention Arms	Nutrient/diet methodology	Well-being parameter	Study Results Post-Intervention	Overall Conclusion
5 [65]	Intervention: Even Distribution of high protein beef containing foods (33/33/33%) Control: Typical American distribution of beef high protein containing foods (15/20/65%) Protein sources including beef, eggs, and dairy	UNEVEN control group consumed 1.1g protein/kg body weight/day in an uneven pattern (15/20/65%) comparable to the traditional pattern of meal intake in the U.S for 8-weeks. The EVEN group consumed an equal amount of protein with an even pattern of ~33/33/33% protein for 8-weeks Diets were configured to maintain a stable body weight via Harris-Benedict equation and level of physical activity.	Lean body mass Physical Function	*DEXA Lean body mass (kg)* EVEN Pre: 50.5 ±2.7 vs, EVEN Post: 50.3±3.1 (NS) UNEVEN Pre:47.7±4.2 vs UNEVEN: Post 46.9±4.1 (NS) EVEN Post: 50.3±3.1 vs UNEVEN: Post 46.9±4.1 (NS) *1 RM knee extension, kg* EVEN Pre:59.2±5.6 vs, EVEN Post: 73.1±7.4 * UNEVEN Pre: 45.8±6.1 vs UNEVEN: Post 52.3±8.7 (NS) EVEN Post: 73.1±7.4 vs UNEVEN Post: 52.3±8.7 (NS) *Handgrip strength, kg* Even Pre: 37.5±3.8: vs EVEN Post: 40.7±4.5 Uneven Pre: 33.0±4.6 vs UNEVEN Post: 32.9±3.9 (NS) EVEN Post: 40.7±4.5 vs UNEVEN Post: 32.9±3.9 (NS) *10 m gait speed, s* *Even Pre:5.6*±0.6 *vs* EVEN Post: 5.0±0.4 (NS) *Uneven Pre: 6.2*±0.6 *vs* UNEVEN Post: 6.7±0.6 (NS) EVEN Post: 5.0±0.4 vs UNEVEN Post: 6.7±0.6 (NS) *Sit/Stand 5 reps (s)* *Even Pre:10.4* ±0.9 vs EVEN Post: 8.0±1.0 (NS) Uneven Pre:10.5 ± 1.7 vs UNEVEN Post: 9.7±1.2 (NS)	8-week intervention period of an even or uneven distribution pattern of mixed meals does not significantly affect muscle strength or functional outcomes.

[1]All values are means ± SDs unless indicated as * denoting SEMs. Non-significant p-values are denoted by NS; #ofn: number of nouns; significant p-values are denoted by the given p-value, P=0.05
[2] PLA, placebo.

Table 4. Intervention arms, nutrients consumed, well-being measures, and results (Cont.)

Study ID	Intervention Arms	Nutrient/diet methodology	Well-being parameter	Study Results Post-Intervention	Overall Conclusion
5 [65]	Intervention: Even Distribution of high protein beef containing foods (33/33/33%) Control: Typical American distribution of beef high protein containing foods (15/20/65%) Protein sources including beef, eggs, and dairy	UNEVEN control group consumed 1.1g protein/kg body weight/day in an uneven pattern (15/20/65%) comparable to the traditional pattern of meal intake in the U.S for 8-weeks. The EVEN group consumed an equal amount of protein with an even pattern of ~33/33/33% protein for 8-weeks Diets were configured to maintain a stable body weight via Harris-Benedict equation and level of physical activity.	Lean body mass Physical Function	*Sit/Stand 5 reps (s)* *Even Pre:10.4* ±0.9 vs EVEN Post: 8.0±1.0 (NS) Uneven Pre:10.5 ± 1.7 vs UNEVEN Post: 9.7±1.2 (NS) EVEN Post: 8.0±1.0 vs UNEVEN Post: 9.7±1.2 (NS) *Stair ascend power, Nm/s* *Even Pre: 347.9* ± 15.7 vs EVEN Post:360.3±30.3 (NS) *UNEVEN Pre: 290.6* ± 46.6 UNEVEN Post: 282.7±46.6 (NS) EVEN Post:360.3±30.3 vs UNEVEN Post: 282.7±46.6 (NS) *Stair descend power, Nm/s* *Even Pre: 363.9* ± 16.8 vs EVEN Post: 401.1±32.7 (NS) *UNEVEN Pre: 300.8* ± 53.7 UNEVEN Post: 304.6±58.1 (NS) EVEN Post: 401.1±32.7 vs UNEVEN Post: 304.6±58.1 (NS)	8-week intervention period of an even or uneven distribution pattern of mixed meals does not significantly affect muscle strength or functional outcomes.

[1]All values are means ± SDs unless indicated as * denoting SEMs. Non-significant p-values are denoted by NS; #ofn: number of nouns; significant p-values are denoted by the given p-value, P=0.05

[2] PLA, placebo.

Table 4. Intervention arms, nutrients consumed, well-being measures, and results (Cont.)

Study ID	Intervention Arms	Nutrient/diet methodology	Well-being parameter	Study Results Post-Intervention	Overall Conclusion
6 [79]	Intervention: Folic acid: 1000 μg, Vitamin B-12 (cobalamin) 500 μg, Vitamin B-6 (pyridoxine) 10 mg, tablet Control: MGF, tablet	Participants had high fasting homocysteine concentrations of at least 13 μmol per liter and were otherwise healthy. Participants orally consumed a daily treatment or control capsule for 2 years in a double-blind, placebo-controlled, randomized manner.	Cognitive Function/ Mood	*Mini-Mental State Examination (pts.)* B-VIT: 29.29±1.41 vs PLA: 29.32±2.10 (NS) *Wechsler Paragraph Recall test (pts)* B-VIT:18.67±6.55 vs PLA:20.76±7.21 (NS) *Category Word Fluency test (# of words)* B-VIT: 65.72±14.96 vs PLA:68.78±13.71 (NS) *Rey Auditory Verbal Learning (# of words)* B-VIT:43.90±9.70 vs PLA: 44.22±9.90 (NS) *Raven's Progressive Matrices (pts)* B-VIT: 11.60±2.92 vs PLA: 11.90±3.05 (NS) *Controlled Oral Word Association test (# of words)* B-VIT: 40.11±14.08 vs PLA: 41.00±12.44(NS) *Part B of the Reitan Trail Making Test (sec to completion)* B-VIT: 114.40±84.23 vs PLA:98.96±40.75 (p=0.007)	2-year oral supplementation of B-vitamins does not significantly affect cognitive performance.

[1]All values are means ± SDs unless indicated as * denoting SEMs. Non-significant p-values are denoted by NS; #ofn: number of nouns; significant p-values are denoted by the given p-value, P=0.05

[2] PLA, placebo.

Table 4. Intervention arms, nutrients consumed, well-being measures, and results (Cont.)

Study ID	Intervention Arms	Nutrient/diet methodology	Well-being parameter	Study Results Post-Intervention	Overall Conclusion
7 [64]	Intervention: oral amino acid (AA) mixture 12 g/day+ 12.21 g of glucose, liquid Control: glucose 12.21 g/day, liquid <u>AA</u> <u>Composition/day:</u> L-leucine: 3.8 g L-lysine 2 g L-isoleucine 1.9 g L-valine 1.9 g L-threonine: 1.1 g L-cystine: 0.4 g L-histidine: 0.4 g L-phenylalanine: 0.3 g L-methionine: 0.2 g L-tyrosine: 0.1 g L-tryptophan 0.1 g	Participants with reduced physical activity consumed an oral amino acid mixture or placebo 3-times daily for 3 months as snacks at 10:00am., 4:00p.m., and 10:00 p.m. in a single-blind, placebo-controlled, randomized manner. Participants were instructed to reduce their usual dietary intake by 450kcal per day to compensate for the supplements.	Physical Function	*Ambulatory function: 6 min walk distance (m)* AA: 268.8±34.9 vs PLA: 212±40 ($p<0.001$) *Self -reported ambulatory ability: distance (%)* AA:68.3±12 vs PLA: 53±14.8 ($p<0.001$) *Self -reported ambulatory ability: speed (%)* AA: 72.2±14.4 vs PLA: 52.8±12 ($p<0.001$) *Self -reported ambulatory ability: stairs (%)* AA: 98.2±24 vs PLA: 72.4±22 ($p<0.001$) *Maximal Isometric muscle strength; Right hand (kg)* AA: 20.2±2 vs PLA: 14.38 ($p<0.001$)	3-months of oral amino acid mixture significantly improved ambulatory capacity, maximal isometric muscle strength, and myocardial ability in elderly subjects without affecting tested metabolic parameters.

[1]All values are means ± SDs unless indicated as * denoting SEMs. Non-significant p-values are denoted by NS; #ofn: number of nouns; significant p-values are denoted by the given p-value, P=0.05

Table 4. Intervention arms, nutrients consumed, well-being measures, and results (Cont.)

Study ID	Intervention Arms	Nutrient/diet methodology	Well-being parameter	Study Results Post-Intervention	Overall Conclusion
8 [84]	Intervention: Zinc gluconate 15 mg/d Zinc gluconate 30 mg/d Control: placebo pill	Participants in four European centers (Northern Ireland, Clermont-Ferrand, Rome, Grenoble) consumed 15mg, 30mg, or a placebo pill with breakfast daily for 6 months in a double-blind placebo-controlled, randomized manner	Cognitive Function/ Mood	*Positive and Negative Affect Scale (PANAS): 55-70 yrs old/Coleraine and Clermont-Ferrand (pts)* *Sum of 4 consecutive days (upon rising, breakfast, lunch, after dinner and before going to bed)* *Positive affect* Zn(15mg) 28.70±6.09 vs PLA: 26.86±5.20 (NS) Zn (30mg): 29.06±5.54vs PLA 26.86±5.20 (NS) *Negative affect (pts)* Zn(15mg): 12.22±3.30 vs PLA 11.84±2.89 (NS) Zn (30mg): 11.22±1.95vs PLA 11.84±2.89 (NS) *Positive and Negative Affect Scale (PANAS): ≥ 70 yrs old/Rome and Grenoble* *Sum of 4 consecutive days (upon rising, breakfast, lunch, after dinner and before going to bed)* *Positive affect* Zn(15mg) 23.20±6.50 vs PLA: 24.36±9.02 (NS) Zn (30mg): 23.59±7.68 vs PLA: 24.36±9.02 (NS) *Negative affect (pts)* Zn(15mg): 13.27±4.90 vs PLA 12.29±3.02 (NS) Zn (30mg): 12.81±4.26.95vs PLA 12.29±3.02 (NS)	6-months of oral zinc supplementation does not significantly affect mood in healthy elderly European adults

[1]All values are means ± SDs unless indicated as * denoting SEMs. Non-significant p-values are denoted by NS; #ofn: number of nouns; significant p-values are denoted by the given p-value, P=0.05

[2] PLA, placebo.

Table 4. Intervention arms, nutrients consumed, well-being measures, and results (Cont.)

Study ID	Intervention Arms	Nutrient/diet methodology	Well-being parameter	Study Results Post-Intervention	Overall Conclusion
9 [66]	Intervention : Leucine 2.5 g/main meal and 7.5 g/day, tablet Control: Wheat flour: 2.5 g/main meal and 7.5 g/day, tablet	Participants consumed 5 capsules of leucine or placebo with each main meal daily for 3-months in a double-blind placebo-controlled randomized manner.	Physical Function Lean body mass	*1RM leg press* *Leucine:170±8 vs Placebo:172±6 (NS) **1RM leg extension* *Leucine: 85±3vs Placebo: 85±3 (NS) **Lean Mass (Kg)* *Leucine: 55.0±1.5 Placebo: 56.2±1.1 (NS)	3-months of leucine supplementation with each main meal does significantly affect muscle mass and strength in healthy elderly men

[1]All values are means ± SDs unless indicated as * denoting SEMs. Non-significant p-values are denoted by NS; #ofn: number of nouns; significant p-values are denoted by the given p-value, P=0.05
[2] PLA, placebo.

Table 5. Meta-Regression Results for Continuous Moderators

Aggregation	*n*	Estimate	SE	95 CI		*p*	Heterogeneity		
				Lower	Upper		Q_b	*df*	*p*
Age	55						260.24	53	<0.01
Intercept		1.93	0.75	0.42	3.43	0.01			
Age (slope)		-0.02	0.01	-0.04	-0.003	0.02			
BMI	33						148.26	31	<0.01
Intercept		8.43	3.17	1.96	14.90	0.01			
BMI (slope)		-0.30	0.11	-0.55	-0.06	0.01			
Length of time in weeks	52						219.51	50	<0.01
Intercept		0.46	0.11	0.24	0.68	<0.01			
Length (slope)		-0.01	0.002	-0.01	-0.003	<0.01			
Publication year (centered at 2005)	55						270.97	53	<0.01
Intercept		0.18	0.10	-0.02	0.38	0.07			
Year (slope)		0.004	0.02	-0.03	0.04	0.82			

Table 6. Modeling Results for Overall Effects and by Moderator Categories

Aggregation	*n*	Effect Size		95 CI		*p*	Heterogeneity		
		g	SE	Lower	Upper		Q_b	*df*	*p*
Fixed effects	55	0.08	0.03	0.03	0.13	<0.01	270.97	54	<0.01
Random effects	55	0.20	0.07	0.05	0.34	0.01	270.97	54	<0.01
Well-being Outcome							22.33	2	<0.01
Lean body mass	2	-0.06	0.29	-0.63	0.52	0.85			
Cognitive Function	36	-0.01	0.03	-0.07	0.06	0.81			
Physical Function	17	0.83	0.17	0.49	1.17	<0.01			
Sex							9.32	2	0.01
Female	4	0.19	0.13	-0.06	0.45	0.13			
Male	3	-0.11	0.08	-0.28	0.05	0.16			
Both	48	0.22	0.08	0.06	0.38	0.01			
Beef and Beef's Nutrients							106.06	6	<0.01
AA	5	1.53	0.25	1.04	2.03	<0.01			
Arginine	4	0.19	0.13	-0.06	0.45	0.13			
B-12	25	0.01	0.04	-0.07	0.08	0.83			
B-12 +B-6 +FA	7	-0.14	0.04	-0.22	-0.06	<0.01			
Leucine	3	-0.11	0.08	-0.28	0.05	0.16			
Protein/Beef	7	0.71	0.10	0.52	0.90	<0.01			
Zinc	4	0.21	0.12	-0.02	0.44	0.07			

[1] FA, folic acid.

Figures

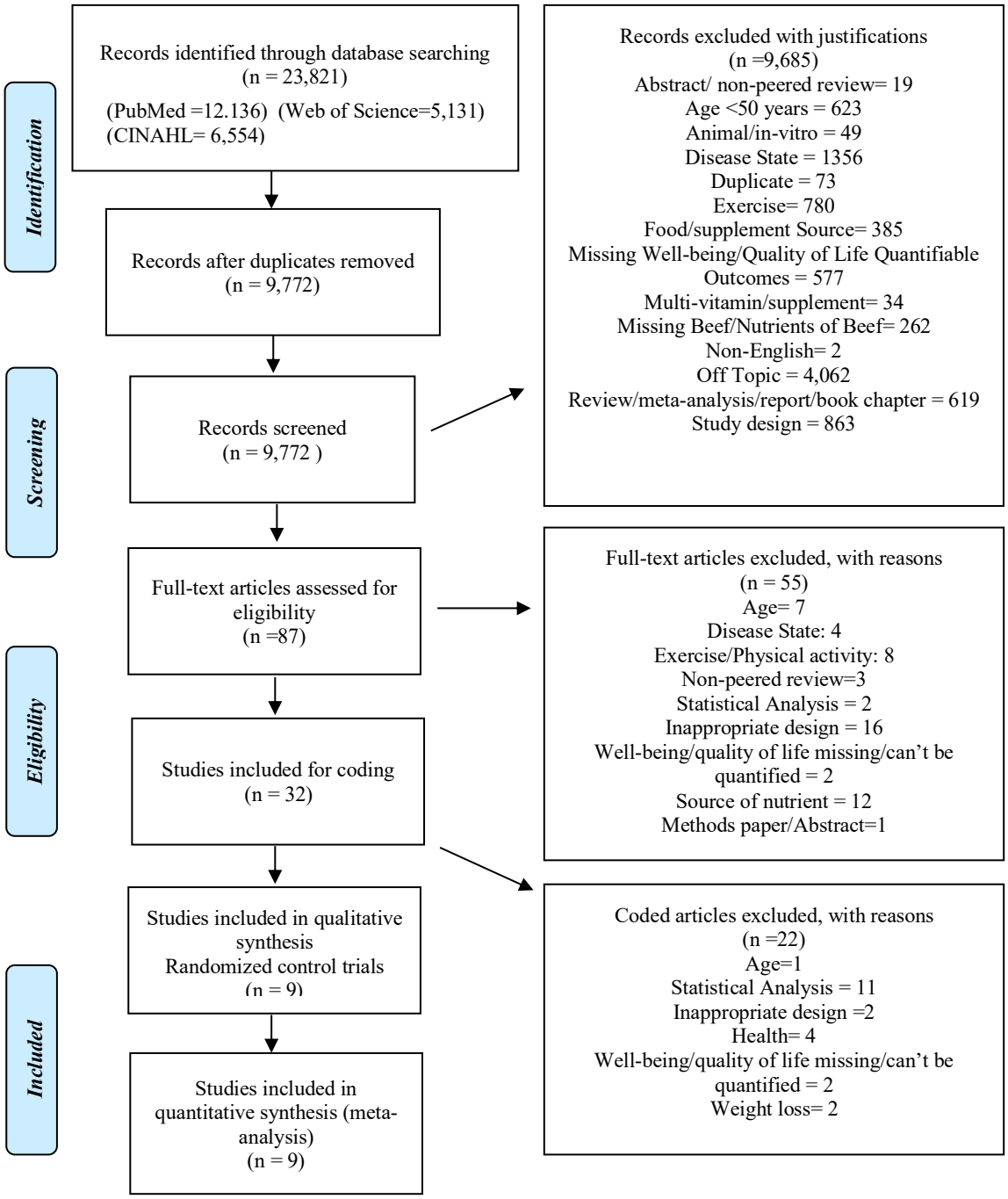

Figure 1. Literature search: Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram

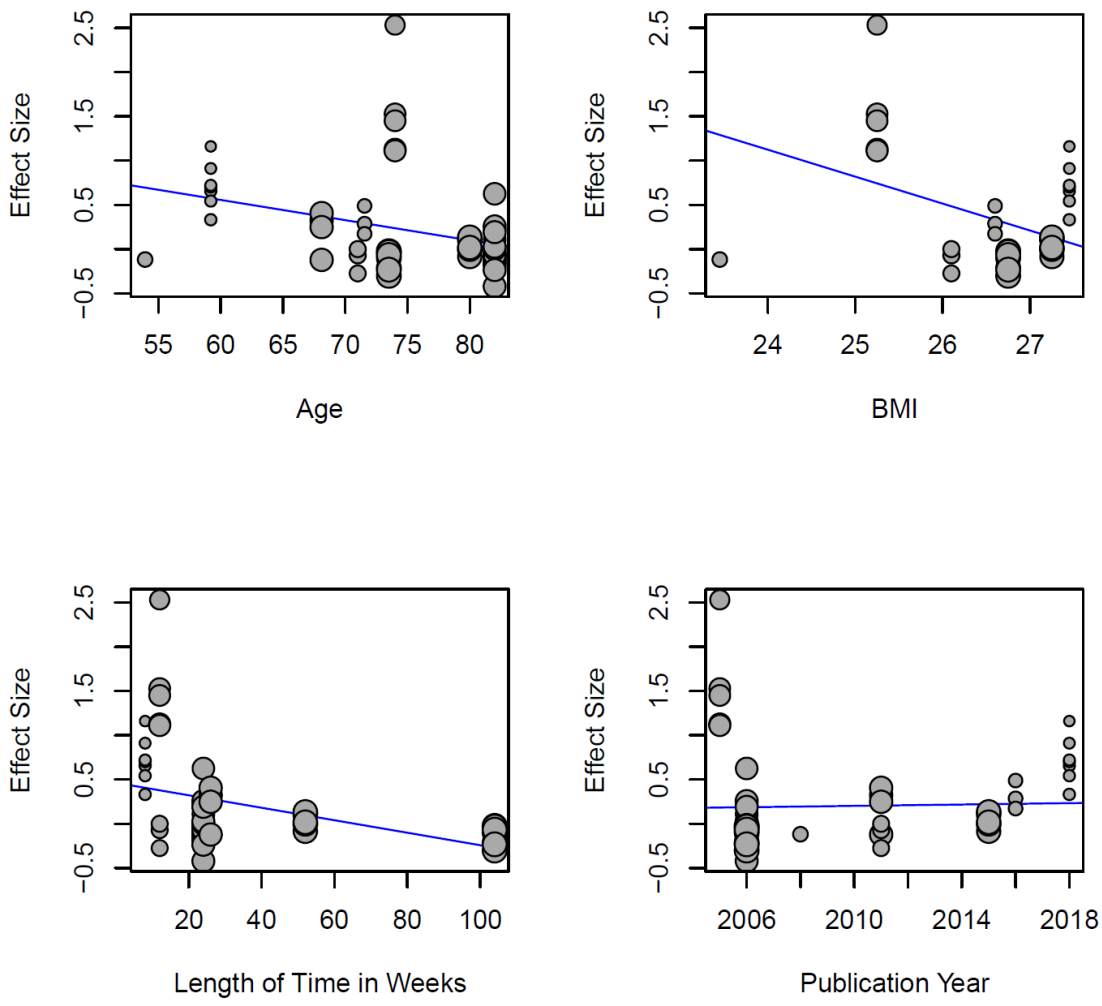

Figure 2. Scatterplots that display the relationship between the effect size and the continuous moderator
Note: the bubble size is proportional to the sample size

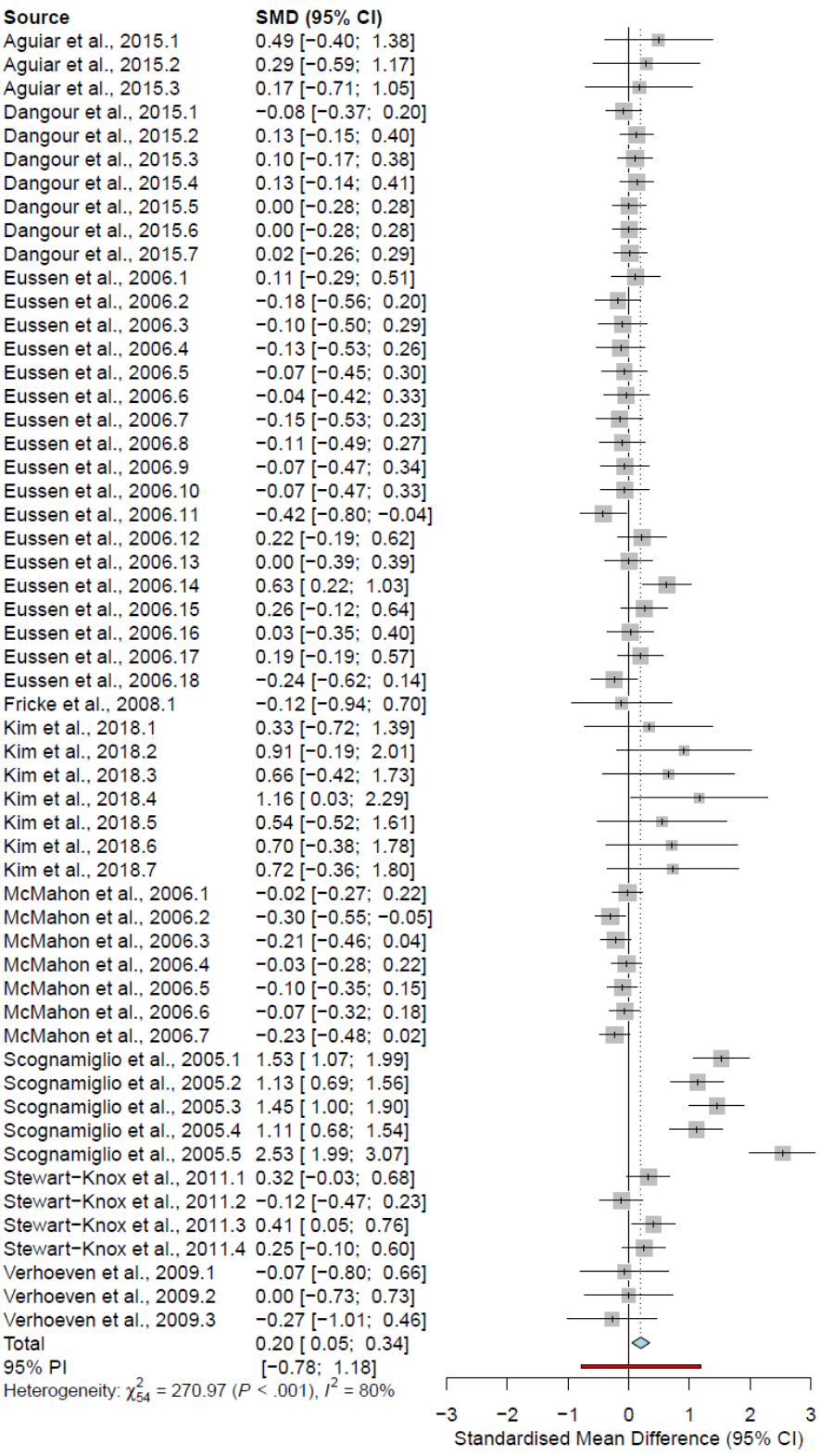

Figure 3. Forest plot for the random-effects mode

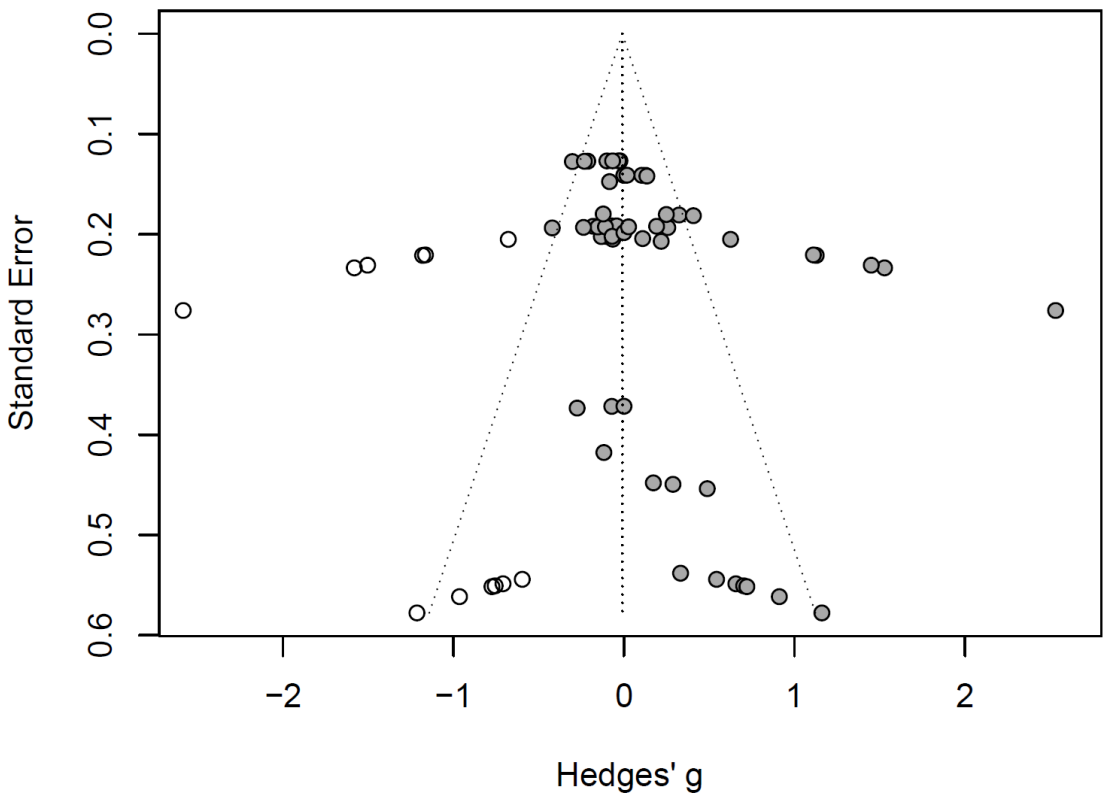

Figure 4. Funnel plot that displays publication bias

CHAPTER 4. The Short-Term Effect of Whey Versus Pea Protein on Appetite, Food Intake, and Energy Expenditure in Young and Older Men

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Abbreviations

niAUC; net incremental area under the curve

OM; older men

PFC; prospective food consumption

PPI; pea protein isolate

REE; resting energy expenditure

SO; substrate oxidation

TEF; thermic effect of feeding

VAS; visual analog scales

WPI; whey protein isolate

YM; young men

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Conflict of Interest and Funding Disclosure

Aubree L. Hawley, Edward Gbur, Angela M. Tacinelli, Sam Walker, Allie Murphy, Regan Burgess, and Jamie I. Baum have no conflicts of interest to report

Abstract

Background: Diets higher in protein have been reported to improve age-related changes in body composition via increased energy expenditure, shifts in substrate oxidation, and decreased appetite. However, how protein source (e.g. animal versus plant protein) impacts energy expenditure, appetite and food intake as we age is unknown. Objective: The objective of this study was to evaluate the effect of protein source as part of a high protein breakfast on appetite, food intake, energy expenditure, and fat oxidation in young men compared to older men. Methods: This study used a randomized, single-blinded crossover design, with a one-week washout period between testing days. Fifteen young (YM; 25.2 ± 2.8 years) and fifteen older (OM; 67.7 ± 4.5 years) healthy, adult men participated in the study. Participants arrived fasted and consumed an isocaloric, volume-matched, high-protein (40g) test beverage made with either an animal (whey protein isolate; WPI) or plant (pea protein isolate; PPI) protein isolate source. Markers of appetite and energy expenditure were determined at baseline and over four hours postprandial. Results: There was a significant effect of time, age, and protein source on appetite ($p < 0.05$). There was no effect of protein source on plasma markers of appetite, food intake, energy expenditure, and substrate oxidation. After controlling for body weight OM had decreased energy expenditure ($p < 0.05$) and lower fat oxidation ($p < 0.001$) compared to YM. Conclusions: This study indicates that a high protein breakfast containing WPI or PPI exerts comparable effects on appetite, energy expenditure, and 24-hour energy intake in both young and older healthy adult men. This trial was registered at clinical trials.gov as NCT0339981

Introduction

Life expectancy continues to increase in the United States and adults 65 years of age and older are projected to more than double from 600 million to 1.6 billion between 2015 and 2050 [1]. Successful aging is commonly defined by high levels of physiological function [2], which is strongly associated with body composition, strength, and appetite [3, 4]. Skeletal muscle mass and strength begin to decrease in the third decade of life and these losses are accelerated in the sixth decade of life [5]. In the midst of skeletal muscle loss, older adults commonly experience concurrent fat mass gain [6]. These shifts in body composition are often accompanied by changes in energy homeostasis via decreased energy expenditure [7], shifts in substrate oxidation [8], and decreases in appetite [9]. Age-related shifts in appetite contribute to energy imbalance and altered body composition often observed with age [10]. Age-related decreases in appetite are largely contributed to alterations in appetite hormones [11], changes in gastrointestinal motility [12] and losses in lean body mass [13-15] . Research suggests nutritional strategies focused on higher-protein diets containing high-quality proteins are a potential way to mitigate the decrease in energy expenditure and body composition observed with age [6].

Dietary patterns promoting plant-based protein have gained significant attention in recent years [16]. However, studies examining the effect of plant-based protein sources versus animal-based protein sources markers of appetite, energy expenditure and markers of metabolism offer conflicting results [17-20] . For example, high-protein meals containing varying protein sources have been shown to influence appetite differently [18, 21, 22], albeit previous work from our lab did not see a difference in postprandial appetite responses in participants consuming an animal protein- versus plant protein-based breakfast [17].

Although research exists comparing the effects of protein source on appetite and energy expenditure in healthy young adults, there is little data looking at the effect of animal and plant protein sources on energy expenditure, appetite, and food intake in young versus older men. Therefore, the primary objective of this study was to compare the acute effects of a high-protein breakfast containing either animal protein or plant protein on energy expenditure, appetite, and food intake young versus older men. Whey protein isolate (WPI) was used as the animal protein source due to the high level of branched chain amino acids (leucine, isoleucine, and valine) and its ability to increase satiety in response to a mixed meal [23]. Pea protein isolate (PPI) was used as the plant protein source due to its complete amino acid profile and its potential to suppress appetite compared to animal proteins [24].

Materials and Methods

Participants and Ethical Approval. From December 2017 to May 2018, young men (YM) between 18-29 years of age and older men (OM) 60-85 years of age were recruited to participate in this study. Participants were recruited from the Northwest Arkansas area via the daily University of Arkansas digital newsletter, flyers throughout the community, word-of-mouth, and social media to participate in this study. The initial screening was carried out via phone interview. Participants who consumed protein related supplements, did not regularly consume breakfast (<5 times per week), smoked, had dietary restrictions, disliked chocolate, were actively trying to lose weight, participated in vigorous activity for 4 hours a week or more, were competitive athletes, had any pre-existing metabolic conditions (e.g. type 1 or 2 diabetes, cancer, cardiovascular disease), were taking medications that would influence protein or energy

metabolism, were claustrophobic and/or were uncomfortable with needles were excluded from participating in the study.

Sixty-one men underwent an initial screening, 17 younger and 20 older men met the screening criteria, and 15 young and 15 older men completed all study procedures (May 2018). Of those who did not complete the study, participants dropped out due to claustrophobia under the metabolic canopy hood, time constraints, and personal reasons. The total participant dropout rate was 18.9%. Each individual agreed to participate by signing the study consent form, completed two test days and an additional final body composition assessment. Written consent was obtained from participants prior to starting the study. Ethical approval for the study protocol was approved by the Office of Research Compliance Institutional Review Board of the University of Arkansas (Fayetteville, AR, USA). This trial was registered at clinical trials.gov as NCT03399812.

Study Design. The study was conducted as a single-blinded randomized cross-over design study in which each participant was allocated to YM (18-29 years of age; n=15) or OM (60-85 years of age; n=15) intervention group. Refer to Table 1 for participant characteristics. On the two test days, the participants arrived fasted (10-12 hours) at the Center for Human Nutrition at the University of Arkansas prior to 08:00. for data collection. Each participant followed a randomized crossover comparison design as they received both breakfast beverages, whey protein-based isolate (WPI) and pea protein-based isolate (PPI), on subsequent test days with each participant serving as their own control. A one-week washout period separated the test days. Refer to Figure 1 for study design.

Upon arrival, anthropometrics were recorded and an intravenous catheter was inserted into an antecubital arm vein. Fasting measurements of subjective appetite via visual analogue

scale [25], resting energy expenditure (REE) and substrate oxidation via indirect calorimetry [26], and venous blood via an intravenous catheter were collected prior to the consumption of the protein-based breakfast test beverage. Participants were then served one of two test breakfast beverages. Each protein-based breakfast test beverage was served with a straw inserted into an opaque disposable cup and lid to prevent visual and olfactory influence. Participants consumed the protein-based breakfast test beverage during the next 10 minutes. The cups were evaluated by research staff to confirm the contents were fully consumed. Subsequently, the participants completed a VAS on subjective appetite and for the palatability of the protein-based breakfast test beverage. Assessment of subjective appetite using a VAS was repeated at 30, 60, 90, 120, 180, and 240 minutes after the ingestion of the protein-based breakfast test beverage. Resting energy expenditure (REE), thermic effect of food (TEF), and substrate oxidation (SO) via indirect colorimetry were measured at 30, 60, 120, 180, and 240 minutes after the ingestion of the protein-based breakfast test beverage. In addition, 10 ml of blood were collected via a syringe from an intravenous catheter at 30, 60, 90, 120, and 240 minutes after the ingestion of the protein-based breakfast test beverage. At the conclusion of the 4-hour test day, a 24-hour food log was administered, and detailed instructions were given to participants to record their food intake until 11:59 p.m.

Dietary Intervention. The protein-based breakfast test beverage contained 40 grams of dietary supplementary chocolate WPI or chocolate PPI. The WPI (BiPRO; Davisco Foods International. Le Sueur, MN) and PPI (NOW Foods Bloomingdale, IL, USA; sourced from yellow peas (Lathyrus aphaca species) were commercially purchased. The test beverages were isocaloric, volume matched, and macronutrient matched (refer to Table 2 for nutrient composition of the test beverages). The amino acid profile of the test beverages is listed in

Supplemental Table 1. Palatability of the test beverages was measured using visual analog scales. Viscosity was measured using a Brookfield Synchro-Lectric Viscometer (Brookfield Engineering Laboratories, INC, Stroughton, Massachusetts). Viscosity of the pea and whey protein drinks were measured at ambient conditions in separate 16 oz. opaque serving containers. Samples were thoroughly mixed immediately prior to measurement. The viscosity samples were measured following the immersion of the spindle and a minimum of 5 revolutions. When the motor was activated, the spindle rotated at a constant speed of 4 rpm. The palatability and viscosity of the protein-based breakfast test beverages can be found in Table 2.

Anthropometric Measurements. Height was measured to the nearest 0.01 cm using a standard stadiometer (Detecto, St. Louis, MO) without shoes, in the free-standing position. Body weight was measured to the nearest 0.05 kg using a calibrated scale (Detecto, St. Louis, MO) in the fasted state. Body composition was determined using duel energy X-ray absorptiometry (DXA) analysis (Lunar Prodigy, GE Healthcare, Madison, WI, USA) at the Exercise Science Research Center at the University of Arkansas.

Appetite Response. Subjective appetite and palatability were assessed using a traditional 100-mm VAS [25] with opposing anchors at 0, 15, 30, 60, 90, 120, 180, and 240 minutes postprandial. Participants were asked to place an "X" on the 100-mm VAS that most accurately reflected their perceived feeling of appetite according to a series of seven questions (e.g., "How HUNGRY do you feel at this moment" and "How FULL do you feel at this moment").

Dietary Records and Assessment. Participants completed a total of two 24-hour food logs, one following each test day. The energy and macronutrient composition of the test breakfast beverages and the remaining 24-hours of the test day were analyzed using the Genesis R&D nutrient analysis software package (version 9.10.2, ESHA Research, Salem, OR, USA).

Energy Expenditure and Substrate Oxidation. Resting energy expenditure (REE; kcal/min), thermic effect of feeding (TEF; kcal/min), and substrate oxidation (SO; kcal/min) were measured by indirect calorimetry using the validated [26] ventilated hood technique with the TrueOne 2400 metabolic cart (Parvo Medics, Sandy, Utah, USA; [27]).

Plasma Biomarkers. Six blood samples (10mL/sample, 60mL/testing day) were collected following a 10-12 hour fast and during the four-hour postprandial meal time response period. The samples were collected in EDTA vacutainer tubes. Samples were immediately centrifuged at 4ºC for 15 minutes at 1800 x g. The plasma was separated and stored at -80 ºC until analysis. Plasma glucose (mg/dl), cholecystokinin (CCK) (pg/ml) and peptide YY (PYY) (pg/ml) levels were determined via colorimetric (Cayman Chemical Company, Ann Arbor, MI, USA), and Enzyme Immunoassay (RayBiotech, Inc) using commercially available kits per manufacture instructions.

Statistical Analysis

Summary statistics were calculated for all data and data are expressed as means ± standard deviation (SD). Two-sample independent t-tests were used to analyze baseline measurements of participant characteristics and body composition. The two factor repeated measures design was analyzed as a generalized linear mixed model with protein source and age as fixed effects and subjects as a random effect nested within age categories. Appetite ratings, REE, substrate oxidation, food intake, and metabolic biomarker levels (glucose, PYY and CCK), that could only take on positive values were assumed to follow a gamma distribution. Thermic effect of food was analyzed as a proportion and was assumed to follow a beta distribution. For appetite ratings, energy expenditure, substrate oxidation, and plasma markers of glucose, PYY,

and CCK there was a third main effect of time. In our model we analyzed main effects of time, age, and protein source. Where appropriate, two-way and three-way interaction of age x protein source, age x time, and protein source x time and age x protein source x time respectively were tested for significance. Where appropriate, follow-up least squares mean comparisons for protein source, age and time main effects were declared significantly different if the corresponding analysis of variance F statistic was significant. For any significant interactions mean comparison were carried using the protected least significant difference (LSD). Subjective rating of palatability was analyzed as a generalized linear mixed model with protein source and age as fixed effects and subjects as a random effect nested within age categories without repeated measures. Viscosity of the test beverages were analyzed using independent t-tests. Net incremental area under the curve (niAUC) was calculated for appetite ratings, REE, TEF, SO, and metabolic biomarker levels. Where significance was found, follow-up least squares mean comparisons for protein source and age categories. For any significant interactions mean comparison were carried using the protected leas significant difference (LSD). Statistical analyses involving generalized linear mixed models were performed using PROC GLIMMIX in SAS version 9.4. All graphs were made using GraphPad Prism Software version 7.0 (GraphPad Software, La Jolla, CA, USA). $p < 0.05$ was considered significant. To verify the appropriateness of the sample sizes we carried out a post-hoc power analysis using the SAS procedure PROC POWER with the paired t-test option. The observed sample means and standard deviations were used to determine that 15 participants per group had a statistical power of 0.987 (based on an overall level of significance of 0.05) to detect an accurate postprandial difference in TEF after supplementation of WPI and PPI protein-based breakfast test beverages.

Results

Participant Characteristics. The demographics and physical characteristics of the participants who completed the study are presented in Table 1. The YM and OM had a mean age of 25.2 ± 2.8 years and 67.7 ± 4.5 years, respectively ($p < 0.0001$). There were significant differences in fat mass (FM; $p < 0.01$), body fat percentage ($p < 0.05$), and fat-to-lean ratio ($p < 0.05$) between groups with no significant differences in lean body mass (LBM) and fat free mass (FFM).

Energy Expenditure and Substrate Oxidation. Results for energy expenditure and substrate oxidation are presented in the line (individual time points) and bar graphs (niAUC) in Figure 2. After controlling for body weight (kg), there was a significant effect of age ($p < 0.0001$) and time ($p < 0.0001$) on REE (kcal/min), TEF (kcal/min), and fat oxidation (kcal/min) with no effect of protein source. There was an effect of age on REE, TEF, and fat oxidation with YM having significantly higher REE ($p < 0.0001$), TEF ($p < 0.05$), and fat oxidation ($p < 0.01$) compared to OM. There was a significant age x time interaction on TEF (kcal/min) ($p < 0.01$). All other two- and three-way interactions of REE, TEF, and substrate oxidation were not significant.

Subjective Appetite and Palatability. Results for perceived hunger, perceived fullness, prospective food consumption (PFC), and perceived desire to eat are presented in Figure 3. Fasting values of perceived hunger, fullness, prospective food consumption and desire to eat were not significantly different between the YM and OM when consuming either protein-based breakfast test beverages. There was a significant effect of time, age, and protein source on subjective hunger ($p < 0.01$), fullness ($p < 0.01$), PFC ($p < 0.01$), desire to eat ($p < 0.01$) and desire for a snack ($p < 0.05$). There was a significant interaction effect of age x time ($p < 0.01$)

and protein source x time ($p < 0.05$) on desire for a snack. All other interactions of age x protein source, age x time, protein source x time, and age x protein source x time were not significant. There were no significant differences in the desire for something sweet on time, age, or protein source (Supplemental Figure 1).

However, there was a significant effect of age on the desire for something salty ($p < 0.001$; Supplemental Figure 1) and an age x time interaction ($p < 0.01$) with no significant interaction effect of age x time x protein source. Palatability was higher for the WPI compared to the PPI protein-based breakfast test beverage ($p < 0.01$) with no significant difference between age groups (Table 2).

Plasma Biomarkers. The plasma glucose, CCK, and PYY responses to the test breakfast beverage are depicted in Figure 4. There was an effect of age ($p < 0.05$), but not protein source, with older men having higher concentrations of all tested biomarkers. There was a significant time x age interaction on glucose ($p < 0.05$) with no significant effect of age x time x protein source. All other interactions of age x time, protein source x time, and age x protein source x time interactions of plasma glucose, CCK, and PYY were not significant.

24-hour Dietary Assessment. Twenty-four-hour energy and macronutrient intake are shown in Table 3. No significant differences were observed in 24-hour total food intake between either protein source or age groups.

Discussion

To our knowledge, this is the first study to examine the short-term effect of a high-protein breakfast from plant or animal derived protein sources on energy expenditure and appetite response in healthy, young and older men. The present study tested the hypothesis that WPI,

when compared to PPI, would have a greater effect on energy expenditure and appetite in OM versus YM when supplemented as a 40-gram protein-based breakfast beverage. Collectively, the results of this study suggest that age, not protein source, effects postprandial energy expenditure and appetite responses.

A breakfast containing high-protein foods has been shown to increase energy expenditure and fat oxidation in healthy, young adults [18, 27]. However, the impact of protein source as part of a high-protein breakfast on energy expenditure and fat oxidation in aging adults still needs to be established. For example, consumption of whey, casein, and soy protein-based beverages compared to a carbohydrate-based control beverage increased TEF and fat oxidation in young men over a five-hour period [18]. One likely mechanism for the increase in TEF could be due to protein turnover and the favoring of protein synthesis or deamination and urea synthesis associated with protein breakdown [28]. However, in this clinical trial, we did not observe any differences between protein source with respect to energy expenditure and substrate oxidation. This may have been due to the 40 grams of protein used in the test breakfast beverages which was a larger dose compared to the doses used in other studies demonstrating differences in energy metabolism between protein sources [18, 29].

The majority of clinical trials investigating the short-term effect of animal- and plant-based proteins on appetite and food intake use soy as the plant-based protein source [20, 30], whey as the animal-based protein source [18, 31, 32], or a complete mixed meal [30, 33-35]. In agreement with our study, fifteen grams of protein sourced from either whey-, pea-, or a combination of whey and pea protein isolate on appetite, postprandial changes in satiety hormones, and energy intake found that the pea protein resulted in a modest increase in satiety, with no differences in energy intake [21]. In addition, a randomized single-blind cross-over study

investigating the role of a meal preload of twenty grams of casein, whey, pea protein, egg albumin, or maltodextrin compared to water found that casein and pea protein increased satiety significantly more when compared to the other sources of protein [24]. In contrast, casein and pea protein also lowered energy intake, albeit food intake was recorded 30 minutes following the meal preload.

There are a limited number of studies investigating the differences in energy expenditure and substrate oxidation between protein sources. In one study, three isoenergetic 30% protein test meals using meat, dairy, and soy protein sources found no significant differences in energy expenditure, carbohydrate oxidation, or fat oxidation between test meals [30], similar to the results found in this study. In contrast, a second study tested three meals with 50% protein coming from either whey, casein, or soy protein and found that TEF and fat oxidation were greater after the consumption of the whey protein meal [18].

To our knowledge, this is the first short-term meal response study to demonstrate the effect of whey protein isolate and pea protein isolate on energy expenditure and appetite in young versus older men at breakfast. However, there are several limitations to this study. This study had strict inclusion and exclusion criteria and we only recruited healthy young and older men which could be the reason that there was no difference in lean or fat-free mass between the younger and older men. Women were excluded from this study, which means the results may not apply to the overall population. The sample size, although powered correctly, was small. The breakfast test breakfast beverages varied in viscosity which may have contributed to differences seen in participant appetite response [36]. The test beverages also varied in palatability despite controlling for nutrient content and sensory properties of smell and sight, which may have influenced appetite [37]. We also relied on self-reported 24-hour food intake for intake for the

24-hour dietary assessment, which may provide inaccurate measurements of food intake [38]. In addition, we did not provide the pea protein and whey protein in mixed-meal context. Therefore, the results cannot be directly translated into a plant-based or animal-based protein complete diet. Finally, there was a racial imbalance in the young compared to the older participants. The 15 older men were Caucasian as the younger men were Caucasian, Indian, and American Asian/Asian. However, as this was a crossover design the racial imbalance was unlikely to impact our primary outcomes.

In conclusion, an isocaloric, isovolumetric, macronutrient- and fiber-matched protein-based breakfast beverages from an animal-based whey protein isolate and a plant-based pea protein isolate exerts comparable effects on appetite, energy expenditure, and 24-hour energy intake in both young and older healthy adult men.

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Tables

Table 1. Baseline characteristics of the study population by age group [1]

	Young (n=15)	Older (n=15)	p-value
Age, y	25.2 ± 2.8	67.65 ± 4.5	<0.0001****
Anthropometrics			
Height, m	1.8 ± 0.1	1.81 ± 0.1	0.59
Weight, kg	78.4 ± 11.3	88.9 ± 10.4	0.01*
BMI, kg/m^2	25.1 ± 3.3	27.9 ± 3.0	0.02*
DXA			
Total body fat mass, kg	17.5 ± 6.4	26.3 ± 9.8	0.01**
Percent body Fat, %	23.5 ± 7.8	30.5 ± 9.7	0.04*
Total lean mass, kg	57.6 ± 11.1	58.3 ± 7.0	0.84
Total fat-free mass, kg	60.9 ± 11.6	58.0 ± 16.6	0.59
Fat-to-Lean ratio, (total fat mass/ total lean mass)	0.32 ± 0.1	0.46 ± 0.2	0.03*
Ethnicity [2]			
American Asian/Asian	4/15	-	
Indian	1/15	-	
Caucasian	10/15	15/15	

[1] Data are expressed as means ± SDs. Significant differences denoted by ****p<0.0001; **p<0.01; * p<0.05.
[2] Ethnicity is expressed as number of participants within age group.

Table 2. Ingredient composition and nutrient profile of breakfast test beverages
Macronutrient Profile, Palatibility, and Viscosity of Breakfast Test Beverages[1]

	WPI	PPI
Ingredient composition		
Protein isolate, g	50.00	73.33
Cane sugar, g	13.00	-
Canola oil, g	0.75	-
Inulin, g	3.60	-
Water, mL	350.00	350.00
Nutrient profile		
Calories, kcal	265.8	263.8
Protein, g	40.0	40.0
Carbohydrate, g	15.0	15.0
Fiber, g	3.6	3.3
Fat, g	4.4	4.2
Palatability, mm[2]	56.2 ± 16.6	37.9 ± 17.9*
Viscosity, cP	62.5	10,500.0*

[1]Whey protein isolate, WPI; Pea protein isolate, PPI; Centipoise, cP.
[2] Palatability is expressed as means ± SDs. Palatability measurements were collected from participants at time point 15 minutes. Significant differences denoted by * $p<0.05$.

Table 3. 24-hour energy and macronutrient intake post-consumption of test breakfast beverages [1]

	Young		Older	
	WPI (n=15)	PPI (n=15)	WPI (n=15)	PPI (n=15)
Calories, kcal	2248.6 ± 703.0	2328.6 ± 903.7	2078.0 ± 542.3	2120.7 ± 850.1
Protein, g	129.4 ± 44.9	141.4 ± 51.4	117.9 ± 26.3	115.7 ± 29.5
Fat, g	88.7 ± 40.1	87.1 ± 53.8	80.1 ± 31.2	80.0 ± 43.7
Carbohydrate, g	236.5 ± 73.1	244.9 ± 82.7	217.7 ± 87.1	2058.0 ± 92.0
Sugar, g	73.5 ± 26.7	64.0 ± 26.7	94.5 ± 52.2	64.5 ± 40.4
Fiber, g	21.4 ± 7.6	22.3 ± 9.2	19.5 ± 5.3	21.1 ± 11.1
Sodium, mg	3934.3 ± 1937.8	3895.0 ± 1482.4	2577.3 ± 1329.8	3611.3 ± 1976.9
Protein, %	23.2 ± 1	24.97 ± 1	23.40 ± 1	24.1 ± 1
Carbohydrate, %	43.3 ± 1	43.9 ± 1	41.3 ± 1	40.0 ± 1
Fat, %	34.1 ± 1	32.3 ± 1	34.5± 1	32.9± 0

[1] Data are expressed as means ± SDs. Whey protein isolate, WPI; Pea protein isolate, PPI.

Figures

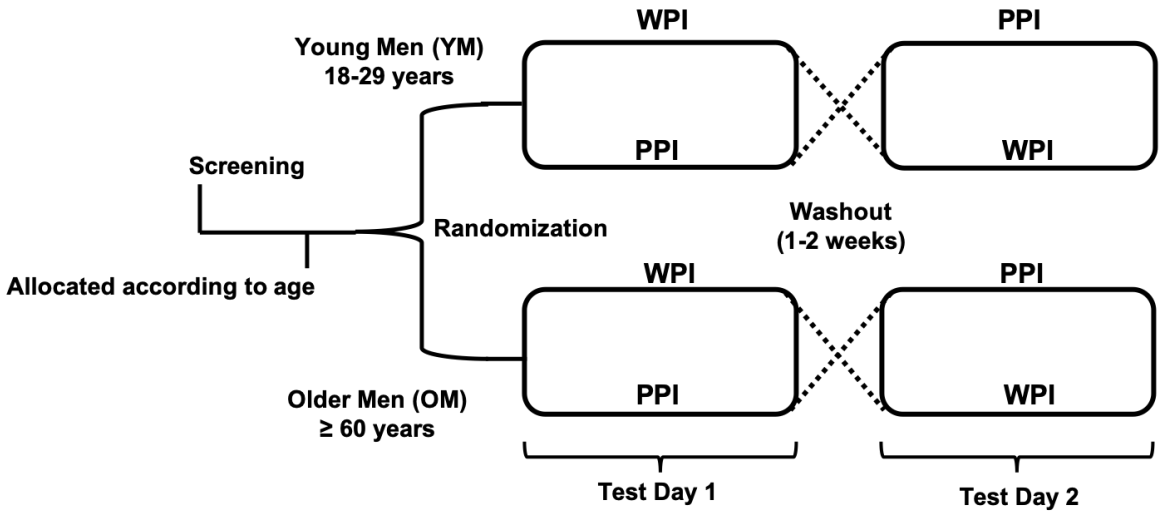


Figure 1. Schematic of randomized, controlled, single-blinded study design

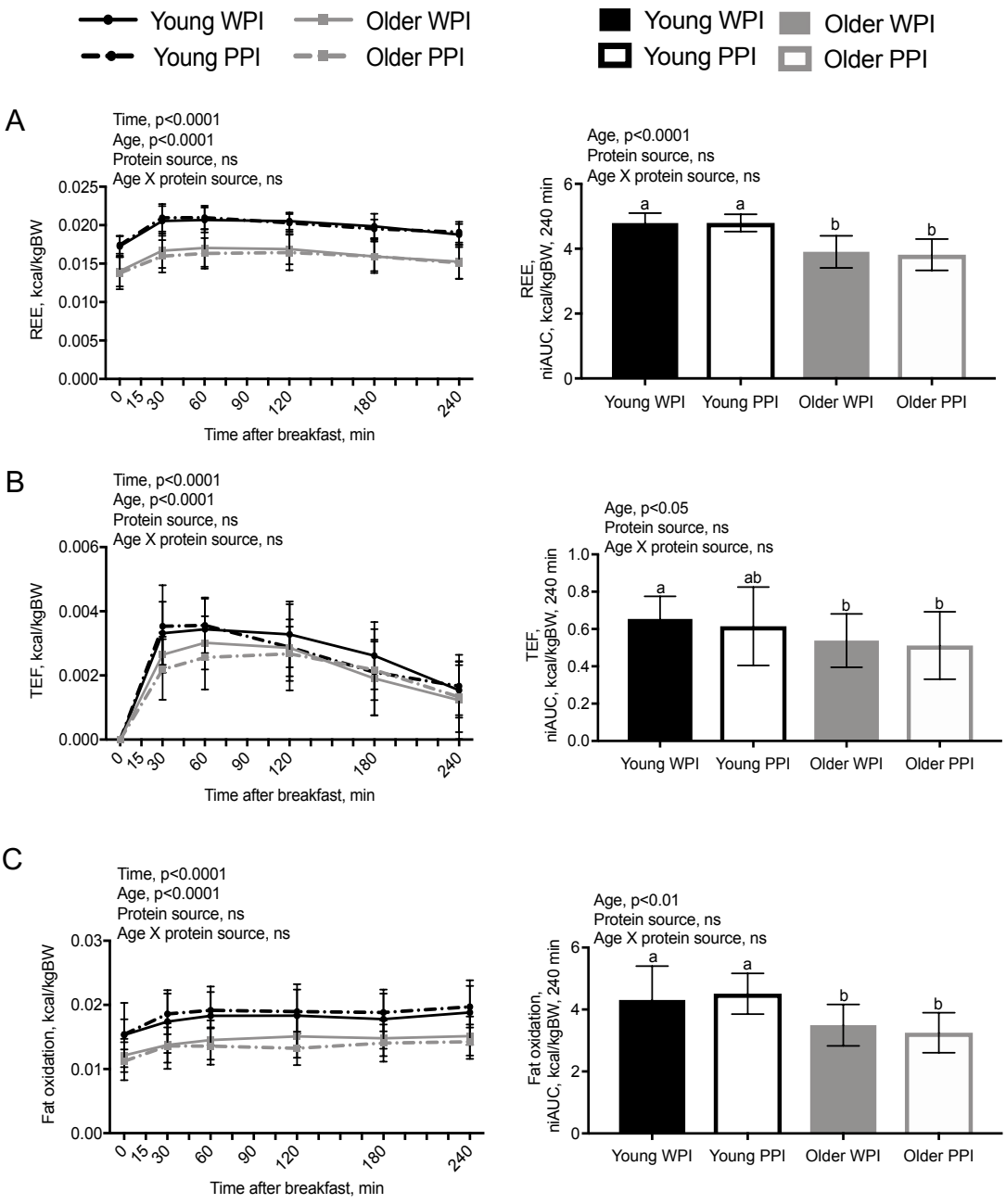


Figure 2. Energy expenditure and substrate oxidation following ingestion of either a whey protein isolate (WPI)-based or pea protein isolate (PPI)-based breakfast test beverage in young (YM, n=15) or older (OM, n=15) men using indirect calorimetry. Data are expressed as means ± SD. Data is controlled for body weight in kilograms (kg). (A) Resting energy expenditure (REE) over time and net incremental area under the curve (niAUC). (B) Postprandial energy expenditure (TEF) over time and niAUC. (C) Fat oxidation over time and niAUC. Data is expressed as means ± SD. Means not sharing the same letter are significantly different ($p < 0.05$).

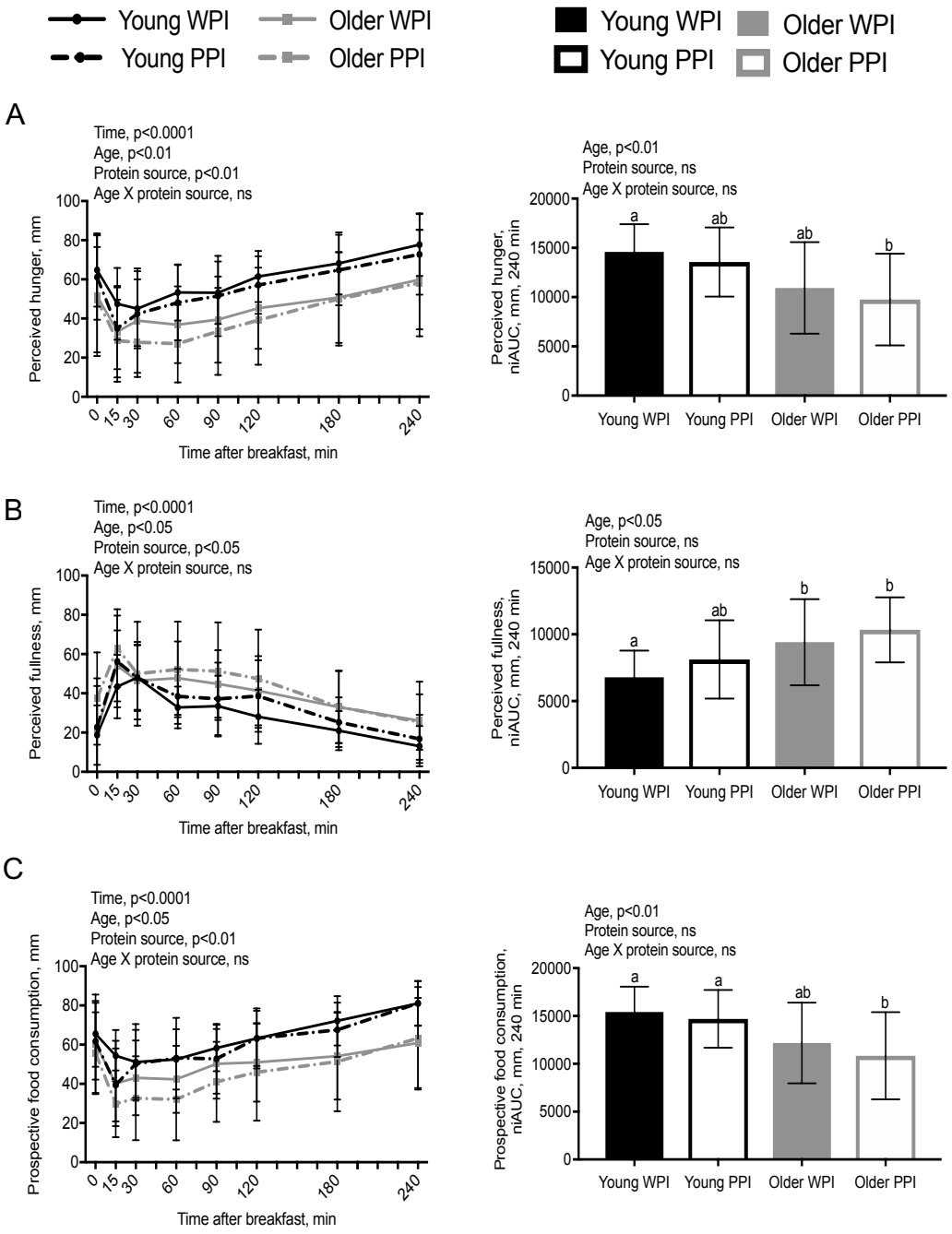


Figure 3. Ratings of perceived appetite assessment following ingestion of either whey protein isolate (WPI)-based or pea protein isolate (PPI)-based breakfast test beverage in young (YM, n=15) or older (OM, n=15) men using visual analog scales. (A) Perceived hunger over time and net incremental area under the curve (niAUC). (B) Perceived fullness over time and niAUC. (C) Perceived prospective food consumption over time and niAUC. (D) Perceived desire to eat over time and niAUC per age and protein source. Data are expressed as means ± SD. Means not sharing the same letter are significantly different ($p < 0.05$).

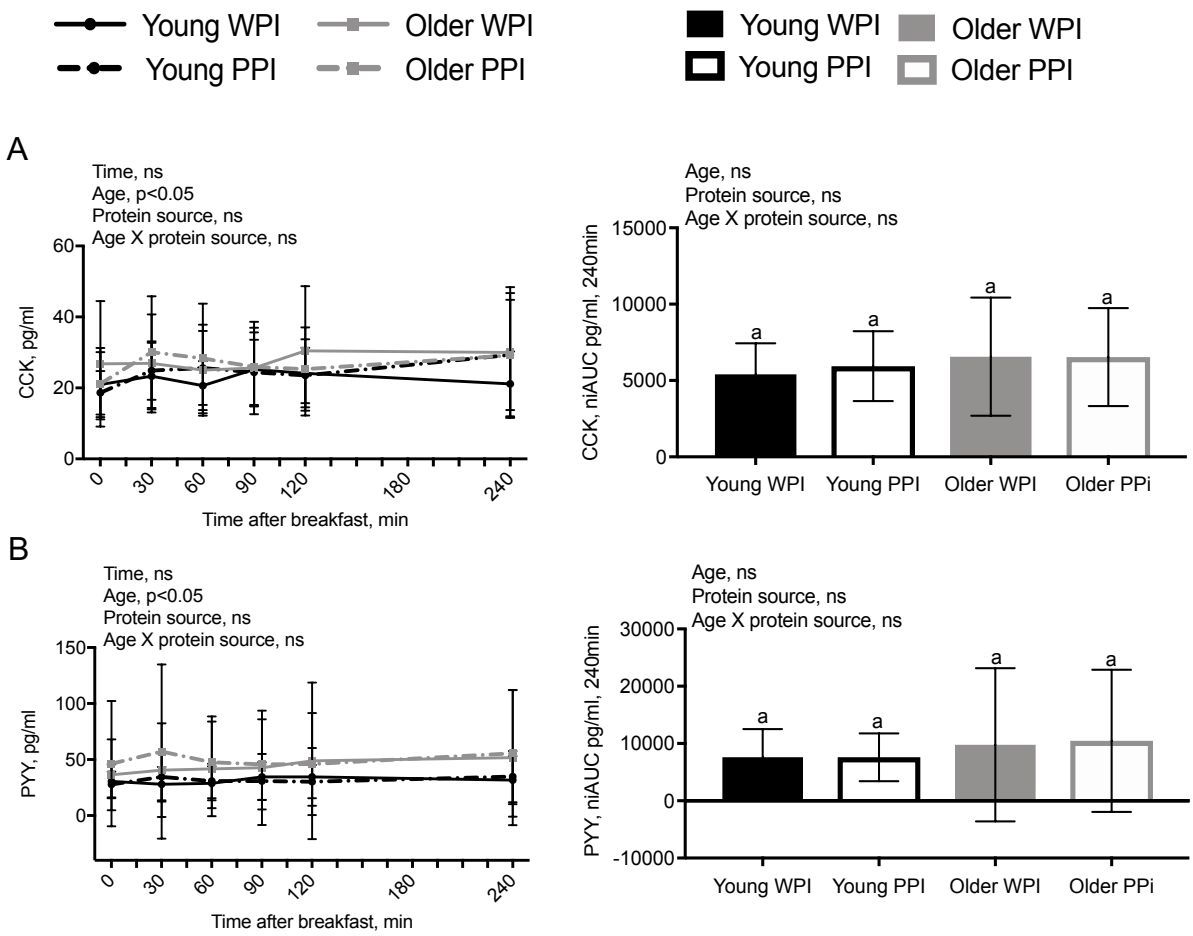
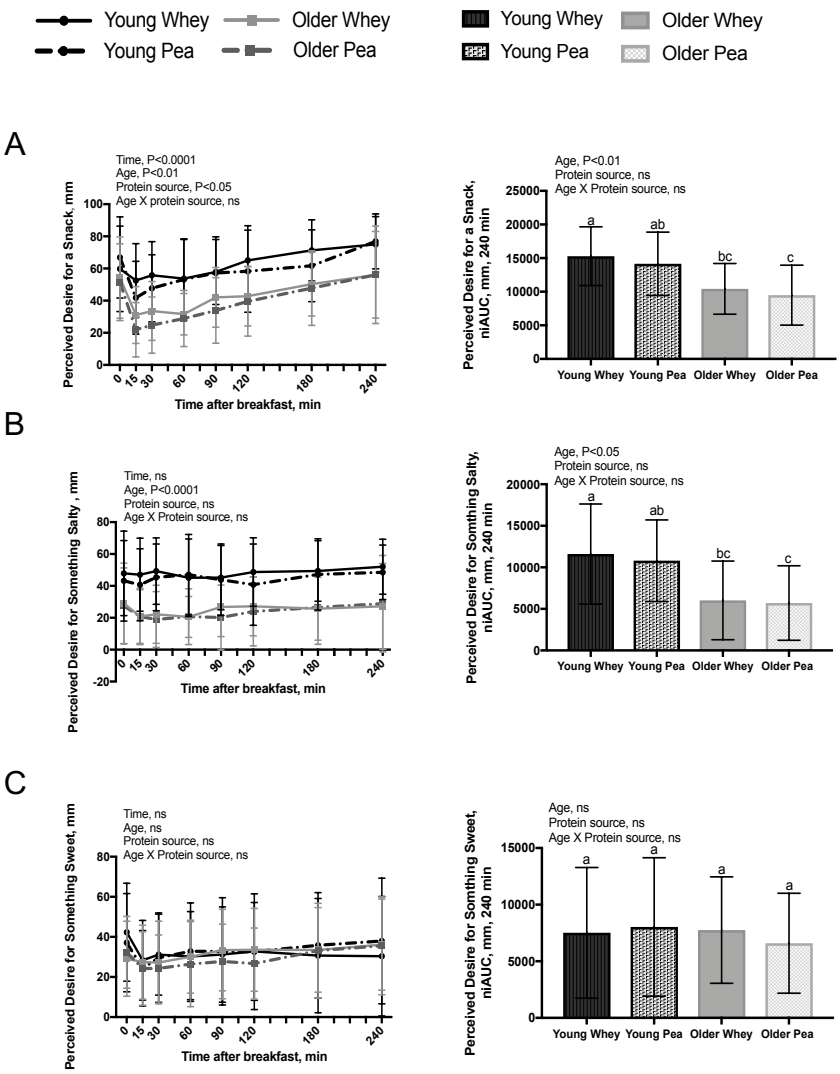
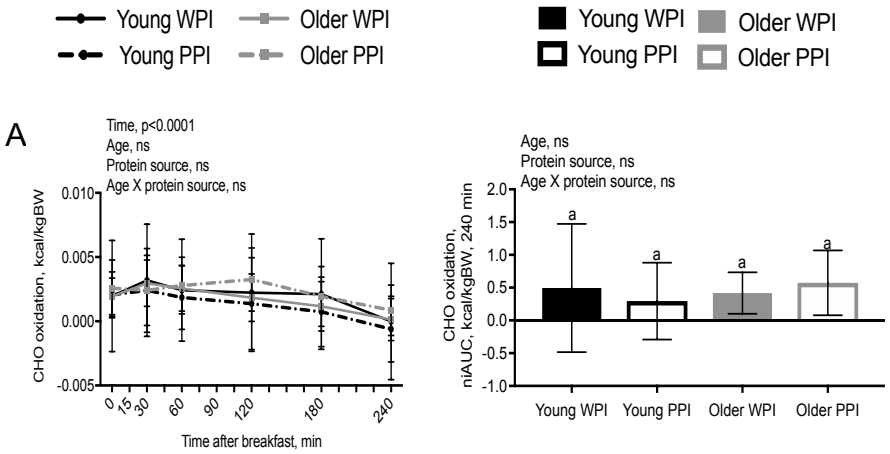


Figure 4. Postprandial peptide YY (PYY), and cholecystokinin (CCK) response following ingestion of either whey protein isolate (WPI)-based or pea protein isolate (PPI)-based breakfast test beverage in young (YM, n=15) or older (OM, n=15) men. (A) CCK response over time and net incremental area under the curve (niAUC). (B) PYYbresponse over time and niAUC. Data is expressed as means ± SD. Means not sharing the same letter are significantly different ($p < 0.05$).

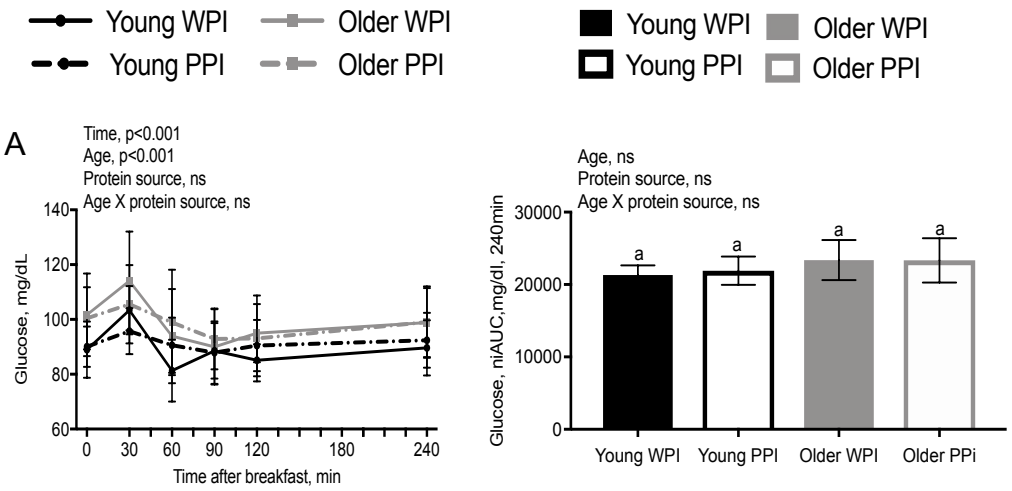
Appendix



Supplemental Figure 1: Ratings of perceived desire for a snack and food cravings Ratings of perceived appetite assessment following ingestion of either whey protein isolate (WPI)-based or pea protein isolate (PPI)-based breakfast test beverage in young (YM, n=15) or older (OM, n=15) men using visual analog scales. Line graphs represent perceived appetite over time and bar graphs represent net incremental area under the curve (niAUC) per age and protein source group (A) Perceived desire to eat; (B) Perceived desire for a snack; (C) Perceived desire for something salty; (D) Perceived desire for something sweet. Data are expressed as means ± SDs. Means not sharing the same letter are significantly different ($p < 0.05$).



Supplemental Figure 2. Carbohydrate oxidation following ingestion of either whey protein isolate (WPI)-based or pea protein isolate (PPI)-based breakfast test beverage in young (YM, n=15) or older (OM, n=15) men using indirect calorimetry. Data is controlled for body weight in kilograms (kg). The line graph represents Carbohydrate (CHO) oxidation over time and the bar graph represents net incremental area under the curve (niAUC). Data is expressed as means ± SDs. Means not sharing the same letter are significantly different ($p < 0.05$)



Supplemental Figure 3. Postprandial glucose response following ingestion of either whey protein isolate (WPI)-based or pea protein isolate (PPI)-based breakfast test beverage in young (YM, n=15) or older (OM, n=15) men. The line graph represents the plasma glucose postprandial response over time and the bar graph represents net incremental area under the curve (niAUC). Data is expressed as means ± SDs. Means not sharing the same letter are significantly different ($p < 0.05$).

Supplemental Table 1. Amino acid composition per serving of breakfast test beverages

	WPI	PPI
Alanine	2.00	1.62
Arginine	1.00	3.38
Aspartic Acid	4.60	4.70
Cysteine	1.20	0.60
Glutamic acid	6.40	7.14
Glycine	0.60	1.64
Histidine	0.80	0.98
Isoleucine	2.20	1.82
Leucine	5.00	3.35
Lysine	4.00	3.00
Methionine	1.00	0.35
Phenylalanine	1.40	2.20
Proline	1.80	1.74
Serine	1.40	2.08
Threonine	1.80	1.56
Tryptophan	1.20	0.35
Tyrosine	1.40	1.49
Valine	2.20	1.86

The amino acid composition of 40 grams of protein in the WPI and PPI breakfast test beverages. WPI, whey protein isolate; PPI, pea protein isolate.

CHAPTER 5. The Effect of Whey Protein Isolate and Omega-3 Fatty Acid Supplementation on Markers of Cardiometabolic Health, Sleep, and Mood in Post-Menopausal Women: A 16-Week Randomized, Controlled Trial

Abstract

Background: Post-menopausal women are at an increased risk for negative health outcomes including cardiometabolic disease, sleep disturbances, and depression. Individual supplementation of protein and omega-3 polyunsaturated fatty acids (n-3 PUFAs) has been shown to mitigate age-related physiological decline with little evidence on well-being. In addition, the combined effect of protein and n-3 PUFAs on successful aging (SA) is unknown. Objective: The objective of this study was to determine the effect of protein and n-3 PUFA supplementation individually and in combination on body composition, cardiometabolic risk, strength, sleep and mood states in postmenopausal women to promote SA. We hypothesized that concomitant protein and n-3 PUFA supplementation would improve body composition, decrease cardiometabolic risk, and increase strength, indexes of sleep, and mood states compared to individual supplementation and would be accompanied by increases in orexin-A (OXA) concentrations. Methods: Thirty-nine postmenopausal women (age: 61.3 ± 8.7 years; BMI: 27.6 ± 6.6 kg/m^2) were randomly allocated to one of 5 groups: 1) control (CON; no intervention free-living; n=6), 2) whey protein isolate (PRO; 25 g/d; n=7), 3) n-3 PUFA (DHA/EPA; 4.3 g/d; n=10), 4) PRO + placebo soybean oil (PRO + PLA; 4.1 g/d; n=7), or 5) PRO + n-3 PUFAs (n=9). Outcome measures of body composition, energy metabolism, metabolic health, sleep, and mood states were assessed every four weeks and compared across all five groups at 0, 4, 8, 12, and 16 weeks, except objective sleep, which was assessed at 0, 8, and 16 weeks, and body

composition and hand-grip strength (HGS) which were assessed at 0 and 16 weeks only. Results: We did not observe a significant treatment effect on anthropometrics, body composition, HGS, resting energy expenditure, mood states, nor subjective or objective sleep quality. We observed a significant treatment effect on OXA ($P < 0.05$). OXA increased significantly in PRO + n-3 PUFA compared to all other groups ($P < 0.05$). Conclusions: Although not significant, the data suggests individual and combined supplementation of protein and n-3 PUFAs have the potential to improve outcomes of SA including cardiometabolic health, mood states, subjective sleep, and OXA levels in postmenopausal women. NCT0303041

Introduction

The older adult population in the United States (U.S.) is a segment of unprecedented growth [1]. This robust shift in demographics emphasizes the importance of independence, quality of life, and health across the lifespan to promote successful aging (SA) [2]. SA can be defined by low cardiometabolic risk, preservation of physical function, and a positive state of well-being, which are strongly associated with body composition [3-9]. Age-related deleterious shifts in body composition, one of the major threats to SA, can lead to sarcopenia, which is the age-related loss of muscle mass, strength, and function [10]. Furthermore, declines in endogenous estrogen production during the menopausal transition are associated with muscle mass loss and increased central adiposity, putting postmenopausal women at increased risk for negative health outcomes such as cardiovascular disease and type-2 diabetes mellitus [11-13]. In addition to cardiometabolic risk, age- and menopause-related reduction in muscle mass and function is associated with decreased well-being such as depression [14] and poor sleep quality [15]. Research suggests nutritional strategies focused on the incorporation of high-quality protein

and omega-3 polyunsaturated fatty acids (n-3 PUFAs) are potential methods to mitigate age-related decline in skeletal muscle mass and gain in fat mass, decreases in metabolic health, sleep, and mood in postmenopausal women to promote SA [16, 17].

Protein is a dietary focal point for SA as the constituent amino acids (AA) are the essential building blocks necessary to sustain life [17]. The benefits of dietary protein intake for older adults above the current recommended dietary allowance (RDA) of 0.8g/kg/day is well established [17, 18], and experts generally recommend a dietary protein intake between 1.2 and 2.0 g/kg/day or higher and ~30 g of high-quality protein per meal to promote skeletal muscle mass and function in older adults [18-25]. A recent cross-sectional analysis found postmenopausal women who consumed ≥ 1.3 g/kg/day had a significantly higher skeletal muscle mass index (appendicular lean mass / BMI) and significantly lower body fat percentage and waist circumference when compared to women who consumed 0.94-1.29 g/kg/day [26].

n-3 PUFAs, eicosapentaenoic acid (EPA; 20:5 n-3), and docosahexaenoic acid (DHA; 22:6 n-3) are also associated with SA [27]. High doses of EPA and DHA (3-4 g/day) [28, 29] may mitigate deleterious characteristics of aging via suppression of chronic inflammation, incorporation into cellular membranes, and via stimulation of muscle growth through the same mechanistic pathway, mechanistic target of rapamycin complex 1 (mTORC1), as dietary protein [30]. Smith et al, demonstrated n-3 PUFAs (in the presence of AA infusion) increased whole-body protein synthesis [30] and that supplementation of 4g/d of n-3 PUFAs for six months increased muscle mass and function in healthy older adult men and women [31]. Similarly, postmenopausal women who consume a diet high in fish rich in EPA and DHA, such as the Mediterranean diet, tend to have higher lean body mass than their counterparts [32] . However, NHANES data demonstrates daily EPA and DHA intake from foods and supplements is well

below recommendations with only ~10% of U.S. adults ≥ 55 years meeting or exceeding the American Heart Association's recommendations of 500 mg/day of EPA and DHA [33].

Approximately 30% of adults ≥ 50 years of age suffer from poor sleep quality and the prevalence of sleep disruption is notably higher in postmenopausal women, with 35 to 60% reporting significant sleep disruptions [34]. Sleep deprivation and low sleep quality are associated with increased energy intake [35], insulin resistance, elevated glucose [36, 37], mood disturbances (e.g., stress, cortisol, and depression) [38, 39], and poor body composition [40, 41]. Cross-sectional studies have found both dietary protein and n-3 PUFAs to independently improve sleep and mood [42-44]. Yet, apart from weight-loss and exercise interventions, few RCTs have investigated the effect of protein or n-3 PUFAs on sleep and mood in adults. Therefore, further research is needed to investigate dietary protein and n-3 PUFAs as moderators of indexes of sleep and mood as well as to further investigate possible mechanisms.

Orexin-A (OXA) and orexin-B (OXB), also known as hypocretin-1 and hypocretin-2, are excitatory neuropeptides solely synthesized in the hypothalamus [45, 46] and project throughout the brain and spinal cord where G-coupled protein receptors, orexin receptor 1 (OXR1) and orexin receptor 2 (OXR2) are located [46, 47]. OXA is a "multi-tasking" neuron and regulates a broad range of physiological functions such as sleep/wake states (rapid eye movement), energy homeostasis (increase in O_2 consumption), excitatory motivational behavior, cognitive function, and affect states [48-50] and has been proposed as a possible mechanism of SA [51]. A lack or deficiency of OXA is associated with daytime sleepiness and nighttime wakefulness (REM disruption), decreased energy expenditure, increased adiposity, decreased mood/motivation [52], decreased motor neuron signaling, and inflammation [46, 47, 53] . Current literature suggests

OXA is a unique endogenous factor that influences SA [51], albeit nutrition-based human research is limited.

The objective of the current randomized, controlled dietary intervention was to assess the individual and combined effect of protein and n-3 PUFAs on body composition, cardiometabolic health, indexes of sleep, and mood states in postmenopausal women to promote SA. This study was also designed to assess the effect of protein and n-3 PUFAs on OXA as a proposed biomarker of SA. We hypothesized that concomitant protein and n-3 PUFA supplementation would improve body composition, metabolic health, indexes of sleep, and mood states compared to individual supplementation and would be accompanied by increases in OXA concentrations.

Materials and Methods

Participant Recruitment and Ethical Approval. From July 2018 to April 2020, postmenopausal women (≥ 12 consecutive months without menstruation) were recruited to participate in this clinical trial. Due to the onset of the COVID-19 pandemic recruitment and enrollment were terminated earlier than expected. Participants were recruited from the Northwest Arkansas area via the daily University of Arkansas digital newsletter, flyers throughout the community, word-of-mouth, and social media to participate in this study. The initial screening was carried out via phone interview. Participants who consumed protein and or n-3 PUFA supplements, consumed fatty fish ≥ two times per week, did not regularly consume breakfast (<5 times/week), smoked, had dietary restrictions, food allergies, were actively trying to lose weight, participated in vigorous activity for ≥4 h/week, had any pre-existing metabolic conditions (e.g., type 1 or 2 diabetes, cancer, cardiovascular disease), were taking hormone replacement therapy and/or medications that would influence protein, n-3 PUFA, or energy metabolism, were

claustrophobic, and/or were uncomfortable with needles, or were unavailable due to travel or work schedule were excluded from participating in the study. At the conclusion of the phone screening participants completed the Pittsburgh Sleep Quality Index (PSQI).
Participants who met all exclusion criteria and scored >5 via the PSQI global score or slept < 7 hours a night qualified for participation in this clinical trial. One hundred seventy women underwent an initial phone screening and 39 eligible women completed all study procedures (July 2020). Written consent was obtained from participants prior to starting the study. Ethical approval for the study protocol was approved by the Office of Research Compliance Institutional Review Board of the University of Arkansas (Fayetteville, AR, USA). This trial was registered at clinical trials.gov as NCT0303041.

Study Design. The study and all measurements were conducted at the Center for Human Nutrition at the University of Arkansas unless otherwise stated. The study was conducted as a randomized parallel design study with one control and four dietary intervention arms via excel complete double randomization of treatment groups and treatment code with an allocation ratio of 1:1. The dietary intervention groups were as follows; 1) control (no intervention, free-living; CON; n=6), 2) whey protein isolate (WPI; 25 g; n=7), 3) n-3 PUFAs, EPA and DHA (n-3 PUFA; 4,300 mg; n=10), 4) WPI + placebo fat (PRO+PLA; 25 g WPI +4,140 g soybean oil; n=7), and 5) WPI + n-3 PUFAs (WPI+n-3 PUFA; 25 g WPI + 4,300 mg of n-3 PUFA; n=9). Refer to Table 1 for participant demographics and baseline anthropometrics for each treatment group. On the basis of previous estimates of variance in triglyceride assessment, we originally aimed to recruit 80 participants as 80 participants (n=16 per dietary intervention) provide 80% power at P < 0.05 for detection of a 17.7 mg/dl change in fasting triglycerides. However, to test the appropriateness of the of the forced sample size (due to COVID-19 ending recruitment early) we

carried out two post-hoc power analyses using the SAS procedure PROC POWER with a one-way ANOVA. First, using the observed sample means and standard deviations we determined 11 participants were needed to reach a statistical power of 0.86 (based on an overall level of significance of 0.05) to detect an accurate 16-week difference in fasting plasma triglyceride concentration. Next, we determined the power of the obtained sample size of 6, 7, 10, 7, and 9 had a statistical power of 0.722.

In this 16-week supplementation intervention all nutritional supplements were consumed daily for 16 weeks. To ensure compliance, participants returned empty containers every four weeks. n-3 PUFA and soybean capsules were stored in pill boxes with AM and PM dividers and WPI was received in 28 individual one-serving bags. At the initial visit, participants signed the consent form and body composition was determined using dual energy X-ray absorptiometry (DXA) analysis (Lunar Prodigy, GE Healthcare, Madison, WI, USA) at the Exercise Science Research Center at the University of Arkansas. Participants also received an ActiGraph sleep monitor (ActiGraph, LLC, Pensacola, FL, USA), sleep diary, and 3-day food records to return at their first clinical test day and the following study materials: a breakfast recipe book with or without the addition of WPI, food scales, measuring cups and spoons, and a Blender Bottle (Blender Bottle Company, Lehi, UT) for protein consumption.

Outcome measures were assessed every four weeks and compared across the four intervention and one control group at 0, 4, 8, 12, and 16 weeks, except objective sleep at 0, 8, and 16 weeks and body composition and strength which was assessed at 0 and 16 weeks only. On the five clinical test days, the participants arrived fasted (10–12 h) at the Center for Human Nutrition at the University of Arkansas at or before 08:00 for data collection. Compliance was

assessed via capsule and empty WPI bag count, completion of weighed food records, and verbal participant confirmation of supplement consumption, and time of consumption.

Dietary Intervention. The n-3 PUFA and placebo soft gels and were supplied by "Nordic Naturals" (94 Hangar Way, Watsonville CA, 95076) and stored in the refrigerator at 4 °C. During the 16-week intervention, participants were instructed to swallow two soft gels containing either n-3 PUFA or placebo fat twice daily with the breakfast and dinner meal. One dose of n-3 PUFAs, two soft gels, contained 1125 mg of EPA and 875 mg of DHA for a daily dose of 4.0 g/day and ratio of 1.3 EPA: DHA. The n-3 PUFAs were sourced from anchovies and sardines. All capsules contained a lemon oil to mask differences in taste and were identical in color and shape. n-3 PUFAs and PLA (4.14 g/d) were administered in a single-blinded manner. The daily supplement of protein contained 25 g unflavored WPI (BiPRO; Davisco Foods International). The WPI was allocated into 28 separate small bags and participants received a new batch every four weeks. Each WPI bag contained one serving of unflavored protein powder and was consumed daily prior to 10:00 am with breakfast. Each daily serving provided 106 kcal, 25 g protein, 3.6 g leucine, 1.6 g isoleucine, 1.5 g valine, 0.4 g fat and 0 grams of carbohydrates. Refer to Table 2 for the nutritional composition of the dietary supplements. Participants were instructed to continue their habitual dietary and physical activity routines for the duration of the clinical trial.

Body Composition and Anthropometrics. Height, weight, and waist-to-hip ratio (WHR) were measured in the fasted state. Body weight was measured to the nearest 0.05 kg using a calibrated scale (Detecto, St. Louis, MO). Height was measured to the nearest 0.01 cm via a standard stadiometer (Detecto, St. Louis, MO) following the removal of shoes and layered clothing, in the free-standing position. Waist circumference measurements were measured by

research personnel at the level of the umbilicus with a 150 cm soft tape measure snug, but no constricting, around the participant's body. Participants were instructed to take normal breaths and relax with their hands at their side, feet positioned closely together, and weight evenly distributed. The measurements were taken at the end of normal expiration or at functional residual capacity, duplicated, and averaged [54]. Body composition was determined via dual-energy X-ray absorptiometry (DXA) analysis (Lunar Prodigy, GE Healthcare) at the Exercise Science Research Center at the University of Arkansas.

Strength Measurements. Isometric grip strength (kg) was measured using a standard hand-grip dynamometer (Takei Scientific Instruments, Niigata-City, Japan). Participants observed a demonstration by the researcher and were properly fitted to the dynamometer so that their middle finger was at a 90-degree angle. In the standing position, participants were instructed to squeeze maximally for 3-seconds. Three trials were completed on each hand, beginning with the dominant hand, with a 60-seconds rest period between trials according to the NHANES Muscle Strength Procedures Manual [55]. Handgrip strength was quantified by the maximal grip force of the dominant hand. Grip strength relative to body weight and lean body mass (LBM) was calculated by dividing grip force by the body mass (kg) and LBM (kg) respectively of the participant at each timepoint.

Energy Expenditure and Substrate Oxidation. Resting energy expenditure (REE; kcal/min) and substrate oxidation (SO; kcal/min) were measured in the fasted state via indirect calorimetry (PARVO Medics, TrueMax 2400 metabolic cart) using the validated ventilated hood technique [56]. A detailed methodology description has been published by Neumann et al [57].

Sleep Assessment. Sleep quality and duration were assessed objectively via an ActiGraph triaxial wrist accelerometer GT3X+, a validated method of sleep assessment [58]. Each participant wore an ActiGraph monitor on the non-dominant wrist for 24-hour per day for seven days (except when bathing or involved in water activities) prior to the start of the intervention, 8-, and 16-weeks. Actigraph monitors were fitted securely on each participants wrist. Participants received sleep diaries to define "time in bed" and "time out of bed". Researchers used the indicated "start" and "end" points to define a sleep region to be analyzed within the ActiGraph software. Sleep outcomes were calculated based on epoch-to-epoch sleep/wake algorithms within the defined sleep period. Data were processed by using the ActiLife Version 6.9.2 software (Pensacola, FL, USA) and sleep was scored via the Cole-Kripke algorithm [59]. The following data was sleep outcomes were recorded: sleep latency (time between lights out and first minute algorithm scores as sleep); sleep efficiency % (total sleep time/total time in bed); total sleep time (TST; total number of minutes scored asleep); time in bed (TIB; total number of minutes in bed); wake after sleep onset (WASO; total minutes awake after sleep onset); awakenings (total and average}; Sleep Fragmentation Index (SFI; degree of sleep fragmentation). A seven-day average was calculated for each sleep outcome.

A subjective measure of sleep quality was assed via the Pittsburgh Sleep Quality Index (PSQI) questionnaire [60]. The 19-item PSQI questionnaire addressed seven components of subjective sleep quality: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleep medications, and daytime dysfunction. In scoring the PSQI, seven component scores are derived, each scored 0 (no difficulty) to 3 (severe difficulty). The component scores are summed to produce a global sleeping score (GSS) with a range of 0 to

21. Higher scores indicate worse sleep quality. A compiled global score of the seven scored components distinguishes good sleepers (≤ 5) from poor sleepers (>5) [61] .

Positive and Negative Affect States. The Profile of Mood States (POMS) questionnaire consists of 65 questions containing a one-word adjective of mood to measure and identify six affective states. The six identifiable mood/affective states are tension-anxiety, depression-dejection, anger-hostility, vigor-activity, fatigue/-inertia, and confusion-bewilderment. Participants were instructed to define their mood on a 5-point Likert scale ranging from 0 to 4. The numbers refer to the following descriptive phrases: 0 = Not at all, 1 = A little, 2 = Moderately, 3 = Quite a bit, 4 = Extremely. Prior to the start of the questionnaire each participant was read the following directions: Describe how you have been feeling during the past week including today by circling the number that best describes your present mood with 0 indicating "Not at all," and 4 indicating "Extremely". A researcher was readily available to answer questions regarding the meaning of a word. POMS was administered in the fasted state at baseline, 4-, 8-, 12- and 16- weeks. To obtain the score for reach identifiable mood/affective state subscale, the sum of the responses for each adjective is calculated. The subscale scores range from 0 up to 36, 60, 48, 32, 28, and 28 for tension-anxiety depression-dejection, anger-hostility, vigor-activity, fatigue/-inertia, and confusion-bewilderment respectively. Higher subscale scores for all affect states, but the vigor domain represent poorer mood. Two adjectives relaxed and efficient were inversely scored from 4 to 0 rather than 0 to 4. Total Mood Disturbance Score (TMD) is calculated by summing the scores across all six factors (weighting vigor negatively). The total mood disturbance (TMD) is calculated by the following equation: TMD = (Tension-Anxiety) + (Depression-Dejection) + (Anger-Hostility) + (Fatigue-Inertia) + (Confusion-Bewilderment) – (Vigor-Activity).

The TMD score is the most reliable outcome of POMS because of the intercorrelations among the six affective factors and ranges from -32 (best possible TMD score) to 200 (worst possible TMD score). The POMS questionnaire has been validated in postmenopausal women [62].

Plasma Biomarkers. Two blood samples (10 mL/sample, 20 mL/testing day) were collected after a 10- to 12-h fast at baseline, 4-, 8-, 12-, and 16-weeks. The samples were collected in EDTA-coated vacutainer tubes. Samples were immediately centrifuged at 4°C for 15 min at 1800 × g. The plasma was separated and stored at −80°C until analysis. Plasma glucose (catalog #: 10009582; mg/dL), triglycerides (TG ; catalog #: 10010303mg/dl), C-reactive protein (CRP; catalog #: 10011236; pg/mL), free-fatty acids (FFA; catalog #: 700310; uM), total cholesterol (catalog #: 10007640, mg/dl), insulin (catalog #: 26619, uUI/mL) concentrations were determined via commercially available kits (Cayman Chemical Company, Ann Arbor, MI, USA). Plasma cortisol (EIA-CORT, ng/mL) and brain-derived neurotrophic factor (BDNF; ELH-BDNF, ng/mL) concentrations were determined via commercially available kits (RayBiotech, Inc, Norcross, GA, USA). Human orexin-A (OXA; LS-F4072; pg/mL) concentrations was determined via commercially available kit (LifeSpan Biosciences, Inc, Seattle, WA, USA). Creatine kinase M (CKM; Ab185988, U/mL) concentrations were determined via commercially available kits (Abcam Cambridge, UK). All kits were performed per the manufacturer's instructions.

Dietary Records and Assessment. Participants completed a five self-administered 3-day weighed food record prior to the intervention and at 4, 8, 12, and 16 weeks (two weekdays and one weekend). Each participant was trained to accurately record quantities of food using a provided food scales (Greater Goods, LLC) and beverages. Participants were instructed to include brand names and methods of food preparation. The 3-day food records were reviewed

with participants on each test day to ensure food intake was properly recorded in detail. The energy, macronutrient, and micronutrient composition of the 3-day food records analyzed using the Nutrition Data System for Research software (NDSR; NDS version 2018, Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN).

Statistical Analysis

Summary statistics were calculated for all data and data are expressed as mean ± SD. One-way ANOVA was used to analyze baseline measurements of participant characteristics and body composition. The one factor repeated measures design was analyzed as a generalized linear mixed model with treatment group and time as fixed factors with time treated as a repeated measures and subjects as random nested within treatment group. Number of levels of time depended on the variable being tested which included 2, 3, and 5 time points. Initially, age and BMI were considered as covariates. BMI was not considered as a covariate when measured as a response or when analyzing body composition variables. All of the response variables, if they could only take on only a positive value and were non-proportion values, were assumed to follow a gamma distribution. Responses that were percentages were converted to proportions and analyzed as a beta distribution. PSQI global score was assumed to follow a Poisson distribution. POMS TMD was assumed to follow a gaussian distribution as TMD includes a range of positive and negative scores. The treatment effect was tested when variables were converted to 16-week change, by subtracting out baseline values (week-16 – baseline) they could take on positive or negative value and were assumed to follow a gaussian distribution.

Where appropriate, follow-up least-squares mean (LS-mean) comparisons for treatment and time main effects were declared significantly different if the corresponding ANOVA F

statistic was significant. For any significant and interaction trends (P <0.1), mean comparisons were carried out using the least significant difference (LSD). Statistical analyses involving generalized linear mixed models were performed using PROC GLIMMIX in SAS version 9.4 (SAS Institute inc., Cary, NC). All graphs were made using GraphPad Prism Software version 7.0 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered significant. As previously mentioned, post-hoc power analyses using the SAS procedure PROC POWER with a one-way ANOVA determined the observed sample means, standard deviations, and sample size of 6, 7, 10, 7, and 9 had a statistical power of 0.722. Therefore, as type-2 error is high trends will be addressed in the subsequent sections.

Results

Subject Flow Chart, Characteristics, and Compliance. Of the 45 women eligible for the study, 39 completed the study resulting in a 13.33 % attrition rate as shown in Figure 1. Reasons for subject withdrawal can be found in Figure 1. Baseline characteristics (sex, age, baseline anthropometrics, and baseline PSQI GSS) of subjects in the four treatment and control groups who completed the study were not statistically different (Table 1). The average compliance of subjects who completed the study in a dietary intervention group, as judged by the leftover capsule and bag count was as follows: PRO: 99.4 ± 0.01%; n-3 PUFA: 98.6 ± 0.02%; PRO + PLA: 98.8 ± 0.02%; and PRO + n-3 PUFA: 99.0 ± 0.02%. 16-weeks of dietary interventions did not significantly affect anthropometric measurements of body weight, BMI, waist circumference, hip circumference, or the waist to hip ratio (Table 3 and Table 4).

Body Composition and Handgrip Strength. The differences in outcomes of body composition and handgrip strength as a result of the dietary intervention are outlined in Table 4. We observed a decreased trend for a group-by-time interaction effect for android fat % (P = 0.07) over the 16-week period. After applying LS-means, the CON and PRO groups had a significant increase and decrease in % android fat from week 1 to week 16 respectively (P < 0.05) with no significant differences between treatment groups. A decreased trend for the treatment effect (16wk – baseline) was observed for android fat % (P = 0.07). Following LS-means, we found android fat % in PRO (-2.5 ± 2.2 %), n-3 PUFA (-0.2 ± 3.2 %), and PRO + n-3 PUFA (-0.1 ± 3.0 %) significantly decreased when compared to the CON group (+2.8 ± 1.8 %) (P < 0.05) with no differences when compared to PRO + PLA (0.2 ± 4.2 %). Although non-significant, we observed a trend towards greater % increase in the treatment effect on total fat mass (kg) in the CON (+4.6 ± 3.6 %) compared to the PRO (-2.4 ± 5.2 %), n-3 PUFA (-0.7 ± 8.6 %), PRO + PLA (+1.3 ± 10.2 %), and PRO + n-3 PUFA (+2.0 ± 6.7 %). Similarly, we observed a non-significant trend in the CON group towards a greater % decrease in FFM (-0.65 ± 2.06 %) compared to PRO (+0.82 ± 1.19 %), n-3 PUFA (+1.25 ± 2.82 %), PRO and PRO + n-3 PUFA (+0.36 ± 3.62) with similar losses compared to PRO + PLA (-0.98 ± 2.93 %). We did not observe any significant effects of the 16-week dietary intervention on FFM, LBM, ALM, Total FM, whole body fat %, android fat %, gynoid fat %, fat-to-lean ratio, or BMD.

We observed an increased trend of treatment effect on high HGS, over the 16-week intervention (P = 0.08). PRO + PLA and PRO + n-3 PUFA supplementation resulted in increases in high HGS by 7.9% and 5.2% compared to 0.3% increase in the control group (P < 0.05).

Energy Expenditure and Substrate Oxidation. The effects of the 16-week supplementation intervention on energy expenditure and substrate oxidation controlled for FFM can be found in Table 5. After controlling for FFM, there was a significant effect of group on fat oxidation (kcal/min), carbohydrate oxidation (kcal/min) and REE (kcal/day). Following LS-mean we observed no significant differences between treatment groups in carbohydrate oxidation nor REE. However, fat oxidation increased in n-3 PUFAs and PRO + n-3 PUFAs from baseline to 16 weeks ($P < 0.05$). After controlling for baseline, an increased trend for fat oxidation treatment effect was observed ($P = 0.06$). Following LS-means n-3 PUFA (+34.6 %; $P < 0.05$) and PRO + n-3 PUFA (+55.6 %; $P < 0.05$) had significantly higher fat oxidation at 16 weeks compared to baseline and PRO had significantly lower fat oxidation (-37.8%; $P < 0.05$) compared to differences in all other treatment groups from baseline to 16 weeks.

Objective and Subjective Sleep. The effects of the 16-week supplementation intervention on objective sleep duration and quality can be found in Table 6. We observed a significant treatment effect ($P < 0.05$) for time in bed with PRO + n-3 PUFA significantly decreasing their bedtime (-42 ± 62.4 min) when compared to n-3 PUFA (+0.6 ± 36.6 min) and PRO (+32.4 ± 18 min). Contrarily, PRO and n-3 PUFAs had a significant increase in bedtime compared to CON (-15 ± 25.8 min). We observed a significant group ($P < 0.05$), but not a treatment effect for WASO and sleep fragmentation with n-3 PUFA displaying a significant increase in WASO and the sleep fragmentation index compared to all other groups. PRO had significantly lower WASO and sleep fragmentation index at baseline ($P < 0.05$) and n-3 PUFA group had significantly higher sleep latency ($P < 0.05$). We found no significant treatment effects of time out of bed, sleep latency, sleep efficiency, sleep duration, nor number of awakenings.

The effects of the 16-week intervention on subjective sleep duration and quality can be found in Figure 2, and seven component scores in Table 7. We observed a significant time effect ($P < 0.05$) on PSQI global scores with no differences between groups, but a significant decrease in GSS from week 1 to 16 ($P < 0.05$). Although not significant, a greater % decrease was observed in PRO (30.3%), n-3 PUFA (23.3%), PRO + PLA (20.2%), and PRO + n-3 PUFA (26.4%) when compared to CON (-17.9%).

Profile of Mood States. The effects of the 16-week intervention on POMS TMD and six-affect state subcomponent scores can be found in Table 8. No significant treatment, group, time, nor group x time main effects were observed for POMS TMD score or subcomponents of depression, anger, and fatigue over the 16-week intervention. However, a significant group and group x time effect was observed for vigor ($P < 0.05$). At week-16 vigor scores were significantly higher following PRO (20.6 ± 9.1) and n-3 PUFA (18.1 ± 8.0) supplementation compared to CON (12.3± 6.4) with no differences compared to PRO + PLA (16.4 ± 6.2) and PRO + n-3 PUFAs (16.3 ± 5.0).

Biomarkers of Metabolic Health and Well-being. The effects of the 16-week intervention on biomarkers of cardiometabolic risk can be found in Table 9. We observed a significant time or group X time effect of the 16-week supplementation intervention on insulin (time: $P < 0.05$), FFA (time: $P < 0.05$), and cholesterol (group X time: $P < 0.05$) with a trend for HOMA-IR (time: $P = 0.09$) and TG (group: $P = 0.05$). Following LS-means we found insulin, HOMA-IR, FFA, cholesterol, and triglycerides decreased over time regardless of group ($P < 0.05$). A significant treatment effect was observed in cholesterol alone ($P < 0.05$) with significant decreases in PRO by -7.3%, n-3 PUFA by -7.9%, PRO + PLA by -1.8% and PRO+n-3 PUFA by -20.6% compared to an increase in CON by +17.8%. Although a treatment effect was not

observed, the percent decrease in triglycerides in n-3 PUFA + PRO was at least -16% greater when compared to subsequent groups. No effect of 16-week intervention was observed on CRP concentrations.

The effects of the 16-week intervention on biomarkers of well-being can be found in Figure 3. We observed a significant effect of time BDNF ($P < 0.05$) and cortisol ($P < 0.05$) with a trend on OXA ($P = 0.07$). However, we observed a significant treatment effect for OXA ($P < 0.05$). After applying LS-means we observed a significant increase in OXA concentration in PRO + n-3 PUFA (Wk16: 28.4 ± 17.5; Δ Wk16: 8.6 ± 9.3 pg/mL) compared to PRO (Wk16: 19.2 ± 9.5; Δ Wk16 : -1.3 ± 7.0 pg/mL), n-3 PUFA (Wk16: 15.7 ± 11.2; Δ Wk16: -0.8 ± 5.9 pg/mL) , PRO +PLA (Wk16: 25.0 ± 16.8; Δ Wk16: 1.5 ± 8.1 pg/mL) , and CON (Wk16: 19.2 ± 10.7; Δ Wk16: 0.8 ± 3.3 pg/mL). Overall, the percent OXA increase in n-3 PUFA + PRO was at least 19.4 % greater than subsequent study groups. We did not observe a treatment effect for CKM, BDNF, or cortisol. OXA, BDNF, CKM, and cortisol raw values can be found in Table 10.

Dietary Intake. The effects of the 16-week intervention on dietary intake of energy and macronutrients, AAs, and lipids can be found in Table 11, Table 12, and Table 13 respectively. We observed no differences in energy intake (kcal/d) at baseline nor a group, time, group X time, or treatment effect. We did not observe a significant treatment effect on total energy (kcal/day) intake, macronutrients total (g/d and % energy), nor protein g/kg/bw. However, at baseline PRO + n-3 PUFA and PRO had a significantly higher protein intake g/day compared to CON ($P < 0.05$). We observed a significant time effect on carbohydrates ($P < 0.05$). Following LS-means total carbohydrates significantly decreased in all groups from week 1 to week 16 ($P < 0.05$) with no significant differences between groups.

We observed a significant increase in EPA (mg/d), DHA (mg/d), % n-3 PUFAs of total energy, and decreased n-6 PUFA: n-3 PUFA ratio ($P < 0.05$) in n-3 PUFA and PRO + n-3 PUFA. We observed a significant treatment effect of cholesterol (mg/d) ($P < 0.05$), but not saturated fat (g/d), with lower cholesterol dietary intake in PRO + n-3 PUFA compared to subsequent groups. PRO + PLA had a significantly lower cholesterol intake at baseline ($P < 0.05$) compared to subsequent groups. No differences in AA intake were observed at baseline. We observed a significant time, group, time X group on total essential amino acids, branched-chain amino acids, tryptophan, and cysteine with increases in the PRO and PRO + n-3 PUFA groups ($P < 0.05$). We observed a significant treatment effect on tryptophan ($P < 0.05$) with an increased trend on total essential amino acids ($P = 0.07$), branched-chain amino acids ($P = 0.05$), and cysteine with increases in the PRO and PRO + n-3 PUFA groups ($P = 0.08$).

Discussion

To our knowledge, this is the first RCT to examine the effect of 16-weeks of dietary protein and/or n-3 PUFA supplementation on body composition, cardiometabolic risk, and well-being effect of in postmenopausal women. The present study tested the hypothesis that combined dietary protein and n-3 PUFA supplementation would have a greater effect on body composition, cardiometabolic risk, and indexes of sleep and mood states in postmenopausal women when supplemented in combination as WPI and n-3 PUFA compared to individual supplementation. Collectively, the results of this study suggest protein and n-3 PUFA combined supplementation when compared to individual supplementation for 16-weeks does not provide additional benefits on body composition, cardiometabolic health, and well-being. However, the results of this study

indicate a trend that individual protein and n-3 PUFA improve different outcomes of SA compared to free-living postmenopausal women.

Diets rich in high-quality protein and n-3 PUFAs, EPA and DHA, are positively correlated with body composition, cardiometabolic health, and well-being in middle-aged and older adults [27, 63, 64]. To our knowledge, RCTs examining the effect of dietary protein and n-3 PUFA combined supplementation have solely been conducted in the context of a multi-nutrient supplement or in combination with caloric restriction or exercise [65-67]. When consumed bi-daily for 6-weeks a multi-nutrient supplement containing WPI, EPA, and DHA increased LBM and strength in older adults [66]. Although not significant, the present study found an increased trend in handgrip strength following PRO + PLA (2.1 ± 2.5) and PRO + n-3 PUFA (1.2 ± 2.5 kg) supplementation with a decrease in CON (-0.3 ± 2.1) from baseline. The observed increases may be functionally relevant as HGS is reflective of physical performance [68]. For example, in older women every 1-kg increase in HGS is associated with a 0.13-s decrease in 3-minute walk time and 1% decrease in chair rise time [69]. Similarly, in the present study non-significant increases in FFM, were found following supplementation with PRO by 0.82%, n-3 PUFAs by 1.25%, and protein + n-3 PUFAs by 0.35% compared to decreases in the control, free-living group by -0.65 %. Although, not statistically significant, in adults ≥50 years of age skeletal muscle begins to significantly decline [70] and annual skeletal muscle loss is estimated to be approximately ~0.5 to 1% which emphasizes the physiological importance for even a small enhancement of FFM preservation [71, 72]. The protective effect of protein and n-3 PUFAs on FFM/LBM is further supported in longitudinal prospective studies in older adults [73, 74], which demonstrates a longer supplementation period may be required to observe changes LBM. Furthermore, a caloric restriction intervention in combination with a high-protein meal replacement (25 g) and fish oil

(2,130 mg) reduced percent of android fat and the prevalence of metabolic syndrome by almost twofold in comparison to caloric restriction alone (>40 years of age) [67]. In the present study we observed a trend for individual PRO supplementation alone to reduce central adiposity. Overall, the results do not indicate a significant effect of supplementation on body composition nor HGS in post-menopausal women.

Protein and n-3 PUFAs may increase whole body REE and fatty acid oxidation, but the results to date are varied. Dietary protein supplementation has been shown to increase REE by preserving LBM primarily under caloric-restriction conditions [75]. When protein is consumed within the AMDR, REE rarely increases after controlling for FFM [76]. For example, a weight-maintenance study following 12-weeks of energy restriction in adults (34-65 y) observed no effect of a high-protein (27% dietary protein) compared to a lower protein (16%) diet [76]. Conflicting results are present in the literature regarding the influence of n-3 PUFAs on REE and substrate oxidation and are conducted primarily in young adults [77-80]. For example, Noreen et al. found six-weeks of fish oil supplementation (1,600mg EPA + 800mg DHA) did not influence REE in adult men and women (34 ± 13 y) [78] and an alternative RCT found 3 g/day EPA and DHA improved REE, but not fat oxidation, over a 12-week supplementation period in young men [77]. However, a seminal study by Couet et al. found supplementation of 6 g/day of fish oil for 3 weeks significantly increased fat oxidation, but not REE after controlling for LBM in young men [79]. More recently, Logen et al. supplemented n-3 PUFAs (2 g EPA, 1 g DHA) for 12-weeks in healthy older women (66 ± 1 y) and found a significant increase in both REE (14%) and fat oxidation (19%) [80]. Our study found no effect of supplementation on REE, but n-3 PUFAs and n-3 PUFAs combined with protein increased fat oxidation over the 16-week intervention by ~34.6 % and ~55.6% respectively after controlling for FFM. Several theories

have been proposed to explain the mechanisms of n-3 PUFAs and protein supplementation on REE and substrate oxidation, although the precise mechanisms are yet to be fully elucidated. A few likely theories include preservation/increases in skeletal muscle mass [81], EPA and DHA incorporation into the phospholipid and mitochondrial membrane [82, 83], and altered gene expression of enzymes involved in fatty acid oxidation [84] and energy metabolism [85] .

Poor well-being characterized by decreased sleep quantity, quality, and mood is independently associated with an increased risk of obesity, sarcopenia, cardiometabolic disease, and functional decline in middle-aged and post-menopausal women [86]. Over the past decade, sleep duration and quality has decreased, and depression has increased in the US. Post-menopausal women report worse sleep quality and higher total mood disturbances compared to the U.S. average [62, 87]. However, a link between diet, sleep, and mood remains inconsistent. Our study results did not indicate a significant effect of supplementation of protein or n-3 PUFAs on indexes of subjective or objective sleep or mood states compared to the free-living control group. Interestingly, we observed improvements in perceived sleep quality and mood states in all arms of the intervention over time. However, as the observed improvements were not significantly greater than the control free-living group benefits cannot be attributed to one or both of the supplemented nutrients. Contrarily, we did not observe an increasing trend in objective sleep quality. In support of our findings, a PSQI validation study identified affect states, opposed to actigraphy sleep parameters as correlates due to the influence of depression and positive outlook on perceived sleep quality [88]. In agreement with the current literature, biomarkers associated with mood states, BDNF and cortisol, increased and decreased respectively with improvements in mood scores [89, 90] . Decreases in cortisol concentrations are reflective of down-regulation of the hypothalamic-pituitary adrenal axis and subsequently

decreases in cardiometabolic health [91]. Collectively we observed improvements in HOMA-IR and decreases in triglycerides, FFA, and cholesterol among treatment groups over time. Similar to our findings, intervention investigating dietary protein and n-3 PUFAs found no effect on mood (POMS), cognitive function [92] or indexes of sleep (accelerometry) [93]. Furthermore, recent meta-analyses concluded n-3 PUFAs decrease depression and anxiety in older adults with, but not without clinical depression [94] and anxiety [95]. Although RCTs have suggested dietary protein and n-3 PUFAs as potential modulators of sleep and mood [96] further data is needed to support these findings.

The orexin system has recently been suggested as "The Key for a Healthy Life" [51]. OXA has an identified role in emotion regulation, energy homeostasis, and sleep and wakefulness [46, 48, 50, 97-99]. However, the effect of nutrients on OXA in humans is largely unknown. Although, open-label, medication clinical trials and cross-sectional analyses have observed a positive association between improvements in metabolic health and psychological outcomes and OXA concentrations [97, 100]. For example, anti-hyperglycemic treatment in type-2 diabetics via metformin improved glycemic control and increased OXA concentration by 26% [97]. Similarly, our results showed that protein and n-3 PUFAs increased OXA concentrations by ~23.9% with <6% change in additional study arms. To our knowledge, comparable dietary interventions have yet to be conducted and a mechanism of action of OXA in cardiometabolic health, physical, and cognitive function, and well-being is yet to be elucidated in humans. Moreover, data from our lab indicate obese Zucker rats assigned a high-protein (40% energy) diet had reduced liver and skeletal muscle lipid deposition, and higher OXA concentrations compared to obese Zucker rats consuming a moderate-protein (20% energy) diet

for 12-weeks [101]. Therefore, there is a need to further assess the effect of dietary protein and n-3 PUFA intake on OXA in post-menopausal women.

There are multiple limitations in the present study. First, only women were included in our study sample to control for sex-specific differences in well-being [102, 103], body composition [104], and strength [105]. Second, in our study, all arms including the control, were associated with significant improvements in sleep quality and duration via the PSQI GSS. The improvements in all groups, despite no changes in objective sleep quality, may be attributed to the placebo effect. The mechanisms of the placebo effect have not been directly established. However, participant expectancy and optimism are significant mediators of subjective outcomes of well-being [106, 107]. To avoid bias, future clinical trials evaluating subjective components of well-being should consider evaluation of expectancy of outcomes post-randomization (e.g., Credibility and Expectance scale) [108]. Third, although we screened for dietary protein supplementation, we did not screen for baseline dietary protein intake. Fourth, a group of participants completed the trial during the COVID-19 pandemic. Although, all supplements were supplied we cannot verify how COVID-19 may have affected food availability, sleep, and stress levels. Lastly, the findings of this clinical trial are based off of a lower than anticipated sample size and may not translate to all post-menopausal women.

The results of this study indicate that concomitant compared to individual supplementation of protein and n-3 PUFAs does not provide significant additional benefits on body composition, cardiometabolic risk, and well-being in post-menopausal women. However, protein and n-3 PUFA have the potential to reduce abdominal adiposity, increase strength, enhance fatty acid oxidation, and to improve subjective mood states and sleep. In addition, a potential additive effect on OXA concentration warrants further investigation. Future research

should evaluate the efficacy of combined protein and n-3 PUFA supplementation over a longer duration and investigate the mechanisms underlying the suggested improvements in cardiometabolic risk, well-being, and OXA to promote SA.

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Tables

Table 1. Demographic and baseline anthropometric characteristics of the study population by treatment group

	CON (n=6)	PRO (n=7)	n-3 PUFA (n=10)	PRO + PLA (n=7)	PRO + n-3 PUFA (n=9)	*P*-value
Age, y	63.0 ± 8.9	61.6 ± 8.4	58.5 ± 12.0	61.2 ± 2.6	63.3 ± 8.4	0.81
Anthropometrics[1]						
Height, m	1.62 ± 0.08	1.63 ± 0.06	1.62 ± 0.05	1.64 ± 0.10	1.65 ± 0.08	0.70
Weight, kg	72.4 ± 15.2	73.4 ± 18.1	76.7 ± 20.3	70.0 ± 19.6	72.8 ± 22.4	0.97
BMI, kg/m^2	27.4 ± 4.6	27.4 ± 4.8	29.5 ± 8.4	25.9 ± 6.0	27.0 ± 8.1	0.86
Waist, cm	91.4 ± 10.1	92.8 ± 17.2	98.0 ±18.0	89.8 ±15.6	89.1 ± 18.8	0.80
Hip, cm	107.3 ± 9.6	108.5 ± 12.1	111.6 ± 17.4	106.2 ± 16.4	110.1 ± 18.0	0.96
WHR	0.85 ± 0.05	0.85 ± 0.07	0.88 ± 0.06	0.84 ± 0.04	0.81 ± 0.06	0.80
PSQI						
GSS, AU	8.3 ± 3.0	6.7 ± 1.6	7.8 ± 2.7	7.7 ± 2.5	8.9 ± 2.8	0.56
Ethnicity [2]						
American Asian/Asian, n (%)	-	-	1 (10.0)	-	-	
Hispanic, n (%)	-	2 (28.6)	-	1 (14.3)	-	
Caucasian, n (%)	6 (100)	5 (71.4)	9 (90.0)	6 (85.7)	8 (89.9)	
Other, n (%)	-	-	-	-	1 (11.1)	

[1] Data are expressed as mean ± SD unless otherwise indicated. Significant differences: * $P < 0.05$. Control, no intervention, free living; whey protein isolate, PRO; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA;

whey protein isolate + placebo soybean oil, PRO + PLA; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA; Waist to hip ratio, WHR; Pittsburgh Sleep Quality Index, PSQI; global sleeping score, GSS.

[2] Ethnicity is expressed in terms of frequency (n) with percentage of participants within treatment group (%).

Table 2. Nutrient composition of dietary supplements[1]

	PRO	n-3 PUFAs	PRO + PLA	PRO + n-3 PUFA
Energy content, kcal	106.4	50	143.7	156.4
Protein, g	25.5	-	25.5	25.5
Leucine, g	3.6	-	3.6	3.6
Isoleucine, g	1.6		1.6	1.6
Valine, g	1.5		1.5	1.5
Fat, g	0.4	5	4.14	5
Total n-3 PUFA	-	4,300	284	4,300
EPA, mg	-	2,250	-	2,250
DHA, mg	-	1,750	-	1,750
Other, mg	-	300	284	300
Carbohydrates, g	-	-	-	-

[1] The PRO represents a single dose of whey protein isolate which participants consumed prior to 10:00 AM with breakfast daily. The n-3 PUFAs and PLA represent a combination of two daily doses. Two capsules of n-3 PUFAs or two capsules of PLAs were consumed with breakfast and with dinner daily. Whey protein isolate, PRO; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA; whey protein isolate + placebo soybean oil, PRO + PLA; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA; soybean placebo, PLA; eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA.

Table 3. Effects of a 16-week supplementation intervention on anthropometrics in dietary intervention and control groups[1]

	Weeks of Intervention					Treatment effect[2]	ANCOVA *P*[3]			
Anthropometrics	0	4	8	12	16	Δ 16 wk	*P*	Group	Time	Group X time
Weight, kg							0.64	0.97	0.11	0.70
CON	72.4 ± 15.2	72.7 ± 15.2	73.0 ± 14.2	73.1 ± 14.2	73.0 ± 14.1	0.57 ± 2.01				
PRO	73.4 ± 18.1	73.3 ± 18.2	73.0 ± 18.4	73.4 ± 19.1	73.2 ± 18.8	-0.23 ± 1.42				
n-3 PUFA	76.7 ± 20.3	76.3 ± 20.9	77.3 ± 20.8	76.9 ± 20.8	76.7 ± 20.8	-0.01 ± 2.52				
PRO + PLA	70.0 ± 19.6	69.7 ± 19.8	70.2 ± 19.3	70.4 ± 19.5	70.2 ± 19.7	0.23 ± 2.06				
PRO + n-3 PUFA	72.8 ± 22.4	73.2 ± 22.4	73.2 ± 21.6	73.8 ± 21.6	74.2 ± 21.7	1.28 ± 2.27				
BMI, kg/m^2							0.46	0.86	0.12	0.58
CON	27.4 ± 4.6	27.6 ± 4.3	27.7 ± 4.3	27.8 ± 4.2	27.7 ± 4.0	0.30 ± 0.79				
PRO	27.4 ± 4.8	27.4 ± 5.0	27.2 ± 4.9	27.3 ± 5.2	27.3 ± 4.9	-0.12 ± 0.52				
n-3 PUFA	29.5 ± 8.4	29.3 ± 8.6	29.6 ± 8.5	29.6 ± 8.6	29.4 ± 8.6	-0.07 ± 0.92				
PRO + PLA	25.9 ± 6.0	25.6 ± 6.1	25.8 ± 5.8	25.9 ± 5.9	25.8 ± 6.1	-0.05 ± 0.89				
PRO + n-3 PUFA	27.0 ± 8.1	27.0 ± 8.2	26.9 ± 8.0	27.3 ± 8.0	27.5 ± 7.9	0.49 ± 0.82				
Waist, cm										
CON	91.4 ± 10.1	91.9 ± 8.3	92.7 ± 8.5	93.2 ± 8.4	92.8 ± 8.6	1.4 ± 3.14	0.36	0.85	0.18	0.42
PRO	92.8 ± 17.2	92.1 ± 16.7	91.9 ± 16.3	91.4 ± 17.1	91.0 ± 16.4	-1.8 ± 3.35				
n-3 PUFA	98.0 ± 18.0	97.7 ± 18.5	98.0 ± 17.2	96.3 ± 16.8	97.1 ± 16.9	-0.8 ± 3.91				
PRO + PLA	89.8 ± 15.6	90.5 ± 16.4	90.4 ± 15.7	88.7 ± 16.8	87.5 ± 15.7	-2.3 ± 3.41				
PRO + n-3 PUFA	89.1 ± 18.8	90.6 ± 18.7	91.8 ± 17.1	91.3 ± 19.2	90.2 ± 18.9	1.0 ± 5.93				

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean

oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$.

[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age as a covariate. P-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 3. Effects of a 16-week supplementation intervention on anthropometrics in dietary intervention and control groups[1](Cont.)

	Weeks of Intervention					Treatment effect[2]	ANCOVA *P*[3]			
Anthropometrics	0	4	8	12	16	Δ 16 wk	*P*	Group	Time	Group X time
Hip, cm							0.99	0.94	0.97	0.94
CON	107.3 ± 9.6	107.2 ± 7.0	107.2 ± 9.1	107.8 ± 9.2	107.4 ± 10.3	0.08 ± 1.46				
PRO	108.5 ± 12.1	108.2 ± 12.5	107.8 ± 13.0	108.6 ± 12.4	108.4 ± 12.5	-0.18 ± 2.12				
n-3 PUFA	111.6 ± 17.4	111.3 ± 16.9	112.6 ± 16.2	111.6 ± 17.4	111.6 ± 16.5	-0.01 ± 3.44				
PRO + PLA	106.2 ± 16.4	105.3 ± 16.7	106.3 ± 14.0	105.9 ± 17.4	106.1 ± 15.3	-0.02 ± 3.52				
PRO + n-3 PUFA	110.1 ± 18.0	111.3 ± 17.8	110.5 ± 17.2	110.5 ± 16.7	110.6 ± 17.5	0.51 ± 3.17				
WHR							0.39	0.34	0.13	0.41
CON	0.85 ± 0.05	0.86 ± 0.04	0.87 ± 0.06	0.86 ± 0.04	0.86 ± 0.04	0.01 ± 0.02				
PRO	0.85 ± 0.08	0.85 ± 0.05	0.85 ± 0.05	0.84 ± 0.08	0.84 ± 0.08	-0.01 ± 0.03				
n-3 PUFA	0.88 ± 0.06	0.88 ± 0.07	0.87 ± 0.06	0.86 ± 0.04	0.87 ± 0.05	-0.01 ± 0.03				
PRO + PLA	0.84 ± 0.04	0.86 ± 0.5	0.85 ± 0.06	0.84 ± 0.06	0.82 ± 0.07	-0.02 ± 0.04				
PRO + n-3 PUFA	0.81 ± 0.06	0.81 ± 0.08	0.83 ± 0.07	0.82 ± 0.08	0.81 ± 0.08	0.01 ± 0.04				

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$.
[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age as a covariate. P-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 4. Effects of a 16-week supplementation intervention on anthropometrics, body composition and handgrip strength in dietary intervention and control groups[1]

	Weeks of intervention		Treatment effect[2]	ANCOVA *P*[3]			
Body Composition	0	16	Δ 16 wk	P	Group	Time[2]	Group X time
Weight, kg				0.64	0.97	0.11	0.70
CON	72.4 ± 15.2	73.0 ± 14.1	0.57 ± 2.01				
PRO	73.4 ± 18.1	73.2 ± 18.8	-0.23 ± 1.42				
n-3 PUFA	76.7 ± 20.3	76.7 ± 20.8	-0.01 ± 2.52				
PRO + PLA	70.0 ± 19.6	70.2 ± 19.7	0.23 ± 2.06				
PRO + n-3 PUFA	72.8 ± 22.4	74.2 ± 21.7	1.28 ± 2.27				
Waist, cm				0.36	0.85	0.18	0.42
CON	91.4 ± 10.1	92.8 ± 8.6	1.4 ± 3.14				
PRO	92.8 ± 17.2	91.0 ± 16.4	-1.8 ± 3.35				
n-3 PUFA	98.0 ± 18.0	97.1 ± 16.9	-0.8 ± 3.91				
PRO + PLA	89.8 ± 15.6	87.5 ± 15.7	-2.3 ± 3.41				
PRO + n-3 PUFA	89.1 ± 18.8	90.2 ± 18.9	1.0 ± 5.93				
WHR				0.39	0.34	0.13	0.41
CON	0.85 ± 0.05	0.86 ± 0.04	0.01 ± 0.02				
PRO	0.85 ± 0.08	0.84 ± 0.08	-0.01 ± 0.03				
n-3 PUFA	0.88 ± 0.06	0.87 ± 0.05	-0.01 ± 0.03				
PRO + PLA	0.84 ± 0.04	0.82 ± 0.07	-0.02 ± 0.04				
PRO + n-3 PUFA	0.81 ± 0.06	0.81 ± 0.08	0.01 ± 0.04				
LBM, kg				0.57	0.99	0.41	0.54
CON	40.3 ± 8.1	40.1 ± 8.8	-0.17 ± 0.99				
PRO	40.2 ± 6.7	40.8 ± 7.5	0.42 ± 0.55				
n-3 PUFA	39.3 ± 5.4	39.9 ± 6.4	0.64 ± 1.46				
PRO + PLA	38.7 ± 6.1	38.6 ± 5.9	-0.13 ± 0.91				
PRO + n-3 PUFA	41.5 ± 7.9	41.8 ± 7.9	0.31 ± 1.25				

[1]

Values are mean ± SD. There were no significant differences between groups at baseline test by one-way ANOVA. Waist-to-hip ratio, WHR; lean body mass, LBM; appendicular lean mass, ALM; fat-free mass, FFM; fat mass, FM; bone mineral density, BMD; handgrip strength, HGS. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=7; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean

oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$.

[3]Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age as a covariate. *P*-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different between groups within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 4. Effects of a 16-week supplementation intervention on anthropometrics, body composition and handgrip strength in dietary intervention and control groups[1] (Cont.)

	Weeks of intervention		Treatment effect[2]	ANCOVA *P*[3]			
Body Composition	0	16	Δ 16 wk	P	Group	Time[2]	Group X time
ALM, kg				0.73	0.90	0.14	0.59
CON	17.0 ± 3.6	17.4 ± 3.7	0.40 ± 0.63				
PRO	17.2 ± 3.5	17.4 ± 3.7	0.09 ± 0.44				
n-3 PUFA	16.7 ± 2.8	16.7 ± 3.1	0.03 ± 0.66				
PRO + PLA	16.2 ± 2.2	16.3 ± 2.7	0.09 ± 1.26				
PRO + n-3 PUFA	17.6 ± 4.0	18.1 ± 4.0	0.45 ± 0.81				
FFM, kg				0.47	0.99	0.94	0.60
CON	42.8 ± 8.4	42.6 ± 9.0	-0.17 ± 0.93				
PRO	42.5 ± 7.0	43.1 ± 7.9	0.37 ± 0.51				
n-3 PUFA	41.6 ± 5.5	42.2 ± 6.5	0.61 ± 1.40				
PRO + PLA	41.1 ± 6.3	40.7 ± 6.5	-0.38 ± 1.16				
PRO + n-3 PUFA	43.6 ± 8.3	43.8 ± 8.3	0.16 ± 1.37				
Total FM, kg				0.80	0.89	0.57	0.45
CON	28.4 ±8.8	29.7 ± 9.6	1.32 ± 1.31				
PRO	30.7 ± 11.4	29.5 ± 12.6	-0.75 ± 1.67				
n-3 PUFA	34.5 ±15.9	33.7 ± 14.7	-0.78 ± 2.67				
PRO + PLA	28.6 ± 15.1	28.6 ±14.9	-0.01 ± 1.57				
PRO + n-3 PUFA	29.1 ±16.3	29.5 ± 15.3	0.42 ± 2.09				
Body fat, %				0.80	0.84	0.61	0.21
CON	41.1 ± 9.5	42.3 ± 9.4	0.48 ± 1.74				
PRO	42.1 ± 6.2	40.5 ± 6.2	-0.39 ± 1.30				
n-3 PUFA	44.8 ± 8.3	44.3 ± 7.4	-0.50 ± 2.08				
PRO + PLA	40.4 ± 10.0	40.6 ± 9.2	0.17 ± 2.21				
PRO + n-3 PUFA	39. 2 ± 10.0	39.5 ± 9.6	0.26 ± 1.72				

Values are mean ± SD. There were no significant differences between groups at baseline test by one-way ANOVA. Waist-to-hip ratio, WHR; lean body mass, LBM; appendicular lean mass, ALM; fat-free mass, FFM; fat mass, FM; bone mineral density, BMD; handgrip strength, HGS. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=7; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean

oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$.

[3]Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age as a covariate. *P*-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different between groups within time point following LS-means; $ significantly different compared to baseline follow LS-means

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Table 4. Effects of a 16-week supplementation intervention on anthropometrics, body composition and handgrip strength in dietary intervention and control groups[1] (Cont.)

	Weeks of intervention		Treatment effect[2]		ANCOVA *P*[3]		
Body Composition	0	16	Δ 16 wk	P	Group	Time[2]	Group X time
Android fat, %				0.06	0.41	0.94	0.07
CON	43.5 ± 11.9	46.3 ± 10.6	2.78 ± 1.82				
PRO	44.5 ± 9.7	40.3 ± 9.7	-2.47 ± 2.24				
n-3 PUFA	49.0 ± 9.0	48.8 ± 8.3	-0.24 ± 3.15				
PRO + PLA	43.8 ± 12.0	44.1 ± 10.7	0.24 ± 4.15				
PRO + n-3 PUFA	38.8 ± 14.2	38.7 ± 14.2	-0.10 ± 3.03				
Gynoid fat, %				0.42	0.75	0.67	0.44
CON	46.7 ± 9.1	47.4 ± 7.8	0.75 ± 3.79				
PRO	49.7 ± 4.1	47.9 ± 5.7	-1.94 ± 1.65				
n-3 PUFA	49.7 ± 7.7	50.3 ± 5.6	0.57 ± 3.10				
PRO + PLA	47.5 ± 7.9	46.8 ± 8.0	-0.66 ± 3.55				
PRO + n-3 PUFA	46.4 ± 6.7	46.5 ± 6.7	0.08 ± 2.35				
Fat-to-lean ratio				0.14	0.68	0.83	0.22
CON	0.73 ± 0.3	0.77 ± 0.3	0.04 ± 0.02				
PRO	0.75 ± 0.2	0.70 ± 0.2	-0.03 ± 0.05				
n-3 PUFA	0. 85 ± 0.3	0.82 ± 0.2	-0.03 ± 0.07				
PRO + PLA	0.72 ± 0.3	0.72 ± 0.3	0.00 ± 0.05				
PRO + n-3 PUFA	0.68 ±0.3	0.69 ± 0.3	0.01 ± 0.05				
BMD, g/cm^2				0.79	0.28	0.61	0.67
CON	1.17 ± 0.07	1.19 ± 0.08	0.02 ± 0.03				
PRO	1.09 ± 0.08	1.09 ± 0.11	-0.01 ± 0.02				
n-3 PUFA	1.11 ± 0.10	1.12 ± 0.10	0.01 ± 0.02				
PRO + PLA	1.12 ± 0.11	1.13 ± 0.11	0.01 ± 0.03				
PRO + n-3 PUFA	1.07 ± 0.10	1.07 ± 0.10	-0.01 ± 0.09				

[1] Values are mean ± SD. There were no significant differences between groups at baseline test by one-way ANOVA. Waist-to-hip ratio, WHR; lean body mass, LBM; appendicular lean mass, ALM; fat-free mass, FFM; fat mass, FM; bone mineral density, BMD; handgrip strength, HGS. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=7; omega-3

polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$.

[3]Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age as a covariate. *P*-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different between groups within time point following LS-means; $ significantly different compared to baseline follow LS-mean

Table 4. Effects of a 16-week supplementation intervention on anthropometrics, body composition and handgrip strength in dietary intervention and control groups[1] (Cont.).

	Weeks of intervention		Treatment effect[2]		ANCOVA *P*[3]		
Body Composition	0	16	Δ 16 wk	P	Group	Time[2]	Group X time
High HGS, kg				0.08	0.65	0.14	0.59
CON	28.2 ± 8.1	27.9 ± 7.0	-0.3 ± 2.1				
PRO	27.4 ± 3.8	27.5 ± 3.1	-1.5 ± 2.3				
n-3 PUFA	25.7 ± 4.2	25.8 ± 4.3	0.1 ± 3.3				
PRO + PLA	28.1 ± 4.9	30.2 ± 4.6	2.1 ± 2.5				
PRO + n-3 PUFA	25.8 ± 4.6	27.0 ± 4.6	1.2 ± 2.5				

[1] Values are mean ± SD. There were no significant differences between groups at baseline test by one-way ANOVA. Waist-to-hip ratio, WHR; lean body mass, LBM; appendicular lean mass, ALM; fat-free mass, FFM; fat mass, FM; bone mineral density, BMD; handgrip strength, HGS. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=7; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$.

[3]Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age as a covariate. *P*-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different between groups within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 5. Effects of a 16-week supplementation intervention on energy expenditure and substrate oxidation controlled for FFM in dietary intervention and control groups[1]

	Weeks of Intervention					Treatment effect[2]	ANCOVA P [3]			
REE and SO	0	4	8	12	16	Δ 16 wk	*P*	Group	Time	Group X time
REE, Kcal/min							0.81	0.76	0.18	0.40
CON	0.022 ± 0.002	0.024 ± 0.005	0.021 ± 0.004	0.023 ± 0.003	0.022 ± 0.003	0.002 ± 0.003				
PRO	0.024 ± 0.003	0.023 ± 0.003	0.024 ± 0.003	0.024 ± 0.002	0.024 ± 0.002	0.000 ± 0.004				
n-3 PUFA	0.023 ± 0.002	0.023 ± 0.002	0.023 ± 0.002	0.023 ± 0.003	0.024 ± 0.003	0.001 ± 0.002				
PRO + PLA	0.022 ± 0.002	0.023 ± 0.003	0.023 ± 0.002	0.022 ± 0.004	0.023 ± 0.002	0.001 ± 0.001				
PRO + n-3 PUFA	0.022 ± 0.002	0.022 ± 0.002	0.022 ± 0.002	0.022 ± 0.002	0.022 ± 0.003	0.001 ± 0.002				
KFAT, Kcal/min							0.06	0.09	0.21	0.03*
CON	0.012 ± 0.004	0.014 ± 0.002	0.010 ± 0.004	0.010 ± 0.004	0.012 ± 0.009	0.001 ± 0.007				
PRO	0.019 ± 0.004	0.016 ± 0.005	0.016 ± 0.007[#]	0.016 ± 0.004[#]	0.012 ± 0.009[$]	-0.006 ± 0.007*				
n-3 PUFA	0.015 ± 0.003	0.013 ± 0.004	0.014 ± 0.006[#]	0.017 ± 0.005[#]	0.020 ± 0.008[$#]	0.005 ± 0.007				
PRO + PLA	0.016 ± 0.001	0.013 ± 0.008	0.017 ± 0.004[#]	0.013 ± 0.006[#]	0.019 ± 0.005	0.002 ± 0.006				
PRO + n-3 PUFA	0.014 ± 0.005	0.014 ± 0.003	0.017 ± 0.003[#]	0.016 ± 0.002[#]	0.018 ± 0.005[$]	0.004 ± 0.009				

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9; rate of fat oxidation (kilocalories per minute), KFAT; rate of carbohydrate oxidation (kilocalories per minute), KCHO.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$

[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. P-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 5. Effects of a 16-week supplementation intervention on energy expenditure and substrate oxidation controlled for FFM in the dietary intervention and control groups[1] (Cont.)

	Weeks of Intervention					Treatment effect[2]		ANCOVA P [3]		
REE and SO	0	4	8	12	16	Δ 16 wk	*P*	Group	Time	Group X time
KCHO, kcal/min							0.22	0.04*	0.28	.50
CON	0.010 ± 0.004	0.010 ± 0.005	0.011 ± 0.005	0.013 ± 0.003	0.010 ± 0.007	-0.004 ±0.004				
PRO	0.005 ± 0.003	0.007 ± 0.004	0.008 ± 0.006	0.008 ± 0.005	0.009 ± 0.009	0.004 ± 0.009				
n-3 PUFA	0.008 ± 0.003	0.010 ± 0.003	0.009 ± 0.004	0.006 ± 0.003	0.004 ± 0.006	-0.004 ± 0.006				
PRO + PLA	0.006 ± 0.002	0.009 ± 0.008	0.006 ± 0.004	0.009 ± 0.003	0.005 ± 0.005	-0.001 ± 0.004				
PRO + n-3 PUFA	0.008 ± 0.005	0.008 ± 0.003	0.005 ± 0.002	0.006 ± 0.003	0.004 ± 0.003	-0.003 ± 0.007				

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9; rate of fat oxidation (kilocalories per minute), KFAT; rate of carbohydrate oxidation (kilocalories per minute), KCHO.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$

[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. P-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 6. Effects of the 16-week dietary supplementation intervention on objective sleep duration and quality in the dietary intervention and control group[1]

	Weeks of intervention			Treatment effect[2]		ANCOVA *P*[3]		
	0	8	16	Δ 16 wk	*P*	Group	Time	Group X time
7-day ActiGraph								
Time in bed					0.03*	0.21	0.21	0.047*
CON	23.1 ± 1.3	22.6 ± 1.4[$]	22.8 ± 1.3	-0.25 ± 0.43				
PRO	22.6 ± 0.8	22.8 ± 1.1	23.1 ± 1.0[$]	0.54 ± 0.65*				
n-3 PUFA	23.2 ± 0.7	23.0 ± 0.4	23.3 ± 0.8	0.14 ± 0.61*				
PRO + PLA	23.0 ± 0.7	23.1 ± 1.0	22.9 ± 0.9	-0.15 ± 0.30				
PRO + n-3 PUFA	22.7 ± 1.0	22.2 ± 0.7[$]	22.0 ± 0.8[$]	-0.70 ± 1.04				
Time out of bed					0.18	0.74	0.31	0.21
CON	6.8 ± 1.2	5.7 ± 2.2	6.2 ± 1.0	-0.57 ± 0.51				
PRO	6.0 ± 1.2	6.2 ± 1.0	6.1 ± 1.1	0.12 ± 0.51				
n-3 PUFA	6.4 ± 1.1	6.4 ± 1.2	6.7 ± 1.4	0.30 ± 0.93				
PRO + PLA	6.8 ± 0.8	6.8 ± 0.7	6.8 ± 1.0	-0.11 ± 0.45				
PRO + n-3 PUFA	6.2 ± 1.4	6.0 ± 1.3	6.0 ± 1.4	-0.21 ± 0.31				
Sleep latency, min					0.95	< 0.01*	0.42	0.33
CON	3.9 ± 3.5	3.7 ± 1.6	3.5 ± 2.0	-0.41 ± 4.78				
PRO	2.0 ± 1.2	2.4 ± 1.3	2.9 ± 2.1	0.92 ± 2.97				
n-3 PUFA	8.5 ± 6.9[$]	4.2 ± 3.7	9.0 ± 11.5	0.46 ± 13.14				
PRO + PLA	3.9 ± 2.1	3.8 ± 2.2	5.9 ± 1.6	2.14 ± 2.96				
PRO + n-3 PUFA	4.4 ± 1.8	6.3 ± 3.3	4.1 ± 3.3	-0.39 ± 3.27				
Sleep efficiency, %					0.31	0.46	0.98	0.41
` CON	85.8 ± 4.5	87.6 ± 4.2	88.7 ± 3.4	2.91 ± 2.89				
PRO	92.6 ± 1.1	91.5 ± 3.7	92.0 ± 3.1	-0.54 ± 2.96				
n-3 PUFA	88.7.1 ± 4.4	86.8 ± 4.4	87.7 ± 5.8	-0.95 ± 6.82				
PRO + PLA	87.0 ± 4.2	88.9 ± 2.3	88.3 ± 1.7	1.23 ± 3.61				
PRO + n-3 PUFA	86.0 ± 5.2	84.8 ± 5.9	87.0 ± 5.8	0.97 ± 2.13				

[1] All baseline, 8, and 16-week values are means ± SD. Control, no intervention and free living, CON, n=5; whey protein isolate, PRO, n=7; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=8. "Time in bed" denotes time from "lights out" to "got up" as indicated by participants in their sleep diary. Time is expressed as hours followed by the proportion of an hour in minutes. "Sleep period" denotes time from "fell

asleep" to "woke up"; "Sleep duration" denotes time spent asleep within sleep period, excluding wake time; WASO denotes time from sleeping to first period of wakefulness; "Sleep latency" denotes time from "lights out" to "fell asleep"; "Sleep duration" (%) denotes the proportion of time spent asleep in the sleep period; "Sleep efficiency" (%) denotes the proportion of time spent asleep of time in bed (100% × sleep duration/the time between bed time and get up time). "Sleep fragmentation index" denotes the number of interruptions of sleep by physical movement calculated as 100 × the number of groups of consecutive mobile 60-s epochs/by the total number of immobile epochs. H:mm, hours: minutes; TST, total sleep time; WASO, wake after sleep onset; SFI, sleep fragmentation index.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$

[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. P-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 6. Effects of the 16-week dietary supplementation intervention on objective sleep duration and quality in the dietary intervention and control group[1] (Cont.)

	Weeks of intervention			Treatment effect[2]		ANCOVA *P*[3]		
	0	8	16	Δ 16 wk	*P*	Group	Time	Group X time
7-day ActiGraph								
TST, min					0.22	0.46	0.98	0.42
CON	395.3 ± 38.1	405.0 ± 25.6	392.3 ± 38.3	-2.94 ± 39.73				
PRO	414.6 ± 47.4	377.9 ± 97.8	388.1 ± 46.2	-26.47 ± 19.92				
n-3 PUFA	381.3 ± 53.9	389.5 ± 57.6	390.3 ± 53.6	9.01 ± 37.70				
PRO + PLA	400.6 ± 35.5	424.4 ± 75.3	409.8 ± 59.6	4.06 ± 36.16				
PRO + n-3 PUFA	384.1 ± 45.2	386.9 ± 56.2	408.6 ± 63.1	24.47 ± 56.30				
Awakenings, #					0.24	0.02*	0.87	0.32
CON	15.9 ± 4.3	15.3 ± 6.1	14.0 ± 4.1	-1.92 ± 2.13				
PRO	10.5 ± 2.8	8.9 ± 2.2	9.4 ± 2.6	-1.15 ± 1.58				
n-3 PUFA	12.6 ± 6.0	16.1 ± 6.0	13.5 ± 6.1	0.89 ± 3.77				
PRO + PLA	16.0 ± 4.1	16.9 ± 2.5	17.0 ± 5.2	0.35 ± 3.77				
PRO + n-3 PUFA	13.4 ± 6.0	14.6 ± 7.6	14.9 ± 7.9	1.54 ± 2.81				
WASO					0.09	0.05	0.76	0.42
CON	61.6 ± 14.7	54.0 ± 19.5	45.7 ± 15.8	-15.88 ± 7.47				
PRO	31.9 ± 4.0	32.4 ± 16.4	31.0 ± 11.6	-0.93 ± 13.22				
n-3 PUFA	40.9 ± 18.8	56.8 ± 20.6	49.7 ± 26.3	8.75 ± 24.01				
PRO + PLA	58.5 ± 25.5	49.7 ± 16.6	48.0 ± 12.9	-10.35 ± 19.53				
PRO + n-3 PUFA	58.1 ± 23.3	62.8 ± 31.2	59.3 ± 34.5	1.23 ± 18.64				
SFI					0.10	0.03*	0.96	0.12
CON	27.3 ± 4.7	24.2 ± 4.1	22.2 ± 7.4	-5.06 ± 4.72				
PRO	16.6 ± 4.4 [$]	19.5 ± 6.6	19.7 ± 6.7	3.01 ± 3.57				
n-3 PUFA	23.6 ± 6.7	25.5 ± 6.2	27.7 ± 10.4	4.18 ± 11.69				
PRO + PLA	27.9 ± 7.3	26.0 ± 4.8	25.5 ± 5.3	-2.48 ± 4.69				
PRO + n-3 PUFA	24.2 ± 7.9	26.0 ± 7.1	22.0 ± 8.4	-2.23 ± 6.79				

[1] All baseline, 8, and 16-week values are means ± SD. Control, no intervention and free living, CON, n=5; whey protein isolate, PRO, n=7; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=8. "Time in bed" denotes time from "lights out" to "got up" as indicated by participants in their sleep diary. Time is expressed as hours followed by the proportion of an hour in minutes. "Sleep period" denotes time from "fell asleep" to "woke up"; "Sleep duration" denotes time spent asleep within sleep period, excluding wake time; WASO denotes time from

sleeping to first period of wakefulness; "Sleep latency" denotes time from "lights out" to "fell asleep"; "Sleep duration" (%) denotes the proportion of time spent asleep in the sleep period; "Sleep efficiency" (%) denotes the proportion of time spent asleep of time in bed (100% × sleep duration/the time between bed time and get up time). "Sleep fragmentation index" denotes the number of interruptions of sleep by physical movement calculated as 100 × the number of groups of consecutive mobile 60-s epochs/by the total number of immobile epochs. H:mm, hours: minutes; TST, total sleep time; WASO, wake after sleep onset; SFI, sleep fragmentation index.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$

[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. P-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means

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Table 7. Mean ± SD are presented from the 16-week supplementation intervention on ratings of subjective sleep quality and duration via the Pittsburg Sleep Quality Index seven subcomponents and GSS in dietary intervention and control groups[1].

	Weeks of Intervention					Treatment effect
PSQI	0	4	8	12	16	Δ 16 wk
Component 1: Sleep Quality						
CON	1.5 ± 0.5	0.8 ± 0.4	1.5 ± 0.8	1.3 ± 0.5	1.0 ± 0.6	-0.5 ± 0.1
PRO	1.3 ± 0.5	0.9 ± 0.4	1.1 ± 0.7	0.9 ± 0.9	0.7 ± 0.8	-0.6 ± 0.3
n-3 PUFA	1.3 ± 0.5	1.2 ± 0.8	0.9 ± 0.3	0.9 ± 0.7	0.8 ± 0.4	-0.5 ± -0.1
PRO + PLA	1.1 ± 0.7	1.0 ± 0.6	0.9 ± 0.4	0.9 ± 0.7	0.9 ± 0.4	-0.3 ± -0.3
PRO + n-3 PUFA	1.4 ± 0.5	0.9 ± 0.6	1.3 ± 0.5	0.9 ± 0.6	0.9 ± 0.6	-0.6 ± 0.1
Component 2: Latency						
CON	1.2 ± 0.8	1.2 ± 0.8	1.0 ± 1.1	1.5 ± 1.0	1.5 ± 0.5	0.3 ± -0.2
PRO	1.1 ± 0.9	1.1 ± 0.9	1.1 ± 0.9	1.1 ± 1.1	0.9 ± 1.1	-0.3 ± 0.2
n-3 PUFA	1.4 ± 1.3	1.2 ± 1.1	0.9 ± 0.9	1.2 ± 0.6	1.2 ± 0.9	-0.2 ± -0.3
PRO + PLA	1.3 ± 0.8	1.4 ± 1.0	1.1 ± 1.1	1.1 ± 0.7	1.1 ± 0.9	-0.1 ± 0.1
PRO + n-3 PUFA	1.4 ± 0.7	1.2 ± 0.7	1.1 ± 0.6	1.3 ± 0.9	1.3 ± 1.0	-0.1 ± 0.3
Component 3: Duration						
CON	1.3 ± 0.5	1.0 ± 0.6	1.5 ± 0.5	1.2 ± 0.4	1.2 ± 0.8	-0.2 ± 0.2
PRO	0.9 ± 0.7	0.7 ± 0.5	0.7 ± 0.8	0.6 ± 0.5	0.9 ± 0.7	0.0 ± 0.0
n-3 PUFA	1.6 ± 0.8	1.3 ± 0.9	1.3 ± 0.9	1.2 ± 0.9	1.2 ± 0.9	-0.4 ± 0.1
PRO + PLA	0.9 ± 0.9	1.0 ± 0.8	0.9 ± 0.7	0.6 ± 0.5	0.7 ± 0.5	-0.1 ± -0.4
PRO + n-3 PUFA	1.3 ± 1.1	1.1 ± 0.8	0.9 ± 0.8	0.8 ± 0.8	0.7 ± 0.7	-0.7 ± -0.4
Component 4: Sleep Efficiency						
CON	0.8 ± 0.4	0.3 ± 0.5	0.7 ± 0.8	0.2 ± 0.4	0.2 ± 0.4	-0.7 ± 0.0
PRO	0.6 ± 0.5	0.3 ± 0.5	0.1 ± 0.4	0.7 ± 1.0	0.4 ± 0.5	-0.1 ± 0.0
n-3 PUFA	0.6 ± 0.8	0.5 ± 0.8	0.6 ± 0.7	0.5 ± 0.8	0.5 ± 1.1	-0.1 ± 0.2
PRO + PLA	0.7 ± 1.1	0.6 ± 0.8	0.3 ± 0.5	0.3 ± 0.5	0.3 ± 0.5	-0.4 ± -0.6
PRO + n-3 PUFA	1.1 ± 0.9	0.7 ± 0.9	1.0 ± 1.0	0.6 ± 0.9	0.6 ± 0.5	-0.6 ± -0.4

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean

oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9. Pittsburgh sleep quality index, PSQI; global sleeping score . GSS.

Table 7. Mean ± SD are presented from the 16-week supplementation intervention on ratings of subjective sleep quality and duration via the Pittsburg Sleep Quality Index seven subcomponents and GSS in dietary intervention and control groups[1] (Cont.)

	Weeks of Intervention					Treatment effect
PSQI	0	4	8	12	16	Δ 16 wk
Component 5: Disturbances						
CON	1.5 ± 0.8	1.2 ± 0.8	1.3 ± 0.5	1.3 ± 0.5	1.3 ± 0.5	-0.2 ± -0.3
PRO	1.6 ± 0.5	1.4 ± 0.5	1.3 ± 0.8	1.1 ± 0.7	1.0 ± 0.6	-0.6 ± 0.0
n-3 PUFA	1.7 ± 0.5	1.5 ± 0.5	1.3 ± 0.5	1.1 ± 0.3	1.3 ± 0.5	-0.4 ± 0.0
PRO + PLA	1.6 ± 0.5	1.3 ± 0.5	1.3 ± 0.5	1.1 ± 0.4	1.3 ± 0.5	-0.3 ± 0.0
PRO + n-3 PUFA	1.8 ± 0.4	1.4 ± 0.5	1.6 ± 0.5	1.6 ± 0.5	1.6 ± 0.5	-0.2 ± 0.1
Component 6: Medications						
CON	1.2 ± 1.2	1.2 ± 1.2	1.3 ± 1.0	1.2 ± 1.2	0.8 ± 1.0	-0.3 ± -0.2
PRO	0.3 ± 0.5	0.1 ± 0.4	0.7 ± 1.3	0.3 ± 0.8	0.1 ± 0.4	-0.1 ± -0.1
n-3 PUFA	0.4 ± 1.0	03 ± 0.9	0.3 ± 0.9	0.2 ± 0.6	0.4 ± 1.0	0.0 ± 0.0
PRO + PLA	1.0 ± 1.2	1.1 ± 1.2	0.6 ± 0.8	0.9 ± 0.9	1.0 ± 1.2	0.0 ± 0.0
PRO + n-3 PUFA	1.0 ± 1.3	0.8 ± 1.3	0.8 ± 1.3	0.8 ± 1.1	0.8 ± 1.3	-0.2 ± 0.0
Component 7: Daytime Dysfunction						
CON	0.8 ± 0.8	1.2 ± 1.0	1.0 ± 1.1	1.0 ± 1.1	0.8 ± 1.0	0.0 ± 0.2
PRO	1.0 ± 0.8	0.7 ± 0.5	0.6 ± 0.5	0.7 ± 0.8	0.6 ± 0.5	-0.4 ± -0.3
n-3 PUFA	0.8 ± 0.6	1.1 ± 0.9	1.1 ± 0.7	0.9 ± 0.6	1.1 ± 0.6	0.3 ± -0.1
PRO + PLA	0.9 ± 0.4	1.0 ± 0.0	0.9 ± 0.4	0.9 ± 0.7	1.0 ± 0.0	0.1 ± -0.4
PRO + n-3 PUFA	0.8 ± 0.4	0.8 ± 0.4	0.9 ± 0.6	0.9 ± 0.6	0.9 ± 0.6	0.1 ± 0.2
Compiled GSS						
CON	8.3 ± 3.0	6.8 ± 2.9	8.3 ± 3.6	7.7 ± 3.5	6.8 ± 3.2	-1.5 ± 0.2
PRO	6.7 ± 1.6	5.3 ± 1.8	5.7 ± 3.7	5.4 ± 4.1	4.6 ± 2.2	-2.1 ± 0.6
n-3 PUFA	7.8 ± 2.7	7.1 ± 4.5	6.4 ± 3.2	6.0 ± 2.4	6.5 ± 2.8	-1.3 ± 0.1
PRO + PLA	7.7 ± 2.5	7.4 ± 2.5	5.9 ± 2.5	5.7 ± 1.4	6.3 ± 2.1	-1.4 ± -0.4
PRO + n-3 PUFA	8.9 ± 2.8	6.9 ± 2.7	7.6 ± 3.2	6.8 ± 3.3	6.7 ± 3.6	-2.2 ± 0.8

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9. Pittsburgh sleep quality index, PSQI; global sleeping score . GSS.

Table 8. Effects of the 16-week dietary supplementation interventions on negative and positive affect states in the dietary intervention and control groups[1].

	Weeks of Intervention					Treatment effect[2]		ANCOVA P [3]		
POMS	0	4	8	12	16	Δ 16 wk	*P*	Group	Time	Group X time
Tension/Anxiety							0.83	0.58	0.28	0.79
CON	4.0 ± 3.3	5.8 ± 7.8	3.7 ± 3.9	4.8 ± 4.4	4.0 ± 3.8	0.0 ± 1.9				
PRO	8.7 ± 6.9	5.6 ± 3.8	7.0 ± 6.4	8.0 ± 5.5	6.4 ± 5.5	-2.3 ± 6.5				
n-3 PUFA	6.7 ± 5.0	3.8 ± 4.0	5.1 ± 4.3	4.0 ± 3.9	6.3 ± 7.0	-0.4 ± 7.6				
PRO + PLA	6.9 ± 3.4	4.9 ± 2.4	6.3 ± 4.5	5.7 ± 4.2	6.3 ± 3.1	-0.6 ± 2.6				
PRO + n-3 PUFA	8.0 ± 6.9	6.0 ± 3.1	7.4 ± 5.5	6.9 ± 3.8	5.8 ± 3.8	-2.2 ± 5.6				
Depression							0.87	0.20	0.84	0.50
CON	3.0 ± 3.0	7.7 ± 13.6	2.7 ± 2.7	5.8 ± 5.8	2.3 ± 2.4	-0.7 ± 0.8				
PRO	7.4 ± 8.3	5.7 ± 5.3	6.7 ± 9.8	7.3 ± 8.0	8.1 ± 8.4	0.7 ± 7.3				
n-3 PUFA	4.1 ± 4.9	2.7 ± 2.7	3.1 ± 3.6	2.7 ± 2.9	2.3 ± 3.4	-1.8 ± 3.6				
PRO + PLA	4.1 ± 4.3	3.4 ± 3.0	2.3 ± 2.2	2.7 ± 1.9	4.6 ± 3.2	0.4 ± 4.4				
PRO + n-3 PUFA	6.7 ± 12.4	3.7 ± 4.0	5.9 ± 4.9	7.0 ± 6.5	6.6 ± 5.2	-0.1 ± 12.1				
Anger							0.89	0.65	0.56	0.12
CON	3.8 ± 5.5	6.7 ± 7.6	1.5 ± 2.5	4.8 ± 6.5	3.0 ± 4.0	-0.8 ± 5.3				
PRO	5.0 ± 3.4	3.0 ± 3.5	5.9 ± 10.4	5.3 ± 8.2	4.4 ± 7.9	-0.6 ± 6.7				
n-3 PUFA	4.1 ± 5.1	3.2 ± 3.4	3.7 ± 4.6	1.8 ± 3.4	1.7 ± 2.1	-2.4 ± 4.5				
PRO + PLA	3.0 ± 1.6	1.9 ± 1.3	1.1 ± 1.3	2.6 ± 2.9	2.7 ± 2.1	-0.3 ± 2.0				
PRO + n-3 PUFA	4.7 ± 5.7	2.6 ± 2.4	5.2 ± 4.3	4.8 ± 6.7	3.1 ± 3.6	-1.6 ± 5.2				
Fatigue							0.42	0.43	0.14	0.41
CON	6.5 ± 8.8	9.3 ± 12.3	6.7 ± 6.1	11.7 ± 8.0	8.5 ± 8.1	2.0 ± 4.6				
PRO	9.1 ± 7.2	7.4 ± 4.9	8.0 ± 6.1	6.4 ± 5.5	4.9 ± 4.5	-4.3 ± 4.6				
n-3 PUFA	5.9 ± 5.0	7.1 ± 5.5	5.7 ± 3.8	4.0 ± 3.6	3.9 ± 3.9	-2.0 ± 5.3				
PRO + PLA	5.6 ± 4.4	6.7 ± 5.0	5.7 ± 4.2	6.6 ± 3.2	5.0 ± 2.9	-0.6 ± 4.4				
PRO + n-3 PUFA	7.8 ± 3.8	9.1 ± 5.9	8.1 ± 5.9	6.0 ± 5.7	6.2 ± 2.2	-1.6 ± 4.7				

[1]Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid,

PRO + n-3 PUFA, n=9. POMS: TMD = (Sum of all subscales except Vigor) minus Vigor, TMD score range (−32) to 200. All subscales are positive.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$

[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. P-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 8. Effects of the 16-week dietary supplementation interventions on negative and positive affect states in the dietary intervention and control groups[1] (Cont.)

	Weeks of Intervention					Treatment effect[2]	ANCOVA P [3]			
POMS	0	4	8	12	16	Δ 16 wk	*P*	Group	Time	Group X time
CON	5.3 ± 3.0	5.8 ± 4.6	5.2 ± 3.8	7.5 ± 6.6	5.7 ± 4.3	0.3 ± 2.0				
PRO	6.6 ± 4.5	4.7 ± 3.8	6.1 ± 4.1	5.0 ± 4.5	3.6 ± 3.9	-3.0 ± 3.7				
n-3 PUFA	5.0 ± 2.7	4.4 ± 3.2	5.3 ± 3.2	4.2 ± 2.6	5.6 ± 4.2	0.6 ± 4.1				
PRO + PLA	4.4 ± 1.7	5.7 ± 2.0	4.1 ± 1.3	5.1 ± 1.5	4.3 ± 2.1	-0.1 ± 2.5				
PRO + n-3 PUFA	5.7 ± 3.2	5.2 ± 2.8	5.6 ± 4.1	4.8 ± 3.4	5.9 ± 4.3	0.2 ± 3.9				
Vigor							0.17	0.01	0.21	0.046
CON	16.3 ± 2.3	17.2 ± 5.5	15.8 ± 5.5	11 ± 7.4	12.3 ± 6.4	-4.0 ± 5.9				
PRO	18.6 ± 6.9	22.0 ± 5.5	23.0 ± 7.3	19.7 ± 9.5	20.6 ± 9.1*	2.0 ± 6.5				
n-3 PUFA	17.4 ± 6.1	18.2 ± 6.3	17.7 ± 7.7	18.9 ± 8.3	18.1 ± 8.0*	0.7 ± 4.7				
PRO + PLA	17.6 ± 5.3	15.6 ± 6.4	18.0 ± 5.1	15.7 ± 5.3	16.4 ± 6.2	-1.1 ± 4.9				
PRO + n-3 PUFA	15.8 ± 6.3	16.3 ± 5.0	15.2 ± 7.1	16.9 ± 4.7	16.3 ± 5.0	0.6 ± 4.3				
TMD							0.71	0.71	0.73	0.11
CON	6.3 ± 19.5	18.2 ± 45.5	3.8 ± 18.1	23.7 ± 33.4	11.2 ± 23.4	4.8 ± 15.6				
PRO	18.3 ± 33.0	4.4 ± 22.4	10.7 ± 37.8	12.3 ± 34.4	6.9 ± 32.6	-11.4 ± 26.1				
n-3 PUFA	8.4 ± 20.7	3.0 ± 19.4	5.2 ± 21.1	-2.2 ± 18.4	1.7 ± 20.2	-6.7 ± 18.2				
PRO + PLA	-0.4 ± 9.1	7.0 ± 9.1	1.6 ± 12.4	7.0 ± 11.5	6.4 ± 10.8	6.9 ± 12.0				
PRO + n-3 PUFA	17.0 ± 31.7	10.2 ± 16.2	17.0 ± 17.4	12.6 ± 15.6	11.2 ± 19.4	-5.8 ± 29.0				

[1]Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9. POMS: TMD = (Sum of all subscales except Vigor) minus Vigor, TMD score range (−32) to 200. All subscales are positive.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$

[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. P-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 9. Effects of the 16-week dietary interventions on fasting plasma concentrations of cardiometabolic risk in the dietary intervention groups and control group[1]

	Weeks of Intervention			Treatment effect[2]	ANCOVA *P*[3]			
Metabolic Biomarkers	0	8	16	Δ 16 wk	*P*	Group[1]	Time[2]	Group X Time[3]
Glucose. mg/dl					0.78	0.34	0.22	0.72
CON	90.1 ± 15.9	98.4 ± 17.1	91.5 ± 14.9	1.5 ± 6.4				
PRO	92.6 ± 11.0	91.7 ± 6.9	88.2 ± 6.6	-4.4 ± 8.6				
n-3 PUFA	93.3 ± 13.2	93.4 ± 12.4	92.0 ± 11.8	-1.3 ± 13.5				
PRO + PLA	88.8 ± 9.6	88.1 ± 12.4	89.9 ± 7.8	1.1 ± 4.8				
PRO + n-3 PUFA	83.7 ± 12.6	86.6 ± 13.4	81.8 ± 5.5	-1.9 ± 9.0				
Insulin, uUI/mL					0.89	0.38	0.07	0.32
CON	10.6 ± 4.1	12.8 ± 9.2	7.5 ± 3.5	-3.2 ± 4.6				
PRO	13.8 ± 9.3	11.9 ± 6.9	11.7 ± 10.4	-2.0 ± 8.0				
n-3 PUFA	22.0 ± 27.1	16.2 ± 13.1	22.4 ± 21.3	0.4 ± 9.5				
PRO + PLA	10.6 ± 8.8	12.8 ± 8.9	10.1 6.4	-0.5 ± 4.3				
PRO + n-3 PUFA	9.6 ± 5.8	11.2 ± 4.7	8.8 ± 4.8	-0.8 ± 6.5				
HOMA-IR, AU					0.98	0.32	0.09	0.54
CON	2.4 ± 1.0	3.3 ± 2.6	1.7 ±1.0	-0.6 ± 0.9				
PRO	3.3 ± 2.5	2.7 ± 1.6	2.6 ± 2.4	-0.7 ± 1.9				
n-3 PUFA	5.2 ± 6.1	4.0 ± 3.7	5.0 ± 4.2	-0.2 ± 3.2				
PRO + PLA	2.5 ± 2.4	3.0 ± 2.5	2.3 ± 1.6	-0.2 ± 1.1				
PRO + n-3 PUFA	2.1 ± 1.6	2.3 ± 1.6	1.8 ± 1.0	-0.3 ± 1.5				
FFA, uM					0.12	0.48	0.02*	0.37
CON	131.5 ± 24.7	144.2 ± 47.2	161.3 ± 42.2	29.7 ± 22.5				
PRO	186.5 ± 36.8	154.9 ± 33.4	147.0 ± 33.4	-39.5 ± 25.1				
n-3 PUFA	187.8 ± 54.2	164.5 ± 68.0	175.4 ± 48.9	-12.4 ± 38.2				
PRO + PLA	175.8 ± 48.2	135.7 ± 54.3	156.8 ± 60.6	-19.1 ± 63.4				
PRO + n-3 PUFA	162.9 ± 71.9	126.1 ± 50.7	137.0 ± 47.0	-26.0 ± 63.0				

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10 *Wk8:n=9; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9. Homeostatic Model Assessment of Insulin Resistance, HOMA-IR; free-fatty acids, FFA; c-reactive protein. CRP.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$.
[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. *P*-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 9. Effects of the 16-week dietary interventions on fasting plasma concentrations of cardiometabolic risk in the dietary intervention groups and control group[1] (Cont.)

	Weeks of Intervention			Treatment effect[2]	ANCOVA *P*[3]			
Metabolic Biomarkers	0	8	16	Δ 16 wk	*P*	Group[1]	Time[2]	Group X Time[3]
Cholesterol, mg/dl					0.01*	0.34	0.58	0.01*
CON	179.5 ± 33.0	200.5 ± 18.1	219.0 ± 24.5[$]	39.5 ± 30.4				
PRO	190.4 ± 30.4	200.0 ± 29.4	178.7 ± 22.9	-11.8 ± 30.8*				
n-3 PUFA	197.5 ± 23.0	212.6 ± 20.9	186.0 ± 32.5	-11.4 ± 24.1*				
PRO + PLA	206.8 ± 19.6	194.4 ± 20.5	204.9 ± 24.7	-1.9 ± 24.8*				
PRO + n-3 PUFA	196.5 ± 28.4	178.0 ± 33.7[$]	171.4 ± 38.2[$]	-25. 1 ± 43.6*				
Triglycerides, mg/dl								
CON	78.1 ± 23.8	66.8 ± 24.6	66.6 ± 16.4	-11.5 ± 26.1	0.25	0.05	0.13	0.71
PRO	118.9 ± 46.2	110.0 ± 51.0	103.5 ± 31.2	-15.4 ± 32.7				
n-3 PUFA	105.8 ± 44.5	92.0 ± 36.0	89.5 ± 36.4	-16.4 ± 29.1				
PRO + PLA	80.4 ± 32.8	74.6 ± 22.2	89.8 ± 29.6	9.4 ± 42.5				
PRO + n-3 PUFA	102.3 ± 36.6	84.0 ± 35.4	76.8 ± 32.8	-25.5 ± 17.0				
CRP, u/L					0.48	0.88	0.99	0.24
CON	.72 ± .43	.70 ± .45	.85 ± .53	0.13 ± .42				
PRO	1.29 ± .60	1.11 ± .61	1.02 ± .72	-0.27 ± .90				
O3FA	1.07 ± .58	1.28 ± .65	1.00 ± .43	-0.07 ± .47				
PRO + PLA	.81 ± .62	.94 ± .61	1.01 ± .76	0.20 ± .33				
PRO + O3FA	1.02 ± 1.09	.82 ± .68	1.04 ± 1.14	0.01 ± .33				

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10 *Wk8:n=9; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9. Homeostatic Model Assessment of Insulin Resistance, HOMA-IR; free-fatty acids, FFA; c-reactive protein. CRP.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$.

[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. *P*-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 10. Effects of the 16-week dietary interventions on fasting plasma concentrations of well-being biomarkers the dietary intervention groups and control group[1]

	Weeks of Intervention			Treatment effect [2]		ANCOVA *P*[3]		
Biomarkers	0	8	16	Δ 16 wk	*P*	Group	Time	Group X time
OXA, pg/mL					0.046	0.39	0.08	0.27
CON	18.4 ± 10.5	17.8 ± 10.3	19.2 ± 10.7	0.8 ± 3.3				
PRO	20.5 ± 15.3	23.6 ± 13.2	19.2 ± 9.5	-1.3 ± 7.0				
n-3 PUFA	16.6 ± 13.6	15.5 ± 9.5	15.7 ± 11.2	-0.8 ± 5.9				
PRO + PLA	23.5 ± 15.7	24.6 ± 18.5	25.0 ± 16.8	1.5 ± 8.1				
PRO + n-3 PUFA	19.8 ± 11.8	25.0 ± 14.0	28.4 ± 17.5[$]	8.6 ± 9.3*				
BDNF, ng/m:					0.39	0.67	0.01*	0.53
CON	562.9 419.9	460.4 101.3	667.7 ± 306.9	104.7 ± 1819.8				
PRO	485.0 ± 148.3	449.8 ± 134.1	452.0 ± 100.7	-32.9 ± 106.0				
n-3 PUFA	528.0 ± 126.7	502.3 ± 142.4	621.1 ± 201.5	93.1 ± 173.6				
PRO + PLA	540.9 ± 241.3	528.0 ± 254.2	611.9 ± 342.1	71 ± 189.9				
PRO + n-3 PUFA	513.6 ± 200.1	400.0 ± 133.2	499.5 ± 179.6	-14.1 ± 1788				
CKM, U/mL					0.32	0.57	0.28	0.40
CON	30.6 ± 16.2	29.4 ± 7.9	33.5 ± 15.0	2.9 ± 11.1				
PRO	27.4 ± 11.6	34.5 ± 17.9	32.5 ± 18.0	5.1 ± 8.6				
n-3 PUFA	30.5 ± 8.6	31.1 ± 17.5	35.7 ± 15.7	5.1 ± 10.0				
PRO + PLA	40.8 ± 13.8	40.1 ± 20.0	37.6 ± 16.6	-3.2 ± 6.5				
PRO + n-3 PUFA	39.6 ± 17.4	40.7 ± 18.9	43.7 ± 20.0	4.2 ± 5.7				
Cortisol, ng/mL					0.47	0.47	0.02	0.16
CON	27.7 ± 18.8	22.0 ± 14.6	24.5 ± 22.2	-3.17 ± 12.57				
PRO	26.0 ± 17.8	26.0 ± 13.5	18.7 ± 13.4	-7.31 ± 15.33				
n-3 PUFA	14.4 ± 6.1	19.4 ± 7.8	15.1 ± 9.9	0.69 ± 6.30				
PRO + PLA	17.3 ± 6.0	16.5 ± 7.4	14.4 ± 4.0	-2.88 ± 4.85				
PRO + n-3 PUFA	15.9 ± 3.1	15.7 ± 3.1	15.2 ± 2.7	-0.70 ± 2.48				

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10 *Wk8:n=9; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9. Orexin A, OXA; brain-derived neurotrophic factor, BDNF; creatine kinase M-type, CKM.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$.

[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. *P*-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 11. Effects of a 16-week supplementation intervention on energy and macronutrient intake in dietary intervention and control groups[1]

	Weeks of Intervention			Treatment effect[2]		ANCOVA *P* [3]		
Energy & Macronutrients	0	8	16	Δ 16 wk	*P*	Group	Time	Group X time
Energy, kcal/d					0.27	0.60	0.73	0.84
CON	1757.3 ± 573.5	1963.3 ± 858.3	1844.6 ± 490.9	87.3 ± 170.8				
PRO	2193.9 ± 1403.0	1560.9 ± 447.1	1560.2 ± 332.1	-124.5 ± 409.9				
n-3 PUFA	2091.7 ± 848.2	1726.5 ± 500.1	1709.6 ± 854.9	-106.2 ± 680.1				
PRO + PLA	1988.1 ± 798.8	1737.0 ± 619.9	1641.4 ± 469.4	-320.7 ± 851.1				
PRO + n-3 PUFA	1881.6 ± 604.5	2006.8 ± 886.1	1799.5 ± 886.0	115.7 ± 597.8				
Protein, g/day					0.51	<0.01*	0.04*	0.12
CON	71.3 ± 18.6	69.9 ± 16.2	72.7 ± 19.7	1.4 ± 18.7				
PRO	87.1 ± 35.9[$]	90.8 ± 32.8	93.9 ± 31.5	6.8 ± 36.5				
n-3 PUFA	76.5 ± 28.2	67.5 ± 21.0	74.3 ± 32.9	-0.9 ± 19.5				
PRO + PLA	73.3 ± 30.6	92.5 ± 29.4	95.2 ± 30.3	25.7 ± 21.6				
PRO + n-3 PUFA	93.2 ± 20.3 [$]	112.6 ± 45.3	95.9 ± 21.1	12.6 ± 30.2				
Protein, %								
CON	16.2 ± 1.6	15.4 ± 2.1	16.1 ± 3.2	-0.07 ± 4.22	0.29	<0.01*	<0.01*	<0.01*
PRO	18.9 ± 6.7	26.6 ± 9.0[$#]	23.6 ± 4.2[$#]	4.70 ± 6.13				
n-3 PUFA	15.2 ± 4.1	15.8 ± 3.1	18.7 ± 6.4	1.14 ± 7.85				
PRO + PLA	15.6 ± 4.9	22.5 ± 5.3 [$#]	24.1 ± 6.6[$#]	9.15 ± 7.02				
PRO + n-3 PUFA	20.8 ± 5.7	23.8 ± 5.0[$#]	23.9 ± 6.7[#]	2.64 ± 3.97				

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$

[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. P-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 11. Effects of a 16-week supplementation intervention on energy and macronutrient intake in dietary intervention and control groups[1] (Cont.)

	Weeks of Intervention			Treatment effect[2]	ANCOVA *P* [3]			
Energy & Macronutrients	0	8	16	Δ 16 wk	*P*	Group	Time	Group X time
Protein, g/kg/bw					0.14	0.01	0.05	0.14
CON	0.99 ± 0.23	1.00 ± 0.27	1.01 ± 0.29	0.018 ± 0.274				
PRO	1.23 ± 0.54	1.32 ± 0.62	1.6 ± 0.54	0.128 ± 0.548				
n-3 PUFA	0.92 ± 0.33	0.79 ± 0.21	0.86 ± 0.24	-0.040 ± 0.256				
PRO + PLA	1.00 ± 0.24	1.28 ± 0.18	1.32 ± 0.23	0.417 ± 0.287				
PRO + n-3 PUFA	1.27 ± 0.26[$]	1.56 ± 0.61	1.35 ± 0.50	0.206 ± 0.442				
CHO, g/d					0.71	0.98	0.03*	0.49
CON	183.8 ± 70.3	204.6 ± 75.1	194.2 ± 46.3	10.3 ± 21.7				
PRO	272.3 ± 202.7	178.2 ± 45.7	175.8 ± 42.3	-96.5 ± 192.8				
n-3 PUFA	242.6 ± 100.2	185.3 ± 85.2	180.4 ± 98.1	-15.4 ± 120.7				
PRO + PLA	235.3 ± 112.5	174.5 ± 83.4	158.9 ± 72.0	-71.9 ± 115.8				
PRO + n-3 PUFA	208.7 ± 92.9	167.8 ± 51.3	190.7 ± 92.2	-17.3 ± 49.8				
CHO, %					0.29	0.72	0.15	0.27
CON	41.4 ± 6.1	42.3 ± 3.7	41.8 ± 9.9	0.36 ± 11.89				
PRO	45.8 ± 11.4	44.5 ± 8.7	47.6 ± 7.3	1.79 ± 6.11				
n-3 PUFA	46.1 ± 7.1	42.7 ± 11.3	41.6 ± 13.7	-0.09 ± 13.63				
PRO + PLA	46.4 ± 10.4	39.6 ± 12.9	37.8 ± 12.2	-7.61 ± 6.25				
PRO + n-3 PUFA	42.4 ± 6.2	35.1 ± 5.4	42.9 ± 4.0	-3.07 ± 8.62				

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$

[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. P-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 11. Effects of a 16-week supplementation intervention on energy and macronutrient intake in dietary intervention and control groups[1] (Cont.)

	Weeks of Intervention			Treatment effect[2]		ANCOVA *P* [3]		
Energy & Macronutrients	0	8	16	Δ 16 wk	*P*	Group	Time	Group X time
Fat, g					0.79	0.65	0.27	0.14
CON	79.0 ± 30.7	83.1 ± 35.7	83.6 ± 33.8	4.6 ± 11.2				
PRO	89.3 ± 64.0	55.8 ± 22.8	55.6 ± 17.6	-33.8 ± 60.4				
n-3 PUFA	93.3 ± 45.2	81.8 ± 26.4	78.6 ± 46.4	-4.2 ± 25.8				
PRO + PLA	81.0 ± 42.5	76.2 ± 32.9	70.2 ± 23.6	-11.2 ± 39.7				
PRO + n-3 PUFA	76.7 ± 25.7	100.4 ± 56.8	76.0 ± 38.4	6.6 ± 28.7				
Fat, %					0.79	0.05	0.38	0.27
CON	39.0 ± 8.3[$]	40.0 ± 5.3	38.2 ±8.3	-0.82 ± 10.87				
PRO	34.8 ± 6.5	30.0 ± 7.2	30.4 ± 4.6	-4.40 ± 3.89				
n-3 PUFA	34.4 ± 8.2	42.8 ± 8.5	40.6 ± 9.9	2.60 ± 6.81				
PRO + PLA	33.9 ± 6.7	38.6 ± 6.6	38.4 ± 5.4	3.37 ± 6.84				
PRO + n-3 PUFA	36.5 ± 7.8	42.4 ± 7.1	37.8 ± 12.6	1.68 ± 8.15				

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$

[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. P-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 12. Effects of a 16-week supplementation intervention on dietary lipid profile in dietary intervention and control groups[1]

	Weeks of Intervention			Treatment effect[2]		ANCOVA *P*[3]		
Dietary Lipid Profile	0	8	16	Δ 16 wk	P	Group	Time	Group X time
n-3 PUFA s								
ALA, g/d					0.82	0.82	0.19	0.77
CON	2.0 ± 1.0	1.79 ± 0.6	2.04 ± 0.9	0.01 ± 1.0				
PRO	1.9 ±1.4	1.55 ± 1.0	1.39 ± 1.2	-0.49 ± 1.5				
n-3 PUFA	1.7 ± 1.1	1.50 ± 0.6	1.47 ± 1.1	-0.25 ± 1.5				
PRO + PLA	1.9 ± 0.9	1.36 ± 0.8	1.12 ± 0.5	-0.79 ± 1.2				
PRO + n-3 PUFA	1.6 ± 0.5	1.97 ± 1.3	1.49 ± 1.3	-0.08 ± 1.4				
EPA, mg/d					<0.01	<0.01	<0.01	<0.01
CON	11.0 ± 5.9	19.6 ± 12.5	52.3 ± 101.8	41.2 ± 102.6				
PRO	61.9 ± 88.3	55.1 ± 87.1	8.6 ± 7.8	-53.3 ± 81.9				
n-3 PUFA	40.5 ± 96.3	2267.0 ± 21.9[#$]	2356.5 ± 314.7[#$]	2316.0 ± 340.5*				
PRO + PLA	10.2 ± 6.3	39.5 ± 72.6	16.5 ± 19.4	6.3 ± 17.7				
PRO + n-3 PUFA	27.6 ± 23.5	2326.5 ± 131.7[#$]	2308.8 ± 107.4[#$]	2281.2 ± 1134.0*				
DHA, mg/d					<0.01	<0.01	<0.01	<0.01
CON	26.2 ± 14.1	46.6 ± 31.5	133.4 ± 247.7	107.2 ± 238.4				
PRO	104.0 ± 115.6	44.9 ± 50.9	34.0 ± 25.2	-123.8 ± 209.6*				
n-3 PUFA	94.6 ± 202.5	1784.1 ± 40.6[#$]	1801.5 ± 71.6[#$]	1706.9 ± 214.5*				
PRO + PLA	30.8 ± 19.0	89.9 ± 157.1	37.0 ± 37.4	6.2 ± 40.9				
PRO + n-3 PUFA	67.6 ± 56.8	1923.2 ± 291.8[#$]	1938.9 ± 330.2[#$]	1871.2 ± 338.5*				
Total n-3 PUFA, g/d					<0.01	<0.01	<0.01	<0.01
CON	2.2 ± 0.9	2.1 ± 1.0	2.5 ± 0.7	0.3 ± 0.9				
PRO	2.2 ± 1.3	1.8 ± 1.0	1.5 ± 1.2	-0.7 ± 1.3				
n-3 PUFA	1.9 ± 1.2	6.0 ± 0.6[#$]	5.9 ± 1.1[#$]	3.9 ± 1.5*				
PRO + PLA	2.1 ± 1.2	1.8 ± 1.1	1.2 ± 0.5	-0.9 ± 1.5				
PRO + n-3 PUFA	1.9 ± 0.6	6.0 ± 0.8[#$]	5.8 ± 1.3[#$]	3.9 ± 1.7*				

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean

oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$.

[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. P-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 12. Effects of a 16-week supplementation intervention on dietary lipid profile in dietary intervention and control groups[1] (Cont.)

	Weeks of Intervention			Treatment effect[2]		ANCOVA *P*[3]		
Dietary Lipid Profile	0	8	16	Δ 16 wk	P	Group	Time	Group X time
n-3 PUFA, %					<0.01	<0.01	<0.01	<0.01
CON	1.33 ± 1.03	1.08 ± 0.67	1.24 ± 0.35	-0.09 ± 0.77				
PRO	1.16 ± 0.69	0.98 ± 0.41	0.79 ± 0.60	-0.36 ± 0.70				
n-3 PUFA	1.03 ± 0.69	3.59 ± 1.05	3.35 ± 0.68	2.33 ± 0.93*				
PRO + PLA	0.93 ± 0.24	0.90 ± 0.52	0.66 ± 0.20	-0.27 ± 0.36				
PRO + n-3 PUFA	1.04 ± 0.61	2.86 ± 0.83	2.91 ± 0.87	1.87 ± 0.80*				
n-6 PUFA / n-3 PUFA Ratio								
CON	10.5 ± 4.4	9.6 ± 2.8	14.9 ± 5.3	4.5 ± 2.8	<0.01	<0.01	<0.01	<0.01
PRO	10.1 ± 2.7	8.1 ±2.6	12.4 ± 8.5	2.3 ± 7.9				
n-3 PUFA	9.7 ± 3.3	2.9 ± 0.7[#$]	2.6 ± 0.9[#$]	-7.1 ± 3.4*				
PRO + PLA	9.3 ± 1.1	10.4 ± 3.8	10.7 ± 1.0	1.5 ± 1.9				
PRO + n-3 PUFA	9.2 ± 3.8	3.5 ± 2.8[#$]	2.7 ± 2.0[#$]	-6.5 ± 3.7*				
Cholesterol, mg/d					0.05	0.96	0.51	0.02
CON	281.7 ± 169.7	268.2 ± 71.5	263.7 ± 158.6	-18.1 ± 171.3				
PRO	287.9 ± 157.5	252.1 ± 139.0	247.0 ± 181.9	-40.9 ± 132.9				
n-3 PUFA	273.8 ± 107.8	211.8 ± 120.2	341.3 ± 191.1[#]	67.5 ± 167.6				
PRO + PLA	188.3 ± 106.1[#]	300.7 ± 187.6	245.8 ± 118.3[$]	57.5 ± 92.9				
PRO + n-3 PUFA	300.8 ± 99.4	280.0 ± 240.0[$]	182.1 ± 145.3[$]	-118.7 ± 160.0				
Saturated fat, g/d					0.79	0.79	0.08	0.56
CON	29.3 ± 6.4	38.4 ± 19.6	26.5 ± 11.5	-2.9 ± 12.8				
PRO	29.0 ± 25.3	19.0 ± 6.8	16.4 ± 6.5	-12.6 ± 24.3				
n-3 PUFA	26.4 ± 14.2	20.3 ± 7.7	21.5 ± 8.4	-4.9 ± 8.3				
PRO + PLA	25.4 ± 15.1	25.5 ± 10.2	21.0 ± 7.6	-4.4 ± 13.7				
PRO + n-3 PUFA	24.3 ± 9.7	32.3 ± 22.2	28.0 ± 18.5	3.6 ± 20.0				

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$.

[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. P-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 13. Effects of a 16-week supplementation intervention on dietary amino acid profile in dietary intervention and control groups[1]

	Weeks of Intervention			Treatment effect[2]		ANCOVA *P*[3]		
Amino Acid Profile	0	8	16	Δ 16 wk [2]	P	Group	Time	Group X time
Leucine, g/d					<0.01	<0.01	<0.01	<0.01
CON	5.41 ± 1.40	5.58 ± 1.55	5.64 ± 1.56	0.23 ± 1.54				
PRO	6.72 ± 2.38	8.70 ± 2.47#$	8.80 ± 2.54#$	2.08 ± 2.96*				
n-3 PUFA	5.40 ± 2.00	4.65 ± 1.57	5.49 ± 2.26	0.09 ± 1.57				
PRO + PLA	5.20 ± 2.39	8.65 ± 2.19#$	8.93 ± 1.83#$	3.74 ± 1.81 *				
PRO + n-3 PUFA	6.57 ± 1.72	9.17 ± 3.92#$	9.10 ± 2.04#$	2.53 ± 2.54 *				
Total BCAAs, g/d					0.05	<0.01	<0.01	<0.05
CON	12.07 ± 3.08	12.48 ± 3.40	12.54 ± 3.54	0.47 ± 3.26				
PRO	15.04 ± 5.38	18.14 ± 5.65	18.38 ± 5.74	3.33 ± 6.75				
n-3 PUFA	12.10 ± 4.44	10.39 ± 3.48	12.08 ± 5.06	-0.03 ± 3.59				
PRO + PLA	11.61 ± 5.40	17.87 ± 4.75#$	18.38 ± 3.67#$	6.77 ± 4.29				
PRO + n-3 PUFA	14.82 ± 3.75	19.59 ± 8.54#$	19.14 ± 4.34#$	4.32 ± 5.49				
Tryptophan, g/d					<0.01	<0.01	<0.01	<0.01
CON	0.83 ± 0.16	0.83 ± 0.21	0.89 ± 0.23	0.06 ± 0.20				
PRO	1.03 ± 0.37	1.58 ± 0.45#$	1.64 ± 0.41#$	0.61 ± 0.46 *				
n-3 PUFA	0.80 ± 0.29	0.70 ± 0.19	0.84 ± 0.30	0.04 ± 0.23				
PRO + PLA	0.75 ± 0.34	1.55 ± 0.31#$	1.63 ± 0.32#$	0.88 ± 0.29 *				
PRO + n-3 PUFA	1.05 ± 0.25	1.60 ± 0.77#$	1.67 ± 0.34#$	0.62 ± 0.37 *				
Methionine, g/d					0.15	<0.05	0.16	0.12
CON	1.75 ± 0.72	1.59 ± 0.40	1.61 ± 0.49	-0.15 ± 0.70				
PRO	2.02 ± 0.80	2.19 ± 0.85#$	2.23 ± 0.86#$	0.21 ± 0.92				
n-3 PUFA	1.58 ± 0.56	1.33 ± 0.47	1.53 ± 0.69	-0.05 ± 0.48				
PRO + PLA	1.49 ± 0.82	2.10 ± 0.73#$	2.23 ± 0.69#$	0.75 ± 0.61				
PRO + n-3 PUFA	1.93 ± 0.52	2.33 ± 1.00#$	2.24 ± 0.56#$	0.31 ± 0.80				

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$.
[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. P-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 13.. Effects of a 16-week supplementation intervention on dietary amino acid profile in dietary intervention and control groups[1] (Cont.)

	Weeks of Intervention			Treatment effect[2]	ANCOVA *P*[3]			
Amino Acid Profile	0	8	16	Δ 16 wk [2]	P	Group	Time	Group X time
Total EAAs, g/d					0.07	< 0.01	<0.05	<0.05
CON	27.03 ± 7.12	27.09 ± 7.20	27.65 ± 7.99	0.62 ± 7.70				
PRO	34.26 ± 11.73	39.58 ± 13.31	40.48 ± 13.73	6.22 ± 15.53				
n-3 PUFA	26.96 ± 10.13	22.88 ± 7.88	26.73 ± 11.64	-0.23 ± 8.07				
PRO + PLA	25.74 ± 12.46	38.58 ± 11.05[#$]	40.22 ± 9.26[#$]	14.48 ± 9.52				
PRO + n-3 PUFA	32.86 ± 8.34	42.29 ± 18.24[#$]	41.42 ± 9.23[#$]	8.56 ± 12.20				
Arginine, g/d					0.9	0.22	0.68	0.96
CON	3.98 ± 1.16	3.70 ± 0.90	3.93 ± 1.11	-0.05 ± 1.62				
PRO	4.65 ± 1.85	4.27 ± 1.93	4.40 ± 1.97	-0.25 ± 2.00				
n-3 PUFA	3.75 ± 1.57	3.21 ± 1.36	3.63 ± 1.58	-0.12 ± 1.10				
PRO + PLA	3.96 ± 1.76	4.10 ± 1.55	4.46 ± 1.68	0.51 ± 1.32				
PRO + n-3 PUFA	4.45 ± 1.12	4.90 ± 1.67	4.76 ± 1.65	0.32 ± 2.35				
Tyrosine, g/d					0.12	<0.01	<0.05	0.11
CON	2.57 ± 0.75	2.55 ± 0.70	2.52 ± 0.75	-0.05 ± 0.68				
PRO	3.01 ± 1.04	3.29 ± 1.11	3.31 ± 1.11	0.30 ± 1.30				
n-3 PUFA	2.41 ± 0.86	2.09 ± 0.68	2.42 ± 0.93	0.02 ± 0.65				
PRO + PLA	2.27 ± 1.05	3.30 ± 1.01	3.42 ± 0.86	1.15 ± 0.82				
PRO + n-3 PUFA	2.96 ± 0.82	3.76 ± 1.82	3.50 ± 0.90	0.54 ± 1.10				
Cysteine, g/d					0.08	<0.01	<0.01	<0.01
CON	0.97 ± 0.29	0.90 ± 0.25	0.94 ± 0.23	-0.04 ± 0.29				
PRO	1.15 ± 0.45	1.59 ± 0.38	1.66 ± 0.43	0.51 ± 0.54				
n-3 PUFA	0.91 ± 0.30	0.76 ± 0.27	1.15 ± 0.67	0.24 ± 0.78				
PRO + PLA	0.90 ± 0.40	1.59 ± 0.35	1.59 ± 0.32	0.70 ± 0.36				
PRO + n-3 PUFA	1.12 ± 0.27	1.66 ± 0.63	1.78 ± 0.35	0.66 ± 0.44				

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$.

[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. P-values are indicated for main effects of group and time and an interaction effect of group X time. * $P < 0.05$ for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-means.

Table 13. Effects of a 16-week supplementation intervention on dietary amino acid profile in dietary intervention and control groups[1] (Cont.)

	Weeks of Intervention			Treatment effect[2]		ANCOVA *P*[3]		
Amino Acid Profile	0	8	16	Δ 16 wk [2]	P	Group	Time	Group X time
Glutamic Acid, g/d					0.59	<0.05	0.08	0.65
CON	13.53 ± 3.31	14.53 ± 4.67	14.60 ± 3.48	1.07 ± 3.02				
PRO	15.98 ± 7.10	16.57 ± 5.26	16.83 ± 5.09	0.85 ± 7.39				
n-3 PUFA	12.94 ± 4.85	11.83 ± 3.66	13.20 ± 4.27	0.27 ± 3.46				
PRO + PLA	13.21 ± 5.18	17.20 ± 4.59	17.23 ± 4.43	4.02 ± 3.59				
PRO + n-3 PUFA	16.49 ± 5.00	18.88 ± 8.18	18.48 ± 4.63	1.99 ± 5.39				
Glycine, g/d					0.64	0.12	0.27	0.80
CON	3.76 ± 2.39	2.77 ± 0.75	2.85 ± 0.87	-0.91 ± 2.33				
PRO	3.90 ± 1.38	3.32 ± 1.55	3.39 ± 1.49	-0.51 ± 1.65				
n-3 PUFA	2.95 ± 1.27	2.45 ± 1.10	2.77 ± 1.50	-0.17 ± 1.18				
PRO + PLA	3.03 ± 1.52	3.03 ± 1.29	3.37 ± 1.25	0.35 ± 1.12				
PRO + n-3 PUFA	3.38 ± 0.90	3.62 ± 1.34	3.52 ± 1.36	0.14 ± 1.81				
Try/LNAAs					0.04	<0.01	<0.01	<0.01
CON	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.00	0.03 ± 0.10				
PRO	0.05 ± 0.00	0.07 ± 0.01	0.07 ± 0.00	0.04 ± 0.61				
n-3 PUFA	0.05 ± 0.00	0.05 ± 0.01	0.06 ± 0.00	0.05 ± 0.04				
PRO + PLA	0.05 ± 0.00	0.07 ± 0.01	0.07 ± 0.00	0.13 ± 0.07*				
PRO + n-3 PUFA	0.06 ± 0.00	0.06 ± 0.01	0.07 ± 0.01	0.10 ± 0.07*				

[1] Values are mean ± SD. Control, no intervention and free living, CON, n=6; whey protein isolate, PRO, n=6; omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, n-3 PUFA, n=10; whey protein isolate + placebo soybean oil, PRO + PLA, n=7; whey protein isolate + omega-3 polyunsaturated fatty acids, eicosapentaenoic acid + docosahexaenoic acid, PRO + n-3 PUFA, n=9.

[2] Treatment effect between groups were tested by one-way ANOVA with baseline measurements subtracted from 16-week values. LS-means significant differences: * $P < 0.05$.

[3] Differences in raw data were analyzed at 0, 8, and 16 using ANCOVA with age and BMI as covariates. P-values are indicated for main effects of group and time and an interaction effect of group X time. * P < 0.05 for main effect of intervention; # significantly different from control within time point following LS-means; $ significantly different compared to baseline follow LS-mean.

Figures

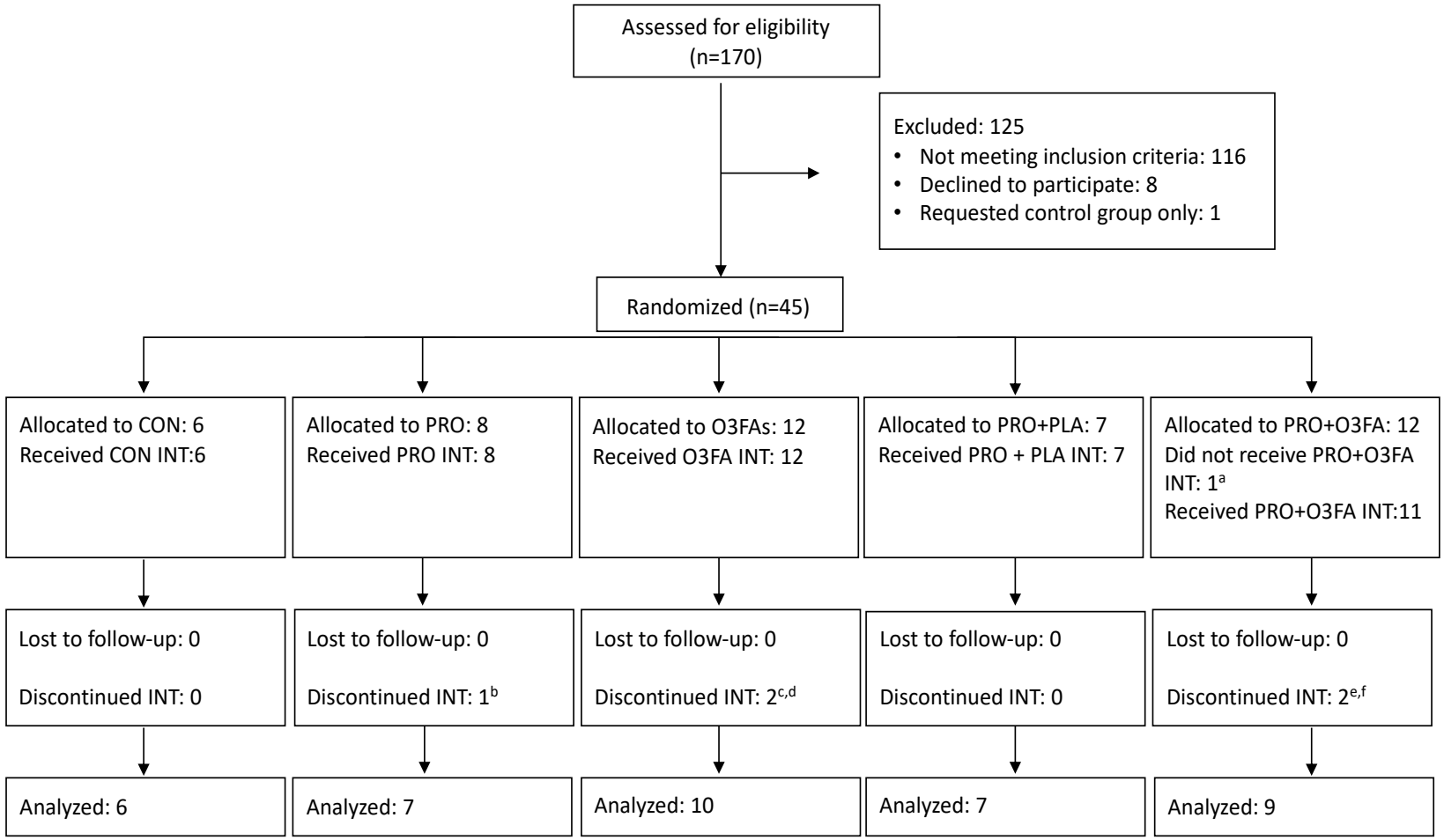

Figure 1: Flow chart showing number of subjects recruited and their attrition patters during the 16-wk intervention study. Int, intervention; CON, control; n-3 PUFA, omega-3 polyunsaturated fatty acids, Eicosapentaenoic acid + docosahexaenoic acid, PRO + PLA, whey protein isolate + placebo soybean oil; PRO + n-3 PUFA, whey protein isolate + omega-3 polyunsaturated fatty acids, Eicosapentaenoic acid + docosahexaenoic acid. Reason for subject withdrawal were as follows: [a] discomfort wearing the ActiGraph

sleep monitor (1), [b] unexpected menstrual cycle (1), [c] fall resulting in injury and pain medication (1), [d] discomfort while swallowing the supplement capsules (1), [e] dislike of the WPI (1), and [F] time constraints (1).

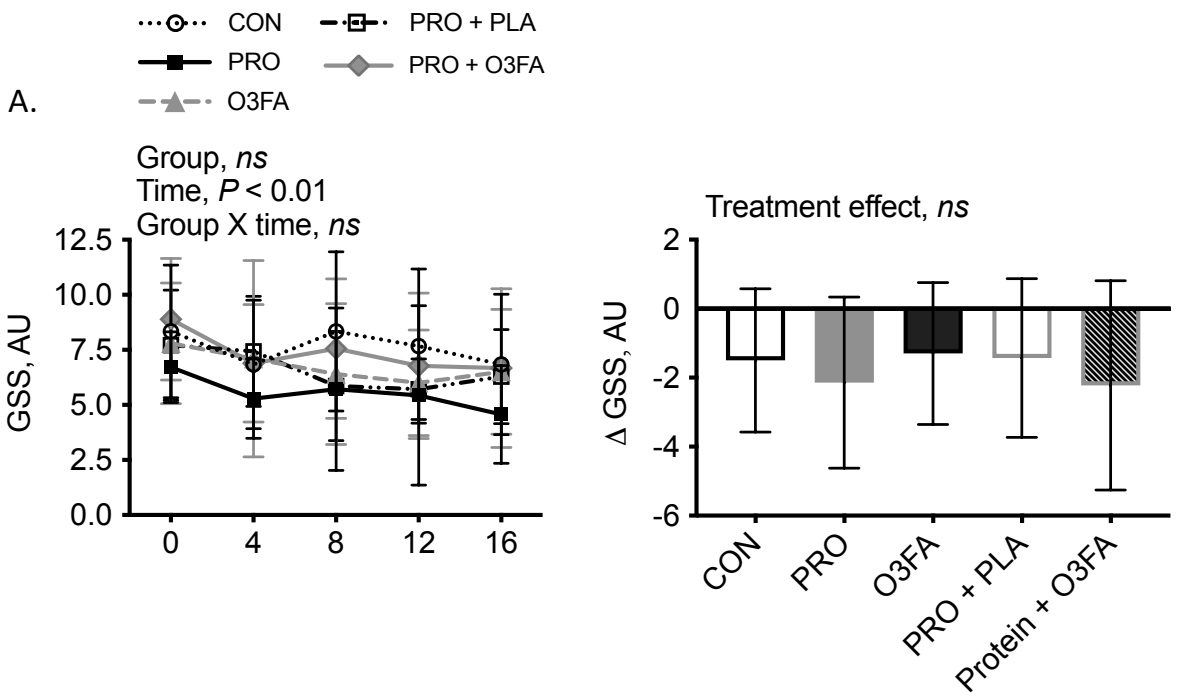

Figure 2: Ratings of subjective sleep quality and duration during and following the 16-week supplementation intervention in the control (CON, n=7), whey protein isolate (PRO, n=7), EPA + DHA (n-3 PUFA, n=10), protein + placebo (PRO + PLA, n=7), and whey protein isolate + EPA +DHA (PRO + n-3 PUFA, n=9) using the Pittsburgh Sleep Quality Index (PSQI) questionnaire. Global sleeping score (GSS) = Sum of seven sub-component scores; range from 0-21. Line graphs represent the GSS over time and bar graphs represent the treatment effect (16-week – baseline values) per treatment group. (A) GSS. Data are expressed as mean ± SD. * $P < 0.05$ is considered significant.

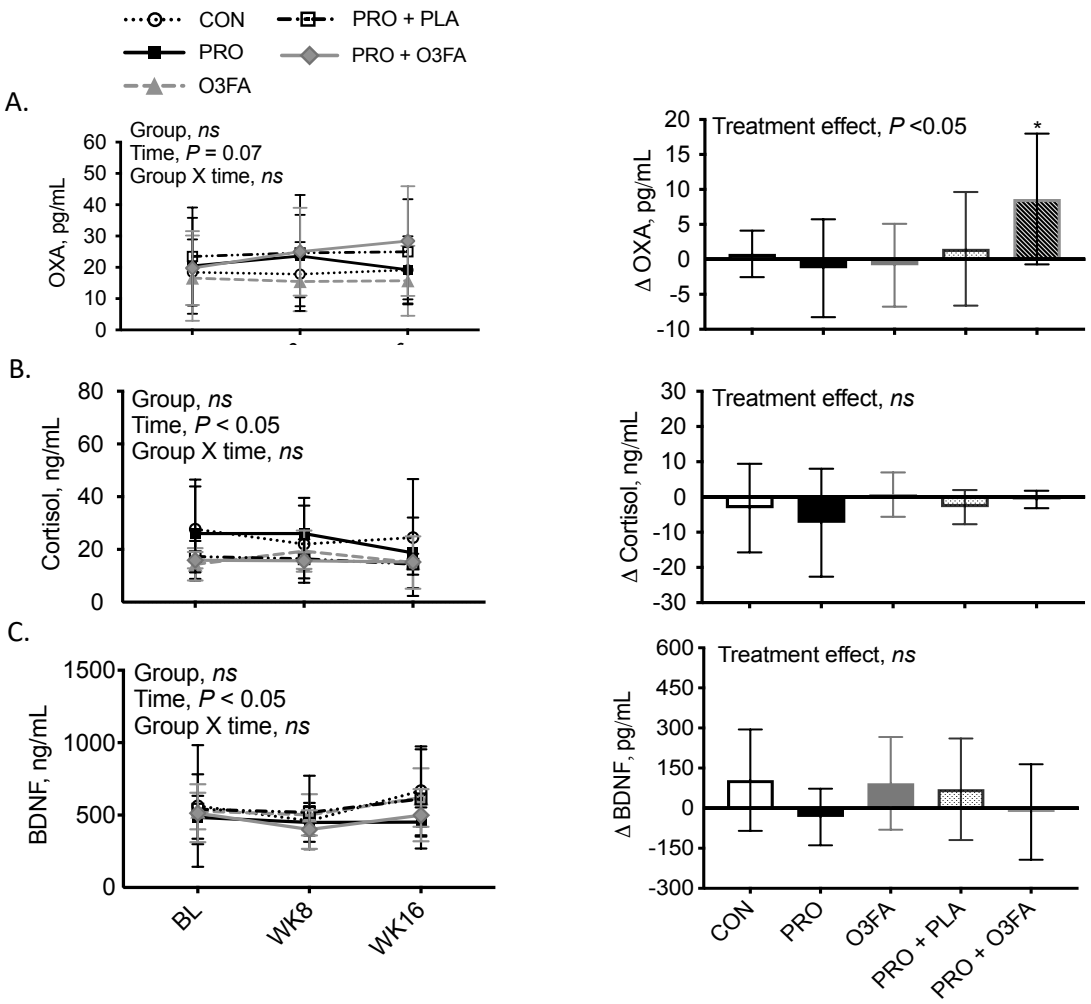

Figure 3. Fasting plasma orexin-A (OXA), cortisol, and brain-derived neurotrophic factor (BDNF) response during and following the 16-week supplementation intervention in the control (CON, n=7), whey protein isolate (PRO, n=7), EPA + DHA (n-3 PUFA, n=10), protein + placebo (PRO + PLA, n=7), and whey protein isolate + EPA +DHA (PRO + n-3 PUFA, n=9). Line graphs represent fasting plasma concentrations over time and bar graphs represent the treatment effect (16-week – baseline values) per treatment group. (A) OXA concentrations; (B) cortisol concentrations; (C) BDNF concentrations. Data is expressed as mean ± SD. * $P < 0.05$ is considered significant.

CHAPTER 6. Conclusion

The current growth rate of the older population is recognized as one of the most substantial demographic trends in United States (U.S.) history [1, 2]. This robust shift in demographics emphasizes the importance of independence, quality of life, and health across the lifespan to promote successful aging (SA) [3]. The concept of SA is associated with longevity, the absence of disease and disability, and a positive state of well-being which are strongly associated with body composition [4-10]. We defined SA as low cardiometabolic risk, preservation of physical function, and a positive state of well-being with nutrition as an integral component. Research suggests nutritional strategies focused on the incorporation of high-quality protein and omega-3 polyunsaturated fatty acids (n-3 PUFAs) are potential methods to mitigate age-related decline in skeletal muscle mass, fat mass gain, cardiometabolic risk, physical function, and well-being in adults to promote SA [11-16]. The overall objective of this dissertation was to determine the effect of nutrition, specifically dietary protein and n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on SA outcomes of cardiometabolic risk, physical function, and well-being. The central hypothesis tested in this dissertation was that increased intake of high-quality dietary protein or n-3 PUFAs would improve SA outcomes of cardiometabolic risk, preservation physical function, and well-being in middle-aged and older adults. This dissertation includes three independent research studies investigating the effect of nutrition on components of SA. Collectively, the results suggest high-quality protein and n-3 PUFAs act as potential regulators of SA.

Study 1, a meta-analysis and systematic review, was designed to evaluate the available evidence of randomized controlled trials (RCTs) incorporating beef and nutrients found in beef, a high-quality dietary protein, on components of SA with a focus on well-being. Nine RCTs were

included in the meta-analysis and an overall positive effect of beef (n=1) and beef's nutrients (n=8) was found on well-being with substantial heterogeneity among sample populations. In this meta-analysis well-being outcomes included LBM, cognitive function, and physical function. Physical function significantly improved following intervention supplementation of beef and beef's nutrients. Although quality of life and subjective well-being outcomes were included in the search processes, RCTs incorporating quality of life and subjective outcomes of well-being did not meet our inclusion criteria. According to the Center for Disease Control and Prevention (CDC) [8], physical well-being and psychological well-being are specific components which are included under the well-being concept. Therefore, although subjective outcomes of well-being were not included, LBM, physical function, and cognitive function components were analyzed within the well-being model. Furthermore, and evident need was identified for additional well-designed RCTs evaluating the efficacy of beef and nutrients found in beef in healthy adults ≥ 50 years of age to promote well-being and SA. Future research should adopt a population representative sample of healthy older adults, absent of chronic diseases, and examine the effect of lean beef on outcomes of well-being. Furthermore, RCTs implementing dietary interventions should incorporate a multidimensional approach with homologous defined functional outcomes of LBM, cognitive function, physical function, and quality of life to advance research in the field of aging, nutrition, and SA in healthy adults.

Study two, a clinical trial with a randomized cross-over design, was designed to investigate the effect of a high protein breakfast containing whey protein isolate (WPI) or pea protein isolate (PPI) on appetite, energy expenditure, and 24-hour energy intake in young compared to older healthy men to decrease cardiometabolic risk and promote SA. To our knowledge, this is the first study to examine the short-term effect of a high-protein breakfast

from plant- or animal-derived protein sources on energy expenditure and appetite response in healthy, young and older men. Collectively, the results of this study suggest an isocaloric, isovolumetric, macronutrient- and fiber-matched protein-based breakfast beverages from an animal-based whey protein isolate and a plant-based pea protein isolate exerts comparable effects on appetite, energy expenditure, and 24-hour energy intake in both young and older healthy adult men. The lack of differences observed between protein source may have been due to the 40 grams of protein used in the test breakfast beverages which was a larger dose compared to the doses used in other studies demonstrating differences in energy metabolism [17, 18] and appetite [19] between protein sources. In addition, we did not provide the pea protein and whey protein in mixed-meal context. Data from the 2017-2018 National Health and Nutrition Examination Survey (NHANES) demonstrate that adults in the U.S. skew protein (and energy) consumption toward the evening meal [20]. Moreover, mean protein consumption for adults aged 20 and over is ~13 grams at the breakfast meal [21]. Therefore, further research is needed to determine the effect of a plant-based compared to an animal-based protein breakfast meal in a comparable quantity to a standard American breakfast of ~13 grams in young compared to older adults.

Study three, a 16-week randomized controlled trial, was designed to investigate the effect of protein and n-3 PUFA supplementation individually and in combination on LBM, physical function, cardiometabolic risk, and well-being in postmenopausal women to promote SA. To our knowledge, this is the first RCT to examine the effect of 16-weeks of dietary protein and/or n-3 PUFA supplementation on LBM, physical function, cardiometabolic risk, and well-being in postmenopausal women. The present study tested the hypothesis that combined dietary protein and n-3 PUFA supplementation would have a greater effect on body composition, cardiometabolic risk, and indexes of sleep and mood states in postmenopausal women when

supplemented in combination as WPI and n-3 PUFA compared to individual supplementation. Collectively, the results of this study suggest protein and n-3 PUFA combined supplementation when compared to individual supplementation for 16-weeks does not provide additional benefits on body composition, cardiometabolic risk, and well-being. However, we observed a potential additive effect of protein and n-3 PUFAs on orexin-A (OXA) concentration. To our knowledge, comparable dietary interventions have yet to be conducted and a mechanism of action of OXA in cardiometabolic risk, physical function, and well-being is yet to be elucidated in humans. Moreover, data from our lab indicate obese Zucker rats assigned a high-protein (40% energy) diet had reduced liver and skeletal muscle lipid deposition, and higher OXA concentrations compared to obese Zucker rats consuming a moderate-protein (20% energy) diet for 12-weeks [22]. There is a need to further assess the effect of dietary protein and n-3 PUFA intake on OXA concentrations in post-menopausal women. Furthermore, a relationship between SA and OXA [23] warrants further investigation.

Collectively, the results of this dissertation suggest high-quality protein and n-3 PUFAs act as potential regulators of SA outcomes. However, additional research is necessary to determine the effectiveness of protein and n-3 PUFA-based nutrition strategies to promote SA. Altogether, further research is recommended to implement RCTs with longer duration and larger study populations to identify the effects of high-quality protein (e.g., whey protein isolate and lean beef) and n-3 PUFAs, EPA + DHA, on middle-aged and older adults to promote outcomes of SA. Moreover, additional research is necessary to determine the effect of dietary protein and n-3 PUFAs on OXA as a potential mechanism of SA. For example, future RCTs should implement WPI and lean beef supplementation alone and in combination with n-3 PUFAs within a multidimensional approach with homologous defined functional outcomes of LBM, physical

function, and well-being to advance research in the field of aging and nutrition. In addition, future studies should investigate the molecular mechanisms underlying the potential effect of dietary protein and n-3 PUFAs, apart from exercise and weight-loss, on OXA and SA in healthy older adults.

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APPENDIX

Chapter 4 IRB committee approval letter

UNIVERSITY OF ARKANSAS

To:	Jamie I Baum FDSC N2216
From:	Douglas James Adams, Chair IRB Committee
Date:	09/20/2017
Action:	**Approval**
Action Date:	09/20/2017
Protocol #:	1708038914
Study Title:	The Effect of Breakfast on Energy Metabolism, Appetite, and Food Intake Men
Expiration Date:	09/12/2018
Last Approval Date:	
Risk Level:	Moderate Risk

The above-referenced protocol has been approved following Full Board Review by the IRB Committee that oversees research with human subjects.

If the research involves collaboration with another institution then the research cannot commence until the Committee receives written notification of approval from the collaborating institution's IRB.

It is the Principal Investigator's responsibility to obtain review and continued approval before the expiration date.

Protocols are approved for a maximum period of one year. You may not continue any research activity beyond the expiration date without Committee approval. Please submit continuation requests early enough to allow sufficient time for review. Failure to receive approval for continuation before the expiration date will result in the automatic suspension of the approval of this protocol. Information collected following suspension is unapproved research and cannot be reported or published as research data. If you do not wish continued approval, please notify the Committee of the study closure.

Adverse Events: Any serious or unexpected adverse event must be reported to the IRB Committee within 48 hours. All other adverse events should be reported within 10 working days.

Amendments: If you wish to change any aspect of this study, such as the procedures, the consent forms, study personnel, or number of participants, please submit an amendment to the IRB. All changes must be approved by the IRB Committee before they can be initiated.

You must maintain a research file for at least 3 years after completion of the study. This file should include all correspondence with the IRB Committee, original signed consent forms, and study data.

cc: Aubree L Worden, Investigator
Hexirui Wu, Investigator
Katie Deanne Cloud, Investigator
Regan K Burgess, Investigator

Page 1 of 1

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Nov 12, 2020

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Licensed content author	Hawley, Aubree L; Gbur, Edward
Licensed content date	Jan 22, 2020
Type of Use	Thesis/Dissertation

UNIVERSITY OF ARKANSAS

To:	Jamie I Baum FDSC N2216
From:	Douglas James Adams, Chair IRB Committee
Date:	03/26/2020
Action:	**Expedited Approval**
Action Date:	03/26/2020
Protocol #:	1708023785A010
Study Title:	The Effect of Protein and Omega-3 Fatty Acid Supplementation on Body Composition, Sleep, Cardiometabolic Health, and Strength in Postmenopausal Women
Expiration Date:	09/11/2020
Last Approval Date:	03/26/2020

The above-referenced protocol has been approved following expedited review by the IRB Committee that oversees research with human subjects.

If the research involves collaboration with another institution then the research cannot commence until the Committee receives written notification of approval from the collaborating institution's IRB.

It is the Principal Investigator's responsibility to obtain review and continued approval before the expiration date.

Protocols are approved for a maximum period of one year. You may not continue any research activity beyond the expiration date without Committee approval. Please submit continuation requests early enough to allow sufficient time for review. Failure to receive approval for continuation before the expiration date will result in the automatic suspension of the approval of this protocol. Information collected following suspension is unapproved research and cannot be reported or published as research data. If you do not wish continued approval, please notify the Committee of the study closure.

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You must maintain a research file for at least 3 years after completion of the study. This file should include all correspondence with the IRB Committee, original signed consent forms, and study data.

cc: Aubree Leigh Hawley, Investigator
Hexirui Wu, Key Personnel
Megan Elizabeth Rosa-Caldwell, Key Personnel
Angela M Tacinelli, Key Personnel
Samuel Preston Belt Walker, Key Personnel
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