RELATIONS AND EFFECTS OF DIETARY PROTEIN AND BODY COMPOSITION ON CARDIOMETABOLIC HEALTH

by

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I have read a few of these dedication sections before, and with a mind towards posterity, I told myself that the primary goal should be to not write anything that would make me cringe in 20 years. However, as I sit down to write this, I realize I have too many people to whom I owe too much to write the dry, stoic *'Acknowledgements'* I had envisioned. Sorry, future Rob.

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ABSTRACT

Obesity has ascended to become the primary modifiable cause of death in the United States. New evidence has called into question the utility of BMI – the typical index of obesity – in predicting cardiometabolic disturbances. The distribution of body fatness may be just as important as the total quantity. Intermuscular adipose tissue (IMAT) has emerged as a distinct subset of adipose in skeletal muscle that may be particularly metabolically deleterious. Typically, sections of either the calf or thigh are used as proxy measurements for whole-body IMAT in investigations. However, IMAT dispersion may not be consistent across tissues, instead infiltrating specific muscle or muscle compartments, and these have may have different metabolic consequences. The study described in Chapter 2 was designed to address this possibility and investigate and compare associations among thigh and calf IMAT stores with indices of cardiometabolic health. The strength of the relationship between IMAT and glucose control-related indices of cardiometabolic health was dependent upon anatomic location. Specifically, thigh IMAT is a better predictor of cardiometabolic risk that calf IMAT.

Skeletal muscle has gained increased recognition in recent years for its importance in promotion of health and wellness throughout the life course. While treatment models addressing issues of declining muscle mass and strength with age previously focused on older adults, the importance of utilizing a life course model to promote skeletal muscle health at all ages was more recently recognized. There is consistent evidence that higher-protein diets modestly improve body composition. However, women are at greater risk for not meeting protein requirements and seem to be less willing to adopt strategies to achieve greater protein intake, such as protein supplementation, for fear that it may cause ‘bulkiness’. Therefore, the study described in Chapter 3 was designed to critically evaluate the effect of whey protein supplementation on body composition changes in women via a systematic review & meta-analysis of published randomized controlled trials. It was hypothesized that whey protein supplementation would moderately improve body composition but would not cause excessive muscle hypertrophy. Consistent with our hypothesis, whey protein supplementation improved body composition by modestly (<1%) increasing lean mass, without influencing fat mass.

Dietary protein and skeletal muscle are conceptually inseparable; protein is often only considered in terms of how it impacts skeletal muscle-related outcomes. However, it is of interest

to determine if the proposed beneficial effects of increased dietary protein consumption extend beyond skeletal muscle. Consumption of higher protein diets result in lower resting blood pressure, but the potential for protein to attenuate acute exercise blood pressure responses is unclear. The study described in Chapter 4 was designed to investigate the effects of meals with different amounts of protein on blood pressure responses to exercise in a randomized, cross-over trial. We hypothesized that consuming the higher-protein meal would attenuate the blood pressure responses to exercise and result in a more robust post-exercise hypotensive response. Contrary to our hypothesis, a higher-protein meal does not attenuate exercise-induced blood pressure responses compared to a lower-protein meal. These findings build upon previous research suggesting that the beneficial effect of chronically elevated protein intake on blood pressure is typically not observed in an acute setting by extending these findings to encompass blood pressure responses to acute responses to exercise.

The three studies packaged herein utilize different techniques and report on different outcomes, but conceptual threads unite these works which augment the collective findings. Future researchers investigating the effects of protein on skeletal muscle anabolism can: 1) learn of the importance of proper reflection on surrogate measures and potential for anatomic-specific effects from the IMAT findings (Chapter 2), 2) appreciate the relevance of energy and training states in modulating responses from the WP meta-analysis (Chapter 3), and 3) recognize the importance of holistic approaches and employing challenges to reveal heterogeneity from the protein and BP trial (Chapter 4). Taken together, the research presented in this dissertation forwards our understanding of the relations and effects of dietary protein with different components of body composition on cardiometabolic health.

CHAPTER 1. LITERATURE REVIEW

1.1. Overweight and obesity of adults in the United States

Obesity is quickly ascending to be the primary modifiable cause of death in the United States [1]. The deleterious effects of overweight and obesity are manifest through health- and lifespan shortening co-morbidities such as cardiovascular disease, type 2 diabetes, and numerous cancers [2]. Obesity has resulted in the greatest number of preventable life years lost, outpacing tobacco, the previous titan of preventable death, by upwards of 47 percent [3]. Indeed, recent epidemiological data suggest that obesity may account for up to 20% of overall mortality [4]. Obesity contributes towards myriad health complications and has resulted in numerous groups calling for integrated efforts to ameliorate the obesity epidemic [5, 6]. However, in order to address the obesity epidemic, we must first be able to define and measure it.

1.2. Assessment of obesity and body composition

Obesity, in the most simple sense, is an excess of body fat. However, it is not a simple task to measure body fat, particularly at a population level. Relative weight indices are useful in this regard to act as a proxy for body fatness. Body mass index (BMI; body mass in kilograms divided by height in meters, squared) is the most widely-used relative weight index to determine presence of overweight or obesity in adult populations [7]. Using this metric, upwards of 70% of Americans are classified as having an overweight (25.0-29.9 kg/m2) or obese BMI (>30.0 kg/m2) [8, 9]. In addition to being the most widely-used metric for obesity, BMI is also the most widely-criticized metric [7]. The utility of BMI in predicting cardiometabolic disturbances is questionable; nearly half of individuals with an overweight BMI present with a healthy cardiometabolic profile, and ~30% of those with a ‘normal’ BMI are cardiometabolically unhealthy [10]. Importantly, BMI was not originally designed to be used as an index of obesity [11]. This is not to say that BMI does not have utility or predictive power as a surrogate marker. Higher BMIs are indeed predictive of deleterious health outcomes and medical expenses [12]. How much of this is a happy accident, or residual confounding, though?

The imprecision of BMI has more recently been highlighted in light of the "obesity-mortality paradox"[13]. This paradox refers to the apparent increased survival rate and improved prognosis of overweight and obese patients with heart failure [12]. One large systematic review and meta-analysis of 97 studies with almost 2.9 million subjects determined that while an obese BMI was predictive of increased risk of mortality when compared to a normal BMI, the optimal survival (6% lower mortality) occurred in those with an overweight BMI [14]. Some of the proposed mechanisms attempting to reconcile this paradox include unmeasured increased metabolic reserves, less cachexia, lower atrial natriuretic peptides, and increased muscle mass and muscular strength of overweight individuals [13]. These mechanistic explanations are fascinating, and may have merit; however, the most prevalent and convincing explanation for the obesity paradox is the poor diagnostic performance of the BMI metric [15]. Indeed, prognostic accuracy is improved in patients with coronary artery disease when measuring body fat percentage via air displacement plethysomography (BOD-POD) versus traditional BMI assessment [16].

We can content ourselves with muddy associations between 'obesity', as determined by BMI, and health, but the theoretical validity remains low when we are actually assessing relative body masses. A direct measurement of body composition – one that directly measures compartments of lean and fat mass – has greater theoretical validity. Body fatness, not body mass (including fat-free mass), is likely the driver of cardiometabolic health disturbances [17]. Markers that more directly assess fat mass compartments, such as the fat mass index (FMI), may be a better metric than the more crude BMI [17]. Similar to BMI, FMI is calculated as fat mass (kg)/height $(m)^2$. Even when assessed by bioelectrical impedance, which is far from a direct measurement of fat mass and has well-documented technical limitations, some evidence indicates that FMI outperforms BMI as a screening tool to predict the presence of metabolic syndrome [18]. However, a preponderance of data suggests limited differential utility between BMI and FMI, with predictive capacity for future obesity and metabolic alterations being largely similar between the two [19, 20].

There is always going to be a trade-off between accuracy and validity of an assessment with the convenience and scalability. This trade-off spectrum ranges from the quick-and-dirty BMI to the burdensome and expensive magnetic resonance imaging (MRI). At one end of the spectrum, BMI is highly convenient and scalable – height and weight are easy to collect at a

standard screening (individuals can even self-report these values) – but lacks theoretical validity. Indexes such as FMI as measured by bioelectrical impedance lie somewhere in the middle, offering greater theoretical validity (actual assessment of fat mass), but require use of more time-consuming and burdensome assessments. Given that the utility of BMI and FMI are largely similar [19], BMI appears to have a superior 'cost-benefit ratio'.

Air displacement plethysmography (ADP) also lies somewhere between BMI and the new 'gold-standard' body composition assessments on the convenience-accuracy spectrum. Briefly, after weighing a subject, ADP involves use of a densitometric technique to measure air displaced by a human subject in a chamber to determine whole-body volume [21]. Importantly, the densitometry principle applied assumes a two-compartment model – estimating fat mass and fat free mass, implications of which will be discussed below. Air displacement plethysmography has been validated against the erstwhile gold-standard adiposity measurement, hydrostatic weighing, and found to be a suitable substitute if ADP subjects are clothed in tight swimsuits [22]. Studies even report greater precision with ADP versus hydrostatic weighing [23]. Air displacement plethysomography has largely supplanted hydrostatic weighing, as it is more rapid, requires less training to operate, and can be used on a wider variety of populations, including physically compromised individuals [23, 24]. However, ADP still falls short in total accuracy and precision when compared with the current gold-standard DXA assessment method [25]. Relative to DXA, ADP underestimates body fat in overweight/obese participants, and overestimates body fat in underweight participants [25].

At the other end of the spectrum lie the highly accurate but costly and inconvenient 'gold-standard' body composition assessments. A recent position paper argued that DXA should be the reference standard for muscle mass analyses due to the feasibility, accuracy, and safety [26]. As such, DXA is currently the most widespread method for measuring body composition [27]. One profound strength of DXA is the high precision; coefficient of variations (CV) for lean body mass are typically ~1% [28]. However, hindering the theoretical validity of air displacement plethysmography and DXA is the use of two-compartment and three-compartment models. An improvement on the two-compartment model utilized by ADP methodologies, DXA is able to use a three-compartment model as it quantifies bone. This still falls short of the classical four-compartment model, which has been a principal in anthropometric quantifications of skeletal muscle mass for almost 100 years [29].

Putting aside the theoretical validity issue inherent to assessing proxies of skeletal muscle mass, distribution of muscle and fat tissues is an extremely important factor to consider. The tremendous heterogeneity in the deleterious metabolic effects of obesity may be due to different distributions of adiposity amongst individuals [30]. Three-dimensional imaging techniques are required to quantify regional muscle and adipose tissue mass with high accuracy [31]. Magnetic resonance imaging (MRI), the preeminent three-dimensional imaging technique, lies at the far end of the convenience-accuracy spectrum. Magnetic resonance imaging is extremely expensive, involves long acquisition times, and requires specialized training to operate. Counterbalancing this, MRI is the most accurate and precise three-dimensional imaging technique for characterizing tissues on the basis of biochemical and physical properties [32]. Some evidence indicates that not all adipose tissue is equally harmful, so increasing use of MRI to add a dimension of localization to body composition analyses represents a major advancement in the field [33].

1.3.Not all adipose tissue confers similar metabolic risk

1.3.1. Differential effects of adipose based on distribution

Adipose tissue was once thought of as a simple storage depot devoid of appreciable metabolic activity. This assumption is understandable, as 95% of the adipocyte is dominated by the lipid droplet. Indeed, the lipid droplet is relatively inert – serving as a storage vessel for triglycerides. The perils of ignoring the remaining 5% of cellular mass were more recently made manifest and adipose is now recognized as the body's largest endocrine organ [34, 35]. Adipocytes exert metabolic activity through the release of a distinct class of cytokines known as adipokines. These adipokines, including leptin and adiponectin, act in an autocrine, paracrine, and endocrine fashion to influence both local and systemic metabolism [35].

In line with the expanded understanding of the metabolic activities of adipose tissue, there is also considerably more nuance in the categorization of distinct adipose tissues that were previously grouped together. Adipose tissue can be categorized in numerous ways depending on the characteristics in mind and degree of granularity sought. Based upon function and location, adipose tissues are often classified as either white adipose tissue, brown adipose tissue, beige adipose tissue, and marrow adipose tissue [36]. As this review focuses on white adipose tissue,

the interested reader is directed to a comprehensive review of all classifications of adipose tissue, including their similarities and differences [37].

Based specifically upon anatomical location, white adipose tissue can effectively be grouped into subcutaneous, visceral, intermuscular, and intramuscular adipose tissue. Subcutaneous adipose tissue, located primarily in the gluteal region, thighs, and midsection, constitutes the majority of tissue volume at approximately 85% of total white adipose tissue [38]. The remainder of white adipose tissue resides in the viscera (~10%) and other ectopic locations, such as skeletal muscle [39]. These distinctions based upon depot location are important because there is evidence that the metabolic consequences of adipose accumulation may vary by site. In fact, the relative distributions of adipose tissue throughout the body may have greater metabolic implications than absolute quantity of adipose [40].

The seeds of this concept were laid by researchers trying to identify why there was such tremendous heterogeneity in health outcomes associated with obesity. Individuals with the same quantity of total body fat could present with markedly different cardiometabolic health profiles [41]. New imaging techniques revealed that overweight and obese individuals with a worsened cardiometabolic health profile possessed excess visceral adipose tissue, and those with a metabolically healthy profile had less visceral adipose tissue and relatively greater subcutaneous adipose content [41, 42]. Subcutaneous adipose tissue accumulation in the gluteal-femoral region, more typical of women, is actually considered to be protective of cardiometabolic health [43].

The use of computed tomography and MRI represented a critical evolution in thinking because it was shown that individuals with similar waist circumference/BMI can present with very dissimilar distributions of subcutaneous versus visceral fat depots [44]. Since then, accumulating evidence has implicated visceral adipose tissue in a host of negative health outcomes [30, 45-50]. Visceral adipose tissue is associated with decrements in almost every imaginable clinical index, including blood pressure, plasma triglycerides, HDL- and LDL-cholesterol, and the homeostatic model assessment of insulin resistance (HOMA-IR) [51, 52]. Further, visceral adipose is an independent predictor of type 2 diabetes and coronary artery disease [53, 54]. Particularly striking is the profound effect visceral adipose tissue can have on cardiometabolic health despite contributing very little to total body adiposity [55]. Indeed,

visceral adipose tissue typically only makes up less than 10% of total white adipose tissue, yet has an outsized impact on health [39].

1.3.2. Sex differences in fat distribution

There are significant differences in the relative quantity, distribution, metabolism, and endocrine function of adipose tissue between men and women [35]. The fact that women typically have a greater body fat percentage than men and differ in distribution – women partition more fat to the gluteal-femoral region while men store more fat in the abdomen – is well documented. The mechanisms underpinning these sexual dimorphisms are relatively less clear, but are thought to be due to a combination of differences in basal fatty acid oxidation, catecholamine- and insulin-mediated differences in lipolysis regulation, and differential postprandial fatty acid storage [56]. Sex steroid hormones are also implicated in regulating body fat distribution in women. Compared with estrogen non-users, postmenopausal women who use estrogen have less visceral adipose tissue [57]. Further, the sex gap in visceral adipose content is much lessened as women go through menopause, where marked increases in abdominal adiposity are observed [58]. While the phenotypical evidence is compelling, the mechanisms that control body fat distribution remain poorly understood. Other reviews adequately discuss the transferability of research findings in the area of adipose tissue biology between the sexes [59-61].

1.3.3. Mechanisms explaining the relationship between ectopic fat and metabolic aberrations

The precise relationship between ectopic fat and metabolic perturbations has yet to be fully elucidated. The “portal free fatty acid” hypothesis, where excess visceral adipose impairs liver function through dysregulated lipolysis leading to free fatty acid overload, has recently been called into question [62]. Research has indicated that upwards of 80% of free fatty acids in the portal circulation do not originate from local visceral adipose, and instead are the product of adipose dispersed throughout the body [63]. In another prevailing model, the “lipid spillover hypothesis” posits that ectopic fat deposition is a *product* of lipotoxicity-related insulin resistance [64]. Here, excess deposition of fat to ectopic depots represents a failure of subcutaneous adipose tissue to adequately expand to accommodate energy intake [42]. Under

conditions of homeostasis, insulin-sensitive subcutaneous adipose tissue acts as a 'sink' to absorb excess energy, which can safely expand to 'buffer' the body from lipotoxic free fatty acid delivery to nonadipose organs and tissues [45]. However, if the subcutaneous adipose tissue is resistant to insulin or aberrant in some other way, it is not able to act as an energy-sink and lipids are stored in undesirable ectopic depots [65]. Alternative models implicate an overactive hypothalamic-pituitary-adrenal axis [46], dysregulated gonadal steroids [66], and overstimulation of the endocannabinoid system [67]. The number of viable, competing theories speaks to the uncertainty surrounding the relationship between ectopic fat and metabolism, including directionality. It is likely the relationship between ectopic fat and metabolism is bidirectional, with ectopic fat both leading to the development of *and*/*or* acting as a marker of metabolic perturbation [42].

1.3.4. Emerging role of intermuscular adipose tissue in health and disease

Similar to the recent expanded understanding of the dissimilar attributes of visceral and subcutaneous adipose tissue, intermuscular adipose tissue (IMAT), or fat beneath the fascia and between muscle bundles, has been identified as an important ectopic fat depot warranting further scrutiny. Metabolic detriments as a result of IMAT accumulation may be similar to that of visceral adipose tissue [68-70]. While there is no universally accepted model by which IMAT impairs cardiometabolic health, the extensive research of visceral adipose tissue offers a suitable analog. See **Figure 1** for an overview of the effects of IMAT on local tissues. Due to the proximity of IMAT to skeletal muscle, which is the largest site of glucose uptake, IMAT is thought to impair insulin signaling and glucose metabolism [71, 72]. Further, the adipokine secretory profile of ectopic adipose is different from that of subcutaneous adipose tissues [35]. IMAT may alter local and systemic metabolism through release of these proinflammatory adipokines, increasing oxidative damage, and impeding blood flow to muscles [73-75].

In comparisons of IMAT and visceral adipose tissue, some evidence indicates that IMAT is more strongly related to cardiometabolic health [68, 76], while other studies give the edge to visceral adipose tissue [48, 77]. Regardless, IMAT has emerged as important risk factor to monitor as it has been linked to numerous decrements to health. Accumulation of IMAT, independent of BMI and total body adiposity, is associated with metabolic syndrome [49]. The most consistent evidence is suggestive of IMAT being associated with insulin resistance and

impairments in glucose metabolism [68, 69, 73, 78], with less consistent evidence of relations with lipids and lipoproteins [48, 68, 76]. These relations are reliably supported by observations in which there is greater IMAT infiltration in muscles of adults with type 2 diabetes and obesity [78-81]. And in the final piece of the epidemiological puzzle, IMAT accumulation is associated with upwards of a 40% increased mortality risk over a decade [82].

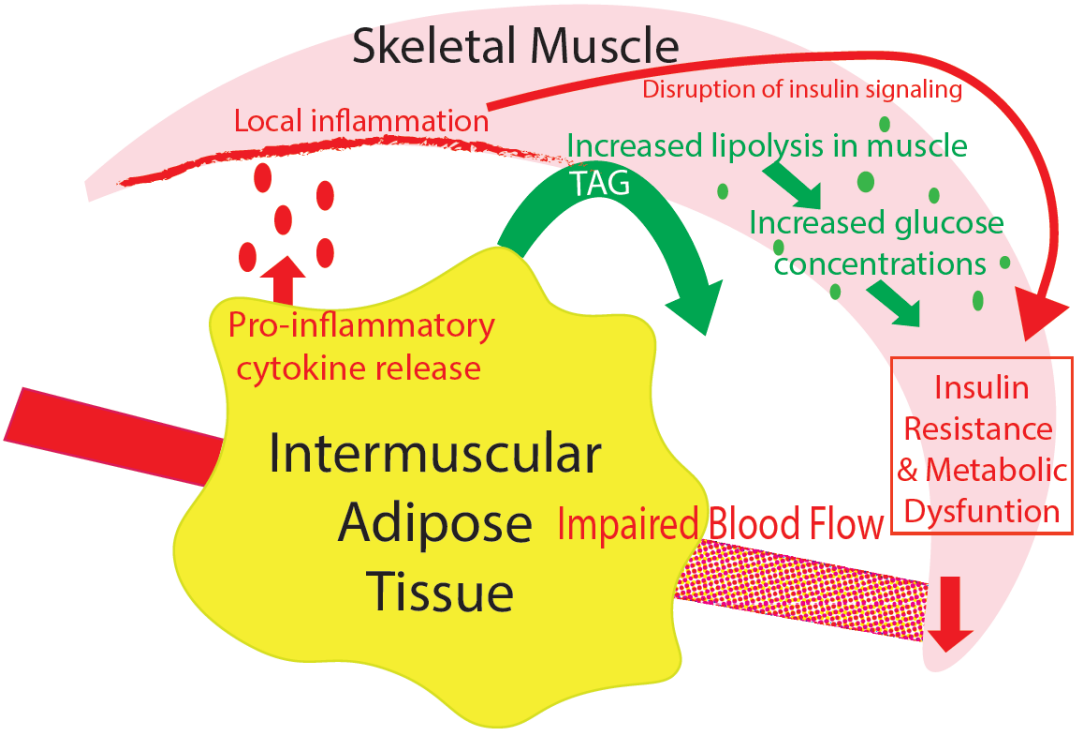

Figure 1.1. Intermuscular adipose tissue disruption of local metabolism. TAG, Triacylglycerol

The importance of documenting and categorizing adipose infiltration in skeletal muscle is now recognized, but there are numerous methodological burdens that still limit our understanding of this ectopic fat depot. Simply, quantification of whole-body IMAT is an expensive and labor-intensive task [69]. MRI is considered the superior technique for quantifying IMAT, as it possesses greater sensitivity than computed tomography and allows direct measurement of tissues [83, 84]. When quantifying IMAT via MRI, manual or computer-assisted segmentation of each slice of interest is performed. In addition to being time-consuming, reliable segmentation across different tissues is challenging. To address this, lower limb segments, typically of the thigh [73, 76, 77, 85, 86] or calf [70, 80, 81, 87], are analyzed and interpreted as being representative of whole-body IMAT infiltration. There is a strong potential

that these surrogate measures may not actually be representative, as IMAT dispersion may not be consistent across tissues, instead infiltrating specific muscles or muscle compartments to greater or lesser degrees [70, 87]. This calls into question the current understanding of IMAT on health, as the current body of literature rests on synthesis of various imaging techniques and extrapolated inferences. The study described in Chapter 2 was designed to address this knowledge gap.

1.4. Significance of lean mass and skeletal muscle in health

1.4.1. A note on terminology

Fat-free mass and lean mass are often (perhaps mistakenly) used interchangeably as proxies for skeletal muscle mass. However, lean mass and fat-free mass are not the same. Lean mass includes the mass contributions from muscle in addition to bone, internal organs, and connective tissue. The key difference between lean body mass and fat-free mass is that lean body mass includes a non-negligible proportion of essential fat found in bones, organs, and muscle. These terms – skeletal muscle mass, lean mass, and fat free mass – will be used interchangeably herein, as there is a strong correlation between densitometric measurements of lean mass and with direct MRI-quantified measurements of skeletal muscle mass [88, 89].

1.4.2. A central role of muscle in health and wellness

Skeletal muscle mass represents the other half of the body composition equation (or body fat percentage), along with fat mass. In addition to the obvious contribution of skeletal muscle towards locomotion, skeletal muscle has other important functions. Skeletal muscle plays a pivotal role in whole-body protein metabolism by functioning as a reservoir of amino acids (AA) to supply to other tissues to maintain protein synthesis in the absence of nutrient supply [90, 91]. Skeletal muscle mass is also central to carbohydrate metabolism as it is the primary site of insulin-mediated glucose uptake. Adequate lean mass promotes glucose disposal and maintenance of euglycemia [92]. Increased lean mass can promote reduced adiposity due to the increased energy required to maintain muscle mass [93]. Resting energy expenditure is the largest component of total daily energy expenditure (except in athletes), and differences in lean mass account for the majority of differences between individuals in resting energy expenditure

[94]. Protein turnover required to maintain lean mass is energetically expensive and inefficient, which is perhaps a good thing in our obesogenic environment.

There is a strong interplay between genetic and environmental factors both in determining peak muscle mass, and in the capacity to maintain mass throughout the lifecourse [95]. Factors that contribute to total muscle mass include height [96], race [97], physical activity [98], hormonal factors [99], and dietary patterns [100]. The importance of skeletal muscle is perhaps best appreciated by the contrapositive – by understanding the causes and consequences of inadequate muscle mass on health and disease.

1.4.3. Sarcopenia and muscle quality

Skeletal muscle mass typically increases throughout the first three decades of life, where muscle mass peaks, thereafter gradual losses can be observed [97]. After age 50, these gradual losses in lean mass are often in the range of 1-2% per year, with proportionately greater losses in strength equating to losses of 1.5 – 5% per year [101]. This acceleration of muscle loss is typically referred to as "sarcopenia". Sarcopenia, as a term, has been used very loosely in the past few decades. Questions such as, 'Since skeletal muscle mass loss occurs in all older adults, do all older adults have sarcopenia?' and 'What are the thresholds we should consider maladaptive?' have afflicted the research and practitioner communities for years and resulted in a lack of study consistency. While the term is still used in myriad ways, there have been recent attempts to reach a consensus definition of sarcopenia that can be used worldwide [102]. Sarcopenia is often accompanied by dynapenia, which denotes a loss of strength or function [103]. The new definition of sarcopenia heavily incorporates aspects of muscle strength and function, as newer evidence indicates that losses in strength and function better predict adverse outcomes than losses in mass [104, 105]. Therefore, the operational definition of sarcopenia includes three criteria, 1) low muscle strength, 2) low muscle quantity or quality, and 3) low physical performance. Probable sarcopenia is identified by the presence of one criterion, diagnosis is confirmed with two criteria, and sarcopenia is considered severe if all three criteria are documented [102].

Low muscle strength is determined by cut points for grip strength (<27 kg for men and <16 kg for women) and chair stand (> 15 s for five rises). Low performance is determined by cut points for gait speed (≤ 0.8 m/s), the short physical performance battery (≤ 8 point score), timed-

up-and-go test (≥ 20 s), and the 400 m walk test (non-completion or ≥ 6 min for completion). Curiously, there is no cut point to diagnose low muscle quality in the new sarcopenia definition, despite cut points being present for the other criteria. Indeed, the term 'muscle quality' appears with some ambiguity in the literature, with definitions for both ratios of strength (knee-extension strength to thigh muscle cross-sectional area [106]), to ratios of tissue (fat to muscle tissue ratio per a given muscle cross-sectional area [107]). It should not be too surprising that there is no consensus in the literature for the determination of muscle quality, given it is a relatively recently coined term and concept. Given the increasing importance of this concept and its inclusion in the sarcopenia definition, there is even greater need to conduct Study 1 to more fully understand the contributions of intermuscular adipose tissue: skeletal muscle in different depots.

1.4.4. Categorizations of sarcopenia

Given how the early definition of sarcopenia was overly broad and inclusive, sub-categories of sarcopenia were devised. Firstly, there is primary and secondary sarcopenia [108]. Primary sarcopenia, the most common form of sarcopenia, is ascribed when there is no other root cause identified. For example, the losses in muscle mass and strength are attributed to aging. Secondary sarcopenia is when there is an identifiable underlying cause, which is often a chronic systemic disease. Sarcopenia can also be classified as chronic (≥6 months) or acute (<6 months). Acute sarcopenia is often a result of illness or some injury causing an individual to be sedentary or bedridden. Indeed, older adults are even more susceptible to inactivity-related muscle loss; profound losses of skeletal muscle mass are seen in as little as 10 days [109]. Lastly, there is the growing phenomenon of sarcopenic obesity – losses in muscle mass and strength in a context of maintained or increasing adiposity [110]. While sarcopenia is often envisioned as a general progressive loss of body mass (both muscle mass and fat mass), sarcopenic obesity is quickly becoming the norm in older adults [111].

1.4.5. Mechanisms of sarcopenia

The fact that there is no consensus unified model delineating the developmental origins of sarcopenia is unsurprising considering the broad spectrum of potential root causes of muscle mass and strength decline. Sarcopenia has a multifactorial etiology, with hormonal,

immunological, nutritional, physical activity, and neurological factors all potentially contributing to its development [112].

Previously, losses in skeletal muscle mass were thought to be representative of losses in muscle strength and function to the point where mass was largely used as a proxy variable for function. New evidence has overturned this notion, as losses in strength and function far outpace losses in muscle mass with age [113]. Changes in the properties of skeletal muscle and 'muscle quality' with age may cause this proportionately larger decrease in strength and function. Infiltration of adipose into skeletal muscle (IMAT) is increasingly seen in the progression of sarcopenia, constituting upwards of 15% of muscle cross-sectional area in sarcopenic older adults versus ~6% in younger adults [114, 115]. Therefore, solely measuring changes in muscle mass with age may yield erroneous results as progressively larger portions of contractile skeletal muscle mass are replaced with non-contractile adipose tissue, resulting in reduced 'muscle quality'.

In addition to reducing contractile properties, IMAT infiltration in skeletal muscle can result in a state of chronic low-grade inflammation through the release of pro-inflammatory cytokines [116]. This state of chronic inflammation can cause or exacerbate insulin resistance and potentiate decrements in muscle quantity and quality [78]. Insulin is a potent anabolic signal and permissive of muscle protein synthesis (MPS). Therefore, insulin resistant muscles reside in a state favoring catabolism [117]. Indeed, insulin resistance is predictive of reduced muscle strength [118].

Chronic inflammation and insulin resistance are further enhanced in sarcopenic obesity [119]. While aging is associated with reductions in anabolic hormones such as insulin-like growth factor I and testosterone, these anabolic hormones are further suppressed with excess adiposity, as seen in sarcopenic obesity [120, 121]. This creates a double-hit to anabolic hormones, which has been associated with the lower than predicted muscular strength reported in adults with obesity [122].

A host of other interrelated factors converge to further reduce muscular strength in sarcopenia. Muscle strength is diminished by neuropathic processes with age which result in a loss of motor unit recruitment and subsequent force output [123]. The loss of motor units, combined with an documented upregulation of muscle cell apoptosis in sarcopenia, ultimately results in a marked decrease in the total number of muscle fibers in sarcopenia [112].

Interestingly, there is a proportionately greater loss of skeletal muscle mass in the lower limbs, compared to the upper limbs (15% versus a 10% decrease, respectively) [124]. The greater loss of mass in the legs is thought to be due to a detraining effect, as even functionally unimpaired older adults are less physically active than their younger counterparts [125]. Collectively, this underscores the profound interrelations between skeletal muscle health and cardiometabolic health, which research included in this document collectively attempts to bridge. As this review is not exhaustive, a more comprehensive mechanistic overview of sarcopenia can be found elsewhere [112].

1.4.6. Consequences of sarcopenia

Estimates of the prevalence of sarcopenia are mixed, likely due to the range of definitions and inadequate classifications previously discussed. Nonetheless, the prevalence of sarcopenia may exceed 24% in older adults aged 65 to 70 [126]. The physical, social, and mental health costs of sarcopenia are great. Functionally, sarcopenia is associated with a greater risk of falls [127], severely impairs mobility [128], reduces the capacity for older adults to perform activities of daily living [129], reduces independence [130], and generally leads to a reduced quality of life [131]. Clinically, sarcopenia is independently associated with hypertension [132], worsened lipid-lipoprotein profile [133], and greater all-cause mortality (HR = 1.32, 95% CI: 1.04–1.69) [134]. In regards to older adults who are hospitalized, the presence of sarcopenia is associated with poor outcomes in surgical and medical studies [126, 135, 136]. More specifically, sarcopenia is predictive of longer hospitalization duration [137], increased post-surgical complications [138], and greater in-hospital mortality [138]. These undesirable physical outcomes are costly to the medical establishment. Even in hospitalized sarcopenic adults who successfully undergo surgical treatment, more post-operative care is required as sarcopenic adults are typically discharged to outpatient care at an average differential cost of ~$16,500, compared to non-sarcopenic adults [139].

1.4.7. Women and sarcopenia

Women may be at greater risk of experiencing the negative outcomes associated with sarcopenia. Sarcopenia appears to afflict both sexes equally at a population level, but women

lose relatively more lean body mass than men from age 20 to 80 (27% versus 18% loss, respectively) [124]. Interestingly, amongst the 'youngest-old', or adults aged 60-70, there is a markedly higher prevalence of sarcopenia in women [140]. This more rapid transition to a sarcopenic state may mirror the more rapid decline in sex steroid concentrations following menopause, relative to men who experience a more gradual decline [141, 142]. Due in part to a smaller physiologic reserve, women are at greater risk of falling below the disability threshold – the threshold of muscle mass required to maintain independence and mobility [112]. Sarcopenia and sarcopenic obesity may have more severe consequences in women, relative to men [119]. Indeed, the negative impact of sarcopenia may be disproportionately higher in women, as they report greater functional impairment from obesity with age [143]. Further, NHANES data indicate an increased all-cause mortality rate in women with sarcopenia, relative to men [134]. These outcomes may be further highlighted by the greater longevity of women, making it more likely that that they will require institutional care.

1.4.8. Treatment models for sarcopenia

Sarcopenia is largely recognized as a condition afflicting older adults, so treatment models were previously focused solely on intervening in this demographic. However, it is more suitable to use a life course model of sarcopenia, as status of muscle mass and function later in life are not just a product of the rate of loss, but also related to the maximal amounts attained in early adulthood [144]. This life course model may extend even back to birth, as there is consistent epidemiological evidence (replicated in 10 studies in various populations) of programming where low birth weight was predictive of both diminished grip strength and muscle mass in older adulthood [144]. As some degree of muscle mass and strength loss will inevitably occur, developing a larger physiologic reserve earlier in life would allow individuals to decline further before reaching the disability threshold. This underscores the importance of beginning behavioral interventions early and identifying population groups that may be predisposed to severe sarcopenia later in life. The recommended front-line treatment for sarcopenia is physical activity, with the strongest recommendations for resistance exercise, and conditional recommendations for consumption of a protein-rich diet or protein supplementation [145]. The following sections will review the current understanding of protein and amino acid regulation of skeletal muscle.

1.5. Protein and amino acid regulation of skeletal muscle

1.5.1. mTOR-dependent regulation of skeletal muscle

The mTOR pathway (now known as '*mechanistic* target of rapamycin') is more nuanced than originally thought; new information is constantly amending our understanding of this pathway despite being discovered over 25 years ago. mTOR is a serine-threonine protein kinase that interacts with numerous proteins to form either mTOR complex 1 (mTORC1) or 2 (mTORC2). The mTORC1 pathway is sensitive to input from AAs, stress, energy status, oxygen availability, and growth factors, while mTORC2 is only responsive to growth factors [146]. As this section focuses on AA regulation, discussion will center on the mTORC1 pathway. While there are many inputs and potential for cross-communication, upstream activation of mTORC1 can largely be separated into tuberous sclerosis 1 & 2 heterodimer-dependent (TSC1/2) and TSC1/2-independent pathways.

For most inputs, TSC1/2 is the primary upstream regulator of mTORC1. TSC1/2 can stimulate mTORC1 via GTP-bound Rheb interaction, or blunt activation by converting Rheb to its inactive GDP-bound form [147]. Phosphorylation of TSC1/2 inactivates it, allowing GTP-bound Rheb interaction with mTOR. PKB, ERK1, and RSK1 all phosphorylate TSC1/2, thus they are considered to stimulate the mTORC1 pathway [148, 149]. Notably, AAs activate mTORC1 independently of TSC1/2, unlike most of the other inputs described (oxygen, growth factors, energy, stress) [150]. The precise mechanism by which 'AA sensing' occurs is under investigation. AAs uniquely stimulate mTOR by initiating translocation from the cytoplasm to the lysosomal surface [151]. AAs must be present for *any signal* (even ones in the TSC1/2 pathway) to activate mTORC1 because GTP- Rheb only interacts with mTORC1 when at the lysosomal surface [152]. Thus, AAs are necessary for mTORC1 activation; other positive signals cannot override an AA deficit to stimulate mTORC1. This implicates AAs as having a particularly central role in regulation of mTORC1-dependent promotion of skeletal muscle homeostasis.

Once activated, mTORC1 exerts downstream metabolic effects primarily through phosphorylation of S6K1 and 4E-BP1 which ultimately promotes muscle protein synthesis (MPS) via enhanced translation initiation and translation elongation [153]. mTORC1 also increases expression of genes involved in oxidative metabolism and glycolytic flux, consistent with its largely energy-consuming functions previously discussed [154, 155]. Outside of its

directly anabolic functions, mTORC1 is indirectly anabolic by downregulating cellular degradation via suppression of kinases required to initiate autophagy [156]. Collectively, mTORC1 promotes MPS and skeletal muscle mass accretion.

1.5.2. Amino acid sensing, muscle protein synthesis thresholds, and the role of leucine

The precise mechanisms by which AAs are sensed in the mTORC1 pathway is still under investigation. Nonessential AAs (NEAA) may not be required to stimulate mTORC1 and subsequently MPS. Volpi et al. assessed whether NEAAs are required to stimulate MPS [157]. Groups were given either 18 g EAAs or 40 g balanced AA (18 g EAA + 22 g NEAAs) in small doses in 10-minute increments over a three-hour period. There were no differences between EAA and mixed AA groups with respect to MPS, despite greater AA provision in the balanced AA condition. These findings implicate EAAs are the primary driver of AA-induced stimulation of MPS. An important consideration is that 18 g EAA could maximally stimulate MPS, in which additional AAs (EAA or NEAA) would not be expected to further increase MPS. Indeed, findings from Paddon-Jones et al. indicate that ~15 g of EAAs are required to maximally stimulate MPS [158]. Despite this limitation in the Volpi et al. study, research indicates that EAA-only supplementation can increase MPS to a similar extent as mixed AA solutions [159].

Substrate requirements for acute stimulation of MPS can be further refined. Bolus feeding of BCAAs are capable of stimulating MPS, while bolus feeding under similar conditions with arginine, glycine, and serine do not have the same effect and fail to stimulate MPS [160]. Anthony et al. demonstrated that of all the BCAAS, leucine was unique in stimulating skeletal MPS above control conditions [161]. Leucine-induced elevations in MPS were achieved through greater phosphorylation of eIF, 4E-BP1, and S6K1. Interestingly, leucine continued to stimulate MPS when mTOR was blocked via rapamycin administration, suggesting that leucine-induced elevations in MPS do not occur solely through the mTOR pathway.

For leucine to effectively function as a signaling molecule to increase mTORC1 activity when ingested as part of mixed meal, a minimum threshold must be passed. Evidence for this ‘initiating threshold’ comes from a study by Norton et al. when MPS in the egg-protein feeding condition was 80% higher than that of the soy feeding condition, despite actual leucine content differing by only 10% [162]. This non-linear response (10% higher leucine content, 80% higher MPS) is consistent with a critical minimal threshold being surpassed to initiate MPS. There is

evidence of a minimal threshold for MPS stimulation in response to complete protein ingestion, as well. One study assessed MPS in response to 80 g protein distributed in a bolus pattern (2x40 g protein, every 6 h), intermediate pattern (4x20 g protein, every 3 h), or a pulse pattern (8x10 g protein, every 1.5 h). Results indicated that the intermediate pattern (4x 20 g) had the highest total daily MPS rate. The pulse pattern does not produce a robust enough rise in plasma AA or leucine to initiate MPS, and the bolus pattern does not stimulate MPS often enough. In a similar experimental protocol, West et al. investigated MPS responses to bolus (25 g whey protein) and pulse feeding patterns (10 x 2.5g, every 20 min) [163]. Despite no difference in net AUC for plasma EAAs, there were greater elevations of MPS in the bolus pattern compared to the pulse pattern.

As there is evidence of a minimal threshold of leucine and protein for stimulation of MPS, there is also evidence of a maximal saturating dose. No further increase in MPS was seen when increasing leucine content from 1.7 g to 2.8 g in one acute feeding study [164]. In another study, however, saturation of MPS in a suboptimal protein meal supplemented with leucine was found to occur at 2.5-3 g leucine [165]. In an acute setting, leucine may even be able to increase MPS to a similar extent as complete dietary protein ingestion. Leucine feeding alone (3 g Leu dose; without provision of any other AAs) is has been shown to robustly stimulate MPS in humans [166]. These data indicate that leucine is effective at maximally stimulating MPS in an acute setting through recruitment of intracellular EAAs. However, the effectiveness of leucine at stimulating MPS chronically is questionable as leucine-only feeding induced elevations in MPS likely deplete intracellular EAA pools, mechanistically suggesting transient effectiveness [160]. Long-term regulation of skeletal muscle by leucine and AA feeding is addressed later in this review.

1.5.3. Amino acid kinetics and relationship with muscle protein synthesis

The relationship between AA ingestion, mTORC1 activity, and MPS is more complex than originally thought. The AA profile of a food may not fully explain the degree to which it increases skeletal MPS, and the MPS response to ingestion of EAAs may not mirror mTORC1 substrate activity [167]. Indeed, the digestion speed and rate of appearance of AAs in the blood may influence MPS responses. Rapid aminoacidemia may increase MPS to a greater degree than prolonged, gradual increases in plasma AAs [168]. Leucine and EAA ingestion (not part as a

mixed-meal; rapid digestion) elevates MPS for ~2 h [169]. However, other factors such as gastric emptying, fatty acid content, fiber content, and insulin release must be considered when investigating MPS responses to a mixed meal.

MPS remains elevated in response to a whole-food mixed-meal containing dietary protein, fat, and carbohydrate for approximately three hours [170]. After this three hour period, MPS returned to baseline despite plasma leucine remained elevated three-fold over baseline levels [170]. Similarly, Bohe et al. conducted a study when EAA were infused continuously for six hours and plasma EAA and MPS was measured [169]. Rates of mixed MPS tracked with plasma EAA concentrations for two hours, as would mechanistically be expected. In agreement with Norton et al.[170] MPS returned to baseline after two hours and did not rise again for the remaining 4 hours, despite EAA levels remaining elevated. Results from Wilson et al. support these findings by reporting a significant relationship between mTORC1 machinery and MPS in the first ninety minutes, followed by a rapid decrease in MPS despite elevated AA and mTORC1 activation [171]. This 'refractory' pattern observed in MPS may explain why rapid and robust aminoacidemia may lead to greater composite MPS than gradual sustained elevations in MPS. This concept has been described previously as the 'muscle-full effect', where AA concentrations are no longer coupled with MPS [172]. Indeed, changes in MPS only mirror increases in mTORC1 signaling during the initial rise in MPS.

While the relationship between initial rise in mTORC1 signaling and MPS is from AA ingestion is well defined, the discordance between AA-induced mTORC1 activation and postprandial duration of elevated MPS is yet to be clearly delineated. One explanation for this discordance could be related to reduced insulin signaling over time post-prandially [171]. Wilson et al. conducted a study to determine if MPS duration could be extended by provision of either leucine or carbohydrate two hours after consumption of a meal. Leucine administration 2 h after feeding was able to sustain MPS similar to carbohydrate provision, despite leucine not commensurately increasing insulin concentration in the plasma. The ratio of AMP to ATP and AMPK phosphorylation in skeletal muscle (energy state of muscle) was posited as the primary limiting factor for MPS 180 min post-prandially. This suggests that substrate availability (AAs) and growth factor signaling (insulin) are not the sole determinants in stimulation of MPS. Protein synthesis is an energetically expensive process; cellular energy deficits may explain why MPS responses to feeding are transient.

1.5.4. Leucine regulation of skeletal muscle in non-acute settings

If leucine increases mTORC1 activation and thus upregulates MPS, one would expect that this would promote accretion of skeletal muscle mass over time. The first step in verifying this extrapolation would be to confirm the persistence of the stimulatory effect of leucine on MPS beyond an acute setting, as seen in Anthony et al.[161]. Casperson et al. addressed this question in a practical setting by supplementing adults consuming the RDA for protein with leucine for two weeks [173]. Leucine supplementation increased postabsorptive and postprandial MPS after two weeks. This suggests that leucine chronically increases MPS (for at least as far as two weeks). These findings were confirmed in a meta-analysis of nine studies in older adults which concluded that leucine supplementation increased MPS relative to control [174].

The next question would be if this chronic increase in MPS actually translates to accretion of lean mass. Casperson et al. observed no change in lean mass from leucine supplementation, but that would not be expected given the inherent error in the method used to detect changes in body mass, and the relatively short time frame of two weeks [173]. Likewise, Verhoeven et al. found no difference in skeletal muscle mass in healthy elderly men supplemented with leucine or a control for a longer period of three months [175]. In accordance with these findings, a recent meta-analysis reported no difference in lean body mass in groups supplemented with either leucine or control [174]. The potential anabolic effects of leucine may be particularly important in higher catabolic states such as bed rest. English et al. conducted a randomized trial to assess the effects of leucine supplementation on skeletal muscle over 14 days of bed rest [109]. Results indicated that leucine supplementation protected against skeletal muscle loss after 7 days, but not after 14 days of bed rest. Collectively, this suggests that the MPS-stimulating effects of leucine are transient, not sustained.

1.5.5. Section conclusions

In conclusion, AA regulation of muscle primarily occurs through alterations in MPS. There is strong evidence that minimal thresholds of leucine and EAAs must be met in order to stimulate MPS and promote optimal skeletal muscle health. However, the saturable dose-response seen in AA-stimulation of MPS must be considered when seeking to apply this knowledge in a practical setting. Despite our mechanistic understanding of AA stimulation of mTORC1 and MPS, there

are many instances where these processes become uncoupled. Additionally, poor concordance between acute studies and long-term trials have been observed. Maintenance and growth of skeletal muscle is a highly regulated process, so it is not unexpected that acute findings typically are not congruent with long-term results. This is particularly true of when taking a hyper-reductionist approach relying solely on measures of anabolism, typified by the promising short-term effects of leucine supplementation not translating to improvements in lean mass. More research investigating the effects of whole food and supplemental protein on physiologically relevant outcomes (such as lean mass accretion) is warranted. The study described in Chapter 3 meets the objective.

1.6. Dietary protein needs and quality

Section 1.6. is adapted from the unpublished narrative review. The dissertation author drafted and first-authored the two included sections.

1.6.1. Influence of dietary protein sources on protein needs and utilization

The current EAR and RDA for protein are defined as quantities of "high" quality protein. Indeed, protein requirements were established based upon short-term nitrogen balance studies involving ingestion of single, high-quality protein sources. Classic studies presented in the 1985 World Health Organization report on protein requirements typically utilized eggs, egg whites, beef, casein, or fish as the protein source; collectively the results from these studies provided an estimated mean requirement at 0.63 g protein/kg per day (range of 0.49 - 0.74) [176].

However, people consume dietary patterns worldwide that differ greatly and contain multiple, highly variable predominant sources of protein and other compounds that impact protein digestion and absorption. Protein requirement studies conducted in 8 different countries using typical mixed diets resulted in estimated protein requirements averaging 20% higher than single high-quality protein feeding studies, at 0.75 g protein/kg per day. The estimated requirements varied among the studies/countries from 0.54 to 0.99 g protein/kg per day. The wide range of requirement estimates among countries suggests that usual mixed diets can influence apparent protein requirements and that in addition to the quantity of protein consumed relative to need, it is important to account for other dietary factors inherent to a country's usual

mixed diet (such as fiber content) which influence protein digestibility. Protein quality is especially important when total protein intakes are low and come predominantly from non-animal sources.

Human health is dependent on both the quantity and quality of protein consumed. The Protein Digestibility-Corrected Amino Acid Score (PDCAAS) was created in response to potential short-comings of nitrogen balance methodology in determining protein quality [177]. PDCAAS is based on the assumption that protein quality can be assessed by expressing the quantity of the first limiting indispensable amino acid in the protein product of interest as a fraction of corresponding limiting amino acid in an 'ideal reference protein', and then multiplying this amino acid score by true fecal nitrogen digestibility [178]. Subsequent to the adoption of PDCAAS in 1991, the Digestible Indispensable Amino Acid Score (DIAAS) has been recognized as a superior method in determining protein quality compared to PDCAAS, but sufficient data using this method are not yet available for practical use [179]. Thus, DIAAS-based determinations of the quality of the vast sources of protein are needed. Until these data are available, evaluations of protein quality are still rooted in PDCAAS data. This is an issue because PDCAAS and DIAAS values were found to be significantly different (10% on average) for 11 of 14 protein sources in male rats [180]. Further, there is not a standard correction that can be applied, such as factoring in a 10% reduced estimate on protein quality if PDCAAS consistently overestimated digestibility by 10%. Rather, PDCAAS appears to underestimate high quality protein sources such as milk protein concentrate, and overestimate low quality protein sources such as oats (**Table 1.1.**).

Table 1.1. DIAAS and PDCAAS in growing male rats for 13 protein sources

Protein Source	**DIAAS**	**PDCAAS**
Milk protein concentrate	1.18	1.00
Whey protein isolate	1.09	1.00
Whey protein concentrate	0.973	1.00
Soy protein isolate	0.898	0.979
Pea protein concentrate	0.822	0.893
Cooked peas	0.579	0.597
Cooked kidney beans	0.588	0.648
Cooked rice	0.595	0.616
Cooked rolled oats	0.542	0.670
Wheat bran	0.411	0.525
Roasted peanuts	0.434	0.509
Rice protein concentrate	0.371	0.419
Corn-based breakfast cereal	0.012	0.078

Adapted from: Rutherfurd, S. M., Fanning, A. C., Miller, B. J., and Moughan, P. J. (2015) Protein digestibility-corrected amino acid scores and digestible indispensable amino acid scores differentially describe protein quality in growing male rats. *The Journal of nutrition* **145**, 372-379

When two sources are combined, the amino acid profiles can be added together quite simply, but the digestibility fundamentally changes, and is an unknown until specific testing on that mixture is done. Due to these concerns, and the non-systematic differences in PDCAAS and DIAAS scoring (overestimating some sources and underestimating others), we did not incorporate digestibility as an explicit consideration and included only amino acid scores and comparisons to the ideal reference in the attached files. Further, Millward et al. concluded, "..the truncation procedure and restriction to only the first limiting amino acid are subject to criticism because these latter issues do not allow expression of the power of a high-quality protein to balance the [indispensable amino acid] composition of inferior proteins [177]." For example, lysine is the limiting amino acid in many foods. Lysine is a notable example of an amino acid which is often chemically modified during processing which leads to inaccuracy in digestibility estimates using traditional analysis methods [181]. Digestible lysine is often overestimated. Thus, caution must be exercised when attempting to apply PDCAAS or even DIAAS scores in formulating products, as analysis of specific amino acids may be required [180]. Also, potential applications of these scores should not be extended to special populations and situations where specific amino acids may be required in higher amounts (e.g. aging, pregnancy, lactation, etc.)[178].

The methods used to assign protein quality to sources are nuanced and not without limitations. With the current available data and potential shortcomings, it is challenging to accurately determine protein quality of foods in a mixed diet. The overall protein quality of protein foods is currently best determined by testing the food using DIAAS, of which there is currently limited data, consistent with the 2016 Food and Agriculture Organization report [182].

1.6.2. Effect of protein intake on indexes of human health and function

The RDA for protein provides an estimate of the minimal amount of protein intake recommended for a person to consume daily to maintain nitrogen balance and avoid progressive loss of body protein over time. Consumption of inadequate protein results in negative nitrogen balance and adverse changes in metabolism, body function, and health that in severe cases may become life threatening. Since the RDA is considered a minimal recommended intake, there is great interest in the effects of consuming protein intakes above the RDA. The majority of people in Western societies regularly consume more protein than the RDA, but this is not the case worldwide. Since this review is focused on apparently healthy people, the following information does not address the critical issue of protein supplementation to restore health of undernourished individuals. Also, while the RDA is a widely recognized standard, most research studies do not specifically use it as a "control" or "reference" intake. More commonly, randomized controlled feeding trials describe subject groups as "lower-protein" or "higher-protein", with or without clearly documenting the actual quantities and sources of protein consumed. Thus, it is important to note that this section will disengage from the RDA and assess the effects of relatively higher-protein (HP) or lower-protein (LP) diets on outcomes of interest.

Body composition: With respect to body composition, a recent systematic review and meta-analysis comparing HP diets (median protein intake 27% of total energy) with LP diets (18% of total energy) from 73 randomized controlled trials (RCTs) with a study duration of 28 days to >12 months, reported that individuals who were consuming a HP diet compared to a LP diet without and with purposeful dietary energy restriction had greater reductions in body mass (standardized mean difference:−0.36, 95% confidence interval: −0.56 to −0.17), and waist circumference (−0.43, −0.69 to −0.16) [183]. These findings are in agreement with another meta-analysis of 24 RCTs with a duration of ≥ 4 weeks where individuals consuming HP diets vs LP

diets had greater body mass loss (0.79 kg; −1.50 to −0.08), fat mass loss (−0.87 kg; −1.26 to −0.48), and greater retention of lean mass during energy restriction (+0.43 kg; 0.09 to 0.78)[184]. However, these results are inconsistent with the findings of another comparable meta-analysis of 15 RCTs specifically only including studies with ≥ 12 months' duration which found no differences in body mass and body composition responses over time between HP and LP diets [185]. In addition to duration of studies included, other key criterion in what constitutes a HP or LP diet could explain the discordant findings in these reviews. A recent review proposed that there must be sufficient 'spread' or difference in protein intake between HP and LP diet groups for anthropometric benefits to be apparent [186]. Wycherly et al. [184] only included studies with ≥ 10% difference in protein intake between LP/HP diet groups, while Schwingschackl et al. [185] included studies where the protein intake difference between groups was smaller (≥ 5%). As noted above, Schwingschackl et al. [185] reported no benefit in lean mass retention, or any anthropometric measure, was observed by higher protein intakes. Perhaps the more modest differences in protein intake could explain the more modest results. Collectively, these data provide evidence of potential modest improvements in body composition parameters from HP diets in shorter-duration trials, but these desired changes were not retained in longer-term interventions.

Lipid-lipoprotein profile: Improving serum lipid profile is a primary goal to reduce cardiovascular disease risk. Fasting triglyceride (TG) values are a significant independent predictor of coronary heart disease risk [187]. High CHO diets are well known to increase TG levels [188]. HP diets consistently result in reductions in TG, but whether this effect is due to increased protein intake or substitution of CHO is debatable [183, 189-191]. Adding credence to the notion that replacement of CHO is the primary means of TG reduction, substitution of CHO with dietary fat also reduces TG levels [192]. However, authors of the OmniHeart trial posited that protein may have a direct TG-lowering effect beyond substitution of CHO due to the HP diet reducing serum TG to a greater extent than the unsaturated fat diet [191]. There is no apparent relationship between dietary protein intake and total cholesterol, LDL-C, and HDL-C from four major meta-analyses included in this report [183-185, 190]. Collectively, these data indicate that increased dietary protein intake reduces TG directly or through substitution of CHO, and that dietary protein does not affect total cholesterol, LDL-C, and HDL-C.

Glucose control: Improved blood glucose control is a proposed potential benefit of HP diets. Controlled intake of CHO is always a concern for diabetics, and there is evidence that substitution of protein for CHO is beneficial in reducing postprandial and fasting blood glucose in this population [193]. However, in the general population, results from multiple meta-analyses indicated no difference between HP and LP diets in fasting blood glucose [184, 185, 190]. Likewise, dietary protein intake did not affect fasting insulin [183, 185]. In conclusion, there is inconsistent and limited evidence that HP diets significantly affect glucose control in non-diabetic individuals.

Skeletal homeostasis: Adequate dietary protein is needed to maintain bone turnover since it is not possible to recycle amino acids for the purposes of collagen synthesis [194]. Daily protein intakes below the RDA are reported to stunt bone formation [195, 196], attenuate peak bone mass [197, 198] and increase the future risk of decrements to bone health [199], specifcally in older adults [200, 201]. However, with origins dating back to the Acid-Ash Hypothesis [202], high protein intakes are implicated in decrements to bone health. Despite these long held contentions, nearly all of the studies conducted on the subject matter have indicated a beneficial relationship between dietary protein and bone mineral content [203]. A meta-analysis of 61 studies revealed strong evidence that higher dietary protein intake was associated with higher bone mineral density and bone mineral content [204]. However, these markers were not conclusive in predicting functional outcomes such as fracture risk. The effects of higher total protein intake (>1.2 $g \bullet kg^{-1} \bullet d^{-1}$) on bone are still not fully delineated and may be dependent upon a number of factors such as source of dietary protein, energy status [205], and activity levels [206]. Collectively, these results support the notion that protein intakes below the RDA are detrimental to bone, whereas protein intakes at and moderately above the RDA promote bone growth and maintenance. The effects of high protein intakes (>2xRDA) on bone, however, require further investigation. **Table 1.2** summarizes the findings described above.

Table 1.2. Summary of the effects of higher-protein diets on indices on indices of health

Health Parameter	Influence of Higher protein diet
BMI, Weight Loss, Waist circumference	Modest improvements over 1-12 months which are not observed beyond 12 months
Triglycerides	Consistent reductions in serum triglycerides*
Total Cholesterol, LDL-C, HDL-C	No effect
Blood glucose, insulin	Limited/inconsistent effect in general population
Bone health	Not harmful, weak/limited evidence of beneficial impact on bone mineral density

**Potential substitution effect (improvement not from increased protein, but from reduced CHO)*

To conclude, higher-protein diets are promoted to improve multiple indices of obesity, body composition, and cardiometabolic health. Results research studies lasting less than 12 months generally support health-promoting effects of higher-protein diets, but not studies greater than 12 months. The impact of higher protein intakes may be direct (mechanistically plausible) or indirect due to concurrent changes in other dietary components (e.g. lower carbohydrate intake). It is also important to note that most of the outcomes described below are indexes of health, not long-term morbidity outcomes or mortality.

1.6.3. Higher protein intake in men & women

There is relatively consistent evidence of modest beneficial effect of higher-protein diets on body composition outcomes across a range of experimental settings [184, 207, 208]. Protein supplementation is promoted as an effective dietary strategy to help adults achieve greater protein intake; evidence indicates that protein supplementation can improve body composition [209-211]. However, women are underrepresented in protein supplementation research, with 15 of 22 studies with male-only populations in the most cited protein supplementation systematic review and meta-analysis [211]. There is some evidence that women may be at greater risk for not reaching protein requirements; the most at-risk demographics being females aged 14-18 y and females ≥71 y (11% and 7% did not reach protein EAR, respectively) [212]. Older adult women not meeting the protein requirements is plainly concerning as they are at a direct increased risk for sarcopenia, and the consequences of sarcopenia are heightened with inadequate protein intake [213]. However, it should be equally concerning that younger women may not be meeting protein requirements, given the new life course paradigm for sarcopenia, discussed earlier. Sufficient protein intake is critical for proper growth and development in adolescents and

younger adults [214]; the importance of building up a physiological reserve of lean mass in younger life cannot be overstated. The public perception that protein supplementation will cause "bulkiness", or excessive body mass, is a real concern that may hinder women from engaging in practices that could help improve their protein-nutrition status and optimal lean mass accretion. Study 2 addresses the issue of protein supplementation and body composition in women in a systematic manner.

1.7. Dietary protein and cardiometabolic health: a closer examination of blood pressure

Thus far, both components contributing to body composition – fat mass and lean mass – and issues surrounding body composition – excess adiposity (obesity, IMAT infiltration) and inadequate lean mass (sarcopenia) – have been discussed. Dietary protein has been highlighted as a nutrient in this discussion that can be modulated to improve health, as mediated *through* changes to lean and fat mass. But what about the effects of dietary protein beyond those on body composition? While the protein literature is rife with studies with skeletal muscle anabolism outcomes, protein receives little attention concerning its potential to impact indexes of cardiometabolic health, relative to dietary fat and carbohydrate.

Hypertension is one of the primary contributors to development of cardiovascular disease. An estimated 1 billion individuals worldwide meet criteria for hypertension, with up to 7.1 million deaths per year attributable to hypertension [215, 216]. The burden of hypertension and often resultant cardiovascular disease is only expected to rise in the following years as our aging population grows. This is due to increasing age being a major factor in hypertension development. The prevalence of hypertension is greater than 50% in Americans aged 60-69, and greater than 75% in those above the age of 70 [217]. For this reason, strategies to prevent the development and effectively treat hypertension are a top priority for many individuals in health-related professions.

Incidence of hypertension has been on the rise during recent decades [216]. However, the impact of hypertension on cardiovascular endpoints has been mitigated by successful screening and pharmaceutical management. It was stated in the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure that, "Between 1960 and 1991, median SBP for individuals ages 60-74 declined by approximately 16mm Hg…Since 1972, age-adjusted death rates from stroke and coronary heart disease have

declined by approximately 60 and 50 percent, respectively" [216]. Studies have reported that even modest reductions in population-wide blood pressure can reduce the burden of blood pressure-related diseases; as little as a 2mm Hg reduction in diastolic blood pressure could result in a 17% decrease in the prevalence of hypertension and a 6% reduction in the risk of coronary heart disease [218].

Despite the marked improvements in treatment of hypertension over the past decades, primary prevention should remain the principal goal. Onset of hypertension is attributable to both genetic and environmental factors. As modulating genetic factors is outside of our control, prevention of hypertension must be accomplished through attenuating environmental risk factors. Diet is potentially the strongest environmental factor influencing blood pressure [219]. This warrants efforts to delineate the impact of diet on blood pressure.

There is an extensive body of literature providing strong evidence of the efficacy of a few dietary and diet-related factors in preventing and treating hypertension. There is strong evidence that increasing potassium intake and reducing intake of both sodium and alcohol reduce blood pressure [219]. Meta-analysis of randomized trials revealed that 5.1 kg weight loss resulted in reductions in systolic and diastolic blood pressure by 4.4 mm Hg and 3.6 mm Hg, respectively [220]. A dose response was apparent as well, as those with greater weight loss presented with greater reductions to blood pressure. Further, these improvements to blood pressure by individual dietary modifications are compounded when combined [221]. Outside of these established factors, the impact of other nutrients of interest such as calcium, magnesium, fiber, and protein on blood pressure are relatively less clear.

While the literature is reasonably consistent on the effect of the aforementioned dietary factors on hypertension, the role of protein in blood pressure regulation is far less clear. Decades ago, protein had been historically linked to increased blood pressure, as noted in a prior review [222]. Considerable interest in protein as an agent to decrease blood pressure originated from epidemiologically derived findings in the mid-1990s [223, 224]. INTERSALT researchers reported that 24-h urinary excretion of total nitrogen and urea nitrogen, a marker of protein intake, were inversely related to systolic and diastolic blood pressure [223]. The Multiple Risk Factor Intervention Trial, which strengthened these relationships by incorporating a more direct dietary link (24-h dietary recall), reported that dietary protein was inversely related to blood

pressure [224]. The stage was set for deeper, evidence-driven research questions to be posed regarding dietary protein and its relationship to blood pressure.

Recent meta-analyses and trials suggest that increased dietary protein results in modest reductions in blood pressure [183, 225-228]. While this is a direct statement, the relationship between dietary protein and BP is quite nuanced. One must consider what we are talking about when we say 'protein'. In the most reductionist manner, we can address this question by delineating the mechanistic underpinnings by which specific amino acids influence blood pressure. Zooming out, we can determine how the macronutrient 'protein' influences blood pressure. However, individuals do not eat isolated macronutrients — they eat food. As such, we may seek to address how protein-rich foods and constituent bioactive peptides, nutrients, and nonnutrients affect blood pressure. Lastly, there is the issue of dietary patterning to consider, in which we would want to answer, "How does higher- vs lower-protein diets influence blood pressure?" in the broadest sense. In the following paragraphs, a background on the physiology of blood pressure regulation (with special consideration towards aging), and a primer on key considerations in interpreting dietary protein and blood pressure research is provided.

1.7.1. Mechanisms of dietary patterning and blood pressure

Increasing intake of dietary protein without concomitant increase in total caloric intake entails that another macronutrient is being substituted. In the Omniheart trial, isoenergetically replacing carbohydrate with protein resulted in significant decreases in blood pressure [191]. However, similar results were obtained in this trial by substitution with monounsaturated fat. This introduced the concept that increased dietary protein intake may not necessarily drive the decrease in blood pressure. Rather, carbohydrate may play an active role in increasing blood pressure and consuming less is antihypertensive regardless of the macronutrient taking its place (fat or protein).

As such, the chronic antihypertensive effect of increased dietary protein is most likely mediated through attenuation of insulin resistance. Insulin resistance and hyperinsulinemia (potentiated by high-carbohydrate diets) will result in chronic elevations in blood pressure [229, 230]. Henceforth, the following paragraphs include findings under the premise that higher-protein diet improves glycemic regulation and thus *indirectly* reduces blood pressure [185, 231, 232].

Mechanistically, higher protein diets shift the regulation of blood glucose to amino acid-mediated hepatic glucose production from insulin-mediated peripheral glucose disposal [233]. This shift is important, because there is less reliance on efficient postprandial peripheral glucose disposal with lower-carbohydrate meals. Large glucose loads from high carbohydrate meals results in robust hyperinsulinemic responses to partition blood glucose. Repeated cycles of this manifests as peripheral insulin resistance. Pancreatic β-cells compensate via hypertrophy and hyperplasia to produce even more insulin, but eventually these cells burn out and β-cell hypotrophy and cell death follow. Thus, the shift away from insulin-mediated glucose disposal towards increased reliance on hepatic gluconeogenesis helps to maintain euglycemia, thereby putting less stress on pancreatic β-cells and preserving insulin sensitivity of peripheral tissues.

It is important to distinguish between the effects of insulin in normal "healthy" individuals and in those with insulin resistance. Insulin is pleiotropic, inducing multiple and often antagonistic physiological effects. For example, insulin has a well-defined capacity to stimulate renal sodium reabsorption which will increase fluid volume and should theoretically *increase* blood pressure [234]. Conversely, insulin is also a potent peripheral vasodilator, resulting in reduced peripheral vascular resistance and should theoretically *decrease* blood pressure [235]. Conceptually, this makes sense because if the function of insulin were to increase glucose uptake in the periphery, increased blood flow would aid in glucose disposal. The net effect of insulin on blood pressure is mediated by insulin sensitivity. In healthy individuals, the net effect of these two competing mechanisms is typically an acute postprandial reduction in blood pressure [236]. However, the sodium-retaining effect of insulin is more pronounced and inappropriately preserved in individuals with insulin resistance, leading to chronically elevated blood pressure [237]. The vasodilatory effect of insulin is blunted in individuals with insulin resistance, contributing to chronic endothelial dysfunction [238]. Endothelial dysfunction in insulin resistance is exacerbated by inherent sustained elevations and activity of endothelin-1, which acts as a vasoconstrictor to increase blood pressure [239].

One potential mechanism by which higher protein diets can indirectly reduce blood pressure is by attenuation of oxidative damage via improvement of insulin sensitivity. Acutely, high carbohydrate meals result in large glycemic oscillations, which is a potent oxidative stressor [240]. Insulin resistance results in an impairment of PI3K-dependent signaling in the endothelium, leading to an imbalance between endothelin-1 and nitric oxide (NO) production

[238]. Tetrahydrobiopterin (BH_4) is a critical cofactor for endothelial nitric NO synthase (eNOS). Hyperglycemia characteristic of insulin resistance results in increased production of reactive oxygen species through a currently unknown mechanism [241]. Increased concentration of superoxide, as seen in insulin resistance, will oxidize BH_4 to the inactive BH_2, thus reducing eNOS activity and NO production.

Individuals with insulin resistance display increased sympathetic nervous system activity, which directly and indirectly influences blood pressure [242]. Directly, SNS overactivity increases cardiac output. Indirectly, increased blood pressure via SNS overactivity is mediated through the renin-angiotensin system (RAS). The SNS and RAS display a positive feedback relationship, such that the chronically increased SNS activity will upregulate RAS activity [237]. This will result in elevated plasma aldosterone, increasing sodium reabsorption and blood volume.

1.7.2. Mechanisms of amino acids influencing blood pressure

Particular amino acids can exert their antihypertensive effect through numerous and diverse mechanisms. Arginine is a prime example of an amino acid that can influence blood pressure both directly and indirectly. Arginine is posited to have the most profound observable effects of all the amino acids on blood pressure, thus arginine will be the focus of this section.

Arginine exhibits direct antihypertensive effects by virtue of it being a key substrate in synthesis of the vasodilator nitric oxide (NO) [243]. NO plays a central role in endothelial function. In response to stimuli which would invoke a vasodilatory response, eNOS produces NO which results in vascular smooth muscle relaxation. Circulating fasting arginine concentrations typically range from 40-100 umol/L, yet 3 umol/L arginine can induce half-maximal activity eNOS activity [244]. Given this information, one may ask how arginine from dietary protein can increase NO-induced vasodilation when the enzyme is saturated at concentrations far below the physiological range. Asymmetric dimethylarginine (ADMA) is a product of post-translational methylation of arginine and acts as a competitive inhibitor of eNOS [245]. This competitive binding of eNOS inhibits NO production and results in vasoconstriction [246]. The ratio between arginine and ADMA is critical to NO synthesis and activity. Thus, arginine intake can improve NO-mediated vascular functions by overcoming competitive inhibition by ADMA [244]. This mechanism is supported by clinical data, as a recent meta-

analysis of randomized controlled trials supplementing arginine reported that arginine significantly lowered systolic blood pressure (-5.39mm Hg, 95% CI: -8.54 to -2.25) compared to control [247]. In addition to serving as a substrate for NO synthesis, arginine also locally and systemically serves as an antioxidant. As previously discussed, BH_4 is an essential cofactor for eNOS that is susceptible to oxidative damage. In the endothelium, arginine functions to prevent BH_4 oxidation to BH_2 and reduces ROS generation from vascular endothelial cells, both supporting eNOS activity [248]. In circulation, arginine can react with hydrogen peroxide to generate NO, which is doubly effective as it decreases an agent that can induce vasoconstriction (ROS) and generates a vasodilator (NO) [249].

Indirectly, arginine can acutely reduce blood pressure due to its insulinotropic effects on pancreatic β-cells [250]. Specifically, arginine stimulates membrane depolarization, permitting Ca^{2+} influx, which activates protein kinase C, and protein kinase A, thereby potentiating glucose-induced insulin secretion [251]. As discussed previously, insulin is a potent vasodilator and decreases postprandial blood pressure. Arginine also decreases activity of the renin-angiotensin system through ACE inhibition, resulting in a decrease in angiotensin II [248].

Other amino acids participate in mechanisms contributing to the modest hypotensive effects of dietary protein, albeit in a less robust and more indirect fashion than arginine. Notably, cysteine and glutamate – as part of the potent antioxidant GSH – contribute to reduced blood pressure through preservation of eNOS, as previously described. Tryptophan can also indirectly lower blood pressure via reduced synthesis of catecholamines, thus reducing cardiac output [252].

In conclusion, with the exception of arginine, the mechanisms by which amino acids influence blood pressure are relatively modest and indirect. The antihypertensive effects of dietary protein likely manifest largely as a result of carbohydrate substitution, with the greatest chronic effects realized by prevention of insulin resistance.

1.7.3. Physiology of blood pressure regulation and aging

The cause of increased systolic blood pressure with increasing age is multifactorial. Some of the factors that contribute to increases in blood pressure are modifiable, while others are inevitable physiological changes that are inherent to aging. Other factors (such as obesity) that contribute to hypertension are not exclusive to age, rather older adults are simply more likely to

become obese than their younger counterparts, thus increasing mean blood pressure. This section will review modifiable factors, inevitable factors, and associative factors in an effort to delineate the relationship between chronological age and blood pressure.

Endothelial Dysfunction in aging

Blood pressure is ultimately a product of cardiac output, blood volume, vessel elasticity, and peripheral resistance. Regarding endothelial function, we are concerned primarily with latter two factors: elasticity and peripheral resistance. In response to the heart contracting and expelling blood (systolic phase), healthy arteries are expected to expand to absorb this shock. In the diastolic phase, healthy elastic arteries will recoil to maintain normal blood flow. In 'unhealthy' arteries that have become rigid, this critical elastic potential is blunted. Hence, the increase in systolic blood pressure and decrease in diastolic blood pressure that typically occurs with increased age can largely be attributed to endothelial dysfunction.

Peripheral resistance, as referenced earlier, also contributes to endothelial function. Peripheral resistance is determined by blood vessel diameter, blood viscosity, and total vessel length. Only blood vessel diameter is will be discussed in this review as the potential for changes in blood viscosity and vessel length are not entirely relevant to endothelial function and thus can be considered outside the scope. Under the same cardiac output, blood pressure will be higher in a smaller blood vessel compared to a larger blood vessel.

NO plays a central role in endothelial function. It has been described as the most important endothelium-derived molecule [253]. In response to stimuli which would invoke a vasodilatory response, endothelial NO synthase (eNOS) produces NO which results in vascular smooth muscle relaxation. This mechanism, known as NO-mediated endothelium-dependent dilation, plays a critical role in maintenance of vascular tone. Generally, NO supports an endothelial environment characteristic of healthy arteries by the balance of molecules in the endothelium inhibiting coagulation, proliferation, inflammation, and vasoconstriction [254]. While age-related decrements to endothelial function can be partially attributable to upregulation of vasoconstrictor molecule production (increased synthesis of endothelin-1 and prostaglandins), reduced NO bioavailability is believed to largely explain this phenomenon [253, 255].

NO production by eNOS decreases with increasing age largely due to oxidative damage. Increased oxidative damage is likely an inevitable consequence of aging as a result increased

superoxide production without commensurate increases in antioxidant defenses [256]. Excessive intracellular superoxide quantities will result in oxidation of BH_4, which is an essential cofactor in eNOS. Without a functional cofactor, eNOS activity will be reduced, ultimately resulting in reduced NO production.

An acute effect of reduced NO bioavailability is increased blood pressure. As NO is also involved in regulation of vascular structure, reduced NO bioavailability can also contribute to vascular remodeling which can occur with aging. The capacity for physiologically appropriate vasodilation and recoil is partially determined by the ratio of elastin to collagen fibers in the media [253]. Repeated cycles of vascular distension and recoil, which naturally occurs throughout the life course, can contribute to fragmentation of elastin fibers in the endothelium. Typically, elastin would be replenished in this normal turnover process. However, during the aging process, the more rigid collagen fibers are increasingly deposited in place of elastin with age. Vascular remodeling is largely a product of the cross talk-between calpain-1 and matrix metalloproteinases (MMPs, MMP2 in particular) [253]. For reasons not entirely clear, calpain-1 expression is upregulated during aging. Again, oxidative damage appears to have a central role driving age-related endothelial changes as MMP activity is increased in the presence of these molecules. The net result is elastin gradually being replaced with collagen with age leading to increasingly rigid, noncompliant arteries.

Increased rigidity of arteries also negatively affects autonomic nervous system regulation of blood pressure. Arterial baroreceptors monitor blood pressure (stretch) and can rapidly and reflexively respond to stimuli to regulate blood pressure via stimulation of either the sympathetic (vasoconstriction, increase heart rate and stroke volume) or parasympathetic (decrease heart rate) systems [257]. Essentially, if the endothelium is not compliant, the baroreceptors cannot properly regulate blood pressure at the autonomic nervous system level. There is some evidence that the blunting of baroreflexes associated with aging is due to decreased cardiac cholinergic responsiveness punctuated by acetylcholine not properly functioning in parasympathetic nervous system to decrease blood pressure [258]. However, a change in mechanical properties with age (loss of elasticity) is likely to be the primary reason for reduced function of a mechanical receptor. These age-related physiological changes result in chronic sympathetic overactivity, leading to increased blood pressure and inflammation.

Metabolic Syndrome in aging

Beyond direct physiological changes that occur with aging, the increased prevalence of the metabolic syndrome and related co-morbidities is highly relevant when investigating causes of hypertension. The prevalence of the metabolic syndrome is <20% among those 20-39 years old compared to nearly 50% in those 60 years or older in the United States [259]. Metabolic syndrome is defined as the presence of 3 or more of the following risk factors: abdominal obesity (waist circumference), elevated triglycerides, low HDL-cholesterol, high blood pressure, or high fasting blood glucose. Considering how this disease disproportionately impacts aging adults, understanding how the metabolic syndrome can impact blood pressure and cardiovascular health has important public health implications.

Metabolic syndrome typically begins with the accumulation of visceral fat (abdominal obesity). While the mechanism is not clear, visceral fat increasingly accumulates with age [260]. Visceral fat can produce adipocytokines such as leptin, tumor necrosis factor-α, and interleukin-6 which induce inflammatory responses [261]. However, the amplified local effect of visceral fat's adipocytokine production on major organs is likely the reason for greater metabolic decrement compared to that of subcutaneous fat.

If abdominal obesity is the primary cause of metabolic syndrome, then insulin resistance is the primary metabolic impairment. The pathogenesis of insulin resistance is multifactorial and not fully elucidated despite strong links to abdominal obesity. As it pertains to hypertension, insulin has been shown to stimulate endothelin-1 production which acts as a vasoconstrictor to increase blood pressure [239]. Individuals with insulin resistance produce an overabundance of insulin, which will lead to sustained elevations in endothelin-1 and thus increased blood pressure. Individuals with insulin resistance display increased sympathetic nervous system activity [242]. Insulin has a well-defined capacity to acutely increase blood pressure by stimulating renal sodium reabsorption which will increase fluid volume and blood pressure [234]. This sodium-retaining effect is inappropriately preserved in individuals with insulin resistance, leading to chronically elevated blood pressure [237].

Inflammation mediates many age-related changes in blood pressure regulation. The increased adiposity of individuals with metabolic syndrome induces chronic low-grade inflammation [262]. Coupled with an increase in inflammation that is characteristic of aging, older adults with obesity are predisposed to a highly pro-inflammatory state. Fluid balance and thus blood pressure

can be impacted by the progressive dysregulation of aldosterone that is characteristic of aging [263]. This will not only cause improper sodium retention but can also increase inflammation. Further, aldosterone dysregulation contributes to sympathetic nervous system overactivity via a positive feedback relationship between these two systems [264]. Collectively, blood pressure regulation is impaired through these various and interconnected pathways in aging adults with metabolic syndrome.

Dietary targets for the maintenance of blood pressure regulation in aging

The first step in determining how diet can positively impact blood pressure regulation with age is to identify mechanisms which are not immutable. Many age-related changes which contribute to hypertension were presented in previous sections, but the origin of many can be traced back to a relatively small set of factors. For endothelial function, we cannot prevent the cycles vascular distension and recoil and subsequent fragmentation of elastin fibers. However, we can use dietary means to improve NO bioavailability and thus better preserve endothelial health.

One way to improve NO bioavailability with age would be to reduce oxidative damage (thus maintaining the essential BH_4 cofactor) through dietary means. Although mechanistically sound, there is little evidence that healthy dietary patterns alone can reduce oxidative damage [265]. However, combination of rigorous low-fat, high-fiber diet and exercise can result in robust improvements in NO bioavailability, oxidative damage, and blood pressure [266]. Another way to overcome NO bioavailability is to supplement or consume high quantities of L-arginine, the amino acid precursor for used for NO synthesis. Arginine supplementation has been proven to be effective in increasing NO availability and endothelium-dependent vasodilation [267]. A meta-analysis of randomized controlled trials supplementing arginine reported that arginine significantly lowered systolic blood pressure (-5.39mm Hg, 95% CI: -8.54 to -2.25) compared to control [247].

It is not possible to completely ablate age-related increases in inflammation and oxidative damage. However, development of abdominal obesity and insulin resistance can be combated effectively through dietary intervention. There is conflicting evidence on the potential for diet composition to differentially impact visceral fat [268]. However, there is experimental evidence that ingestion of sugar-sweetened beverages (such as regular cola products) result in substantial

increases in visceral fat relative to control groups [269]. Dietary- and exercise-induced weight loss has been proven effective in reducing visceral fat and subsequently ameliorating insulin resistance [270]. Dietary interventions to improve insulin sensitivity can curb much of the age-related increases in blood pressure. Dietary and lifestyle modifications to improve insulin sensitivity should be recommended before clinical diagnosis of insulin resistance, as there is evidence that rigorous interventions are required to improve insulin sensitivity in those already with insulin resistance [271]. Collectively, these findings suggest that a lifestyle approach to reduce cardiovascular disease risk should be taken, as improvement in metabolic health becomes increasingly difficult.

1.7.4. Considerations for examining the relationship between dietary protein and blood pressure

While there is a sizable amount of data supporting the inverse relationship between dietary protein and blood pressure [272-277], there is also a significant body of literature which contradicts these findings [278-281]. Despite these discrepancies, there is still a preponderance of evidence that supports a minor favorable effect of dietary protein on blood pressure, as concluded in previous reviews [226, 282, 283]. Evaluation of protein and blood pressure literature is challenging. Stating that dietary protein has a slight favorable effect on blood pressure is perhaps an oversimplification. The impact of dietary protein on blood pressure can vary greatly depending on factors such as hypertension status, age, protein spread, substitution effects, and others. For example, improvements in blood pressure are often markedly greater in hypertensive participants compared to normotensive individuals. The considerations addressed in this section will act as tools to readers to aid in the interpretation of results in the following sections. This will potentially explain some discrepancies, but also is likely to introduce complexity where some relationships seemed apparent.

Protein sources and individual nutrients vs. whole foods

A major consideration that has garnered a significant amount of attention recently has been the attempt to dissect the effects of individual nutrients from those of whole foods and dietary patterns. Particularly, the potential for a differential effect of protein from plant sources or animal sources on blood pressure is of recent interest [284-286]. Many other nutrients and non-

nutrients which demonstrate a capacity to affect blood pressure are captured when one attempts to quantify intake of plant or animal protein. The potential for "plant protein" to be a surrogate marker for other blood pressure-lowering nutrients warrants consideration [225]. One must be careful in attributing the observed differences in markers of cardio-metabolic health to dietary protein quantity alone in these analyses. Blood pressure changes can be ascribed to various factors related to dietary protein (outside of mere quantity), such as specific amino acids [243, 247, 287], peptides [288-290], food sources [281, 291-295], and dietary patterns [191, 296]. Given this potential for blood pressure to be affected at any one of these levels, approaching this research question simply with, "Does protein decrease blood pressure?" does not pay respect to the inherent complexity and can potentially distort interpretations.

Protein spread

Another important factor which may aid in explaining the discordant observed findings is the "protein spread hypothesis" proposed by Bosse & Dixon [186]. This hypothesis was proposed to explain discrepancies in clinical trials examining the impact of protein intake on body composition. The protein spread theory proposed that there must have been "a sufficient spread or % difference in g/kg/day protein intake between groups during a protein intervention to see body composition and anthropometric differences."[186] Authors analyzed 51 studies and reported that on average, anthropometric benefits were observed when the higher protein group presented with a 58.4% g/kg/day between group protein intake spread, while no benefits above control were observed when average between group protein intake spread was 38.8% [186]. As changes to cardiometabolic health typically precede changes to anthropometrics, applying this protein spread theory to blood pressure outcomes seems warranted [297].

Substitution effects

Increasing intake of dietary protein without concomitant increase in total caloric intake entails the substitution of another macronutrient. An important consideration to make when interpreting the literature on protein and blood pressure is that the observed effects may vary according to what macronutrient protein is replacing. The Omniheart Trial is one example of research findings strengthening some relationships while adding complexity at the same time

[191]. While it is true that isoenergetically replacing carbohydrate with protein resulted in significant decreases in blood pressure, similar results were obtained in this trial by substitution of carbohydrate with monounsaturated fat. This introduced the concept that increased dietary protein intake may not necessarily drive the decrease in blood pressure. Rather, carbohydrate may play an active role in increasing blood pressure and consuming less is antihypertensive regardless of the macronutrient taking its place (fat or protein). While the hypertensive effect of elevated carbohydrate intake does not typically manifest in acute settings [236], there are data indicating that insulin resistance and hyperinsulinemia (potentiated by high-carbohydrate diets) result in chronic elevations in blood pressure [229, 230]. Thus, consideration must be given regarding what macronutrient dietary protein is replacing.

Hypertensive status

Another important consideration to make when interpreting the literature on protein and blood pressure is that the observed effects may be mediated through the hypertensive status of the individual or population in question. There are data suggesting dietary protein supplementation is effective in reducing systolic and diastolic blood pressure in subjects with hypertension, but not in those without hypertension [298]. Similarly, the effect of protein on blood pressure is blunted in hypertensives currently taking antihypertensive medications [278]. As older individuals are more likely than their younger counterparts to present with higher blood pressures, the previous findings may be related to the belief that dietary protein may be more effective in reducing blood pressure in older adults [278]. However, arterial stiffening is also a product of aging which may render aged populations less receptive to dietary interventions proposed to improve vascular reactivity [296, 299]. The considerations for aging are currently unclear, as aged populations possess theoretical predispositions to benefit either more or less than younger populations from dietary interventions.

1.7.5. Results from observational studies on the role of protein in blood pressure regulation are inconsistent

Several observational studies have been conducted that address the question of whether dietary protein contributes to blood pressure regulation. While a systematic review of observational research concluded that higher-protein has a modest beneficial effect on blood

pressure, the individual studies within are conflicting [282]. In this section, notable observational studies with conflicting conclusions are presented. Particularly, observational studies containing characteristics which may predispose the studies to certain conclusion are highlighted (e.g. a study including only normotensive participants is more likely to report null findings compared to a study including only untreated hypertensives).

Protein spread in observational studies

The INTERSALT study was foundational to establishing the link between dietary protein intake and decreased blood pressure [223]. Authors reported that systolic and diastolic blood pressure were 3.0 and 2.5 mm Hg lower, respectively, in individuals with 24-h urinary excretion of nitrogen 30% above the group mean in comparison to those 30% below the group mean (~37g more protein/d). There are a few characteristics of this study which could explain these results. The ~37g protein spread (30% above to 30% below mean) used to present the main findings of this manuscript passes Bosse's threshold for protein spread [186]. The group mean protein intake in this study was ~62.2g, which means that those 30% below the mean are consuming around ~43g protein per day. This equates to ~0.63 g pro/kg body weight/d in the lower group (study mean body weight: 67.7 kg) and ~1.19g pro/kg body weight/d in the higher group. The relative importance of the spread of protein intake between groups (and any other variable for that matter) may vary depending upon where in the spectrum of dietary protein adequacy one falls. The lower group is significantly below the established RDA for protein, while the higher group has sufficient protein intake. As a general principle of human nutrition, the relative benefit of a nutrient is likely to be greater when moving from deficiency to sufficiency than from sufficiency to surplus. The authors of INTERSALT reported that the regression coefficients were larger for both older individuals (compared to young) and women (compared to men). These two groups typically consume less protein than younger, male adults. Thus, it is possible that older adults and women presented with stronger regression coefficients because they were more likely to cross the dietary protein adequacy threshold. These considerations could be applied to explain discordance in much of the observational research involving different study populations. This study was among the first to report an inverse association between dietary protein and blood pressure.

Cultural differences in mean protein intake may explain why findings from observational data on the association between total dietary protein intake and blood pressure have been inconsistent – some report an inverse association [274, 276, 300, 301], while others report no association [225, 272, 302, 303]. Notably, mean total protein intake varies greatly in different cultures assessed. For example, Umesawa et al. found that total protein intake was inversely associated with blood pressure in Japanese adults [300]. A significant inverse relationship in multivariable-adjusted systolic blood pressure by quartile (Q) of total protein intake was only observed when comparing Q1 (mean ± SD, 46.8g ± 0.3) to Q3 (78.3g ± 0.2), and Q4 (105.0g ± 0.3). In contrast, researchers of the PREMIER trial reported no association between total protein intake and blood pressure in Americans at baseline and after 18 months of lifestyle intervention [225]. The average protein intake of the American population (~77g) is comparable to the third quartile in the Japanese population (~78g). Thus, the benefit of protein on blood pressure is most robust when comparing inadequate (~48g) intake to sufficient intake (~78g), which is seen in the Japanese cohort. In contrast, due to the high mean protein intake, it is unlikely that many participants in the American cohort are classified as ingesting an inadequate amount of protein and therefore the effect of protein is attenuated. Therefore, total protein intake differences within study populations are a likely cause of inconsistent findings noted in observational research.

Protein source and individual nutrients vs. whole foods in observational studies

In addition to differences in total protein intake, differences in protein sources between cultures can potentially explain some of the inconsistent findings. To this end, recent efforts have been focused towards elucidating potential differential relationships between plant protein and animal protein on blood pressure. Notably, observational research from China and Japan report either no difference between plant and animal protein, or that animal protein is superior to plant protein in reducing blood pressure [300, 301, 304-306]. One observational study even detected a positive association between plant protein intake and systolic blood pressure [300]. Conversely, studies originating in Western countries more often report that plant protein is inversely associated with blood pressure, while animal protein is not [225, 285, 303, 307]. The predominate sources of dietary protein vary considerably in these different cultures. Total protein intake in the United States is comprised of a considerable amount of red meat, while relatively little red meat is consumed in China and Japan. Saturated fat found in red meat has been linked

with increased blood pressure and cardiovascular risk. In contrast, fish is a primary source of animal protein in Japan. Fish intake has been associated with reduced systolic blood pressure and cardiovascular disease risk [291]. These findings suggest that stratifying dietary protein into the two broad categories of 'plant' or 'animal' protein may lack sufficient precision, given the disparate effects attributed to different sources within each category. Collectively, these data suggest that differences in protein sources consumed by different cultures may influence observed associations.

If plant and animal proteins differentially affect blood pressure independent of associated nutrients and non-nutrients within those foods, then constituent amino acid profiles must be the driving force. Different amino acids can exert their antihypertensive effect through numerous diverse mechanisms, as thoroughly reviewed by Vasdev & Stuckless [248]. For example, arginine exhibits antihypertensive properties by virtue of it being a key substrate in synthesis of the vasodilator nitric oxide, while histidine blunts expression of angiotensin converting enzyme mRNA [243, 248]. Tuttle et al. reported that intakes of methionine and alanine were associated with increased blood pressure, while intakes of threonine and histidine presented with inverse associations [277]. As plant proteins consist of lower ratios of methionine and alanine to threonine and histidine, the investigatorss suggested that plant proteins possess a more favorable balance of blood pressure-lowering amino acids. In another study, after researchers detected an inverse relationship between plant (but not animal) protein and blood pressure, they analyzed and compared dietary amino acid contents of those with high vegetable/low animal protein diets (n=491) and low vegetable/high animal protein diets (n=471) [307]. Those who consumed high vegetable/low animal protein diets had greater proportionate intake of serine, phenylalanine, proline, cysteine, and glutamic acid. The implication here was that the differential effect on blood pressure could be attributable to higher proportions of these specific amino acids.

The association between intake of specific amino acids and blood pressure was strengthened by research investigating clusters of amino acids. Jennings et al. identified seven amino acids with established mechanistic links to cardiovascular health and investigated their relationship to blood pressure and cardiovascular risk [243]. Greater consumption of each of the seven amino acids – leucine, arginine, glutamic acid, cysteine, tyrosine, histidine, glycine – was correlated with lower blood pressure. Amino acids from plant sources were correlated with reduced systolic blood pressure, while amino acids from animal sources were correlated with lower pulse wave

velocity – a measure of arterial stiffness and predictive of cardiovascular disease risk. Neither plant protein nor animal protein intake was associated with blood pressure in this study, perhaps suggesting specific amino acid analysis to be a more sensitive measure. Although amino acids from plant and animal proteins differentially influence markers of cardiovascular disease risk, recommendations from the use of this approach are limited. However, it appears that a preponderance of observational evidence supports amino acids in plant proteins having a more beneficial effect on blood pressure than amino acids in animal proteins.

In addition to the different properties of plant/animal proteins and specific amino acids, differences in other nutrients and non-nutrients such as fiber may confound the implications from comparing plant and animal sources. As previously discussed, intake of plant protein may simply be a surrogate marker for other nutrients which may lower blood pressure. One such example is fiber, which has been independently linked with reduced blood pressure [308]. In the INTERMAP study, an inverse relationship between plant protein and blood pressure was detected [307]. Authors reported difficulty in ascribing the results solely to plant protein due to the high correlations between intake of plant protein, fiber, and magnesium. Similarly, authors of the PREMIER study reported that intake of plant protein, but not total or animal protein, was inversely associated with blood pressure [225]. However, statistical significance was lost when fiber was added to the regression model. Buendia et al. reported that total protein, animal protein, plant protein, and fiber intake were associated with lower blood pressure levels [274]. Participants who consumed high amounts of both fiber and protein (animal or plant) presented with the lowest mean systolic and diastolic blood pressures (4.0 mm Hg and 2.3mm Hg lower, respectively). To summarize, determining the independent effects of plant or animal proteins by inclusion of covariates without overcorrecting will continue to be a challenge with this research design. Collinearity is a persistent issue in observational studies investigating whole foods [225]. Clearly, factors other than protein in whole foods can influence blood pressure findings despite rigorous attempts of statistical correction.

Protein and blood pressure – conclusions from observational research

Findings from observational research collectively suggest a modest beneficial effect of dietary protein on blood pressure. The inverse association between dietary protein and blood pressure is most pronounced when there is an adequate spread in protein intake in the population

analyzed, and when there is a low mean protein intake. Observational findings from studies investigating differential responses of plant and amino protein using amino acid and whole food models are conflicting. While there is some evidence that plant protein may be more effective in reducing blood pressure than animal protein, notable design limitations obstruct the ability to make definitive statements.

1.7.6. Results from experimental studies on the role of dietary protein in blood pressure regulation are inconsistent

Results from experimental trials investigating the effect of dietary protein on blood pressure are inconsistent. Five meta-analyses of randomized-controlled trials were conducted in recent years that report either a modest reduction in blood pressure in the group consuming increased protein [183, 226, 227], or no effect by group [184, 185]. Here, it may be beneficial to highlight select studies included in these analyses to shed light on these discrepancies.

Hypertensive Status, Substitution Effects

The hypertensive status of the population and the macronutrient that protein replaces to be considered a 'high-protein diet' can impact blood pressure response. In a recent meta-analysis, authors reported that greater blood pressure reductions were observed in trials where protein was increased at the expense of carbohydrate [227]. Authors of the Optimal Macronutrient Intake Trial to Prevent Heart Disease (Omniheart) were among the first to suggest that carbohydrate substitution could be responsible for reductions in blood pressure [191]. Compared to the high carbohydrate diet, the high protein and high monounsaturated fat diets both similarly lowered blood pressure in participants who were hypertensive. Some key study characteristics which could explain the positive results are the large protein spread between intervention groups (25% total energy from protein vs 15%), and the participants baseline blood pressure (prehypertensive and hypertensive). Due to there being no blood pressure differences between the high protein and monounsaturated fat diets in the full analysis, the potential for carbohydrate substitution to drive these improvements is often cited. However, blood pressure reductions were statistically significant only for the protein diet in subgroup analysis of prehypertensive participants. Prehypertensive participants would be expected to be less responsive to dietary interventions than untreated hypertensives [298]. Significant blood pressure reductions in in this less-

responsive group of prehypertensives argues for some beneficial effect of dietary protein on blood pressure beyond substitution of carbohydrate. The effect of dietary protein independent of carbohydrate substitution is difficult to ascertain as much of the research on protein and blood pressure utilizes carbohydrate as an isocaloric control [278, 296, 298, 309, 310]. Research investigating dietary protein on blood pressure without modifying carbohydrate content of the diet would be required to definitively state that the observed benefit of protein is not due to carbohydrate substitution. Regardless, careful consideration of the specific macronutrient that protein is replacing and the hypertensive status of the population in question must be observed.

Support for the beneficial effect of protein on blood pressure beyond substitution of carbohydrate is found in one study which compared high protein to high fat diets with carbohydrate intake equivalent [311]. Both diets reduced systolic blood pressure, but the high protein diet resulted in a greater decrease. Further, only the high protein diet reduced diastolic blood pressure. Caution must be taken when making generalizations from this study as the population consisted solely of obese individuals with newly diagnosed type 2 diabetes. Hyperinsulinemia plays a direct role in increasing blood pressure by increasing sodium and water retention [242]. The high protein diet reduced hyperinsulinemia to a greater extent than the high fat diet. It is likely the effect of protein on blood pressure was mediated by reductions in hyperinsulinemia, which is more pronounced in this population. There are numerous other mechanisms in which dietary protein is hypothesized to act through to decrease blood pressure [248], but the consistent use of carbohydrate controls make interpretation of these findings challenging. Collectively, there is limited and inconclusive evidence in support of a beneficial effect of protein on blood pressure independent of carbohydrate substitution.

Protein source and individual nutrients vs. whole foods

Another potential source of discordance in meta-analyses of protein and blood pressure trials come from the inclusion or exclusion of supplementation trials. Supplementation trials often report greater improvements in blood pressure relative to the control (typically carbohydrate) when compared to whole dietary interventions [310, 312]. For example, researchers of the PROPES trial investigated the effects of 60 g protein supplementation per day compared to 60 g maltodextrin supplementation on blood pressure for 4 weeks in prehypertensive and untreated hypertensive participants [310]. Systolic blood pressure was reduced by 4.6 ± 1.7mm Hg in the

protein supplemented group relative to the maltodextrin group after just four weeks. Supplementation trials are critical in advancing our understanding of the effects of dietary protein as a macronutrient on blood pressure, as opposed to the effects of protein-rich foods and diets. However, including both whole dietary pattern interventions and supplementation trials in the same meta-analysis may not be appropriate. Notably, both meta-analyses which reported no effect of dietary protein on blood pressure did not include supplementation trials [184, 185]. Conversely, two out of three meta-analyses which did detect a differential improvement to blood pressure in the high protein group included supplementation trials [226, 227]. While both designs are valid, they are pursuing fundamentally different research questions. The potential for nutrients and other characteristics of whole foods to influence blood pressure independently of the protein component of the diet must be considered when interpreting study design.

While most observational evidence suggests that plant protein may be superior to animal protein in reducing blood pressure in Western cultures, most experimental trials do not support this claim [281, 296, 312]. However, there are some limitations in the available research that may bias findings towards the null. Hill et al. compared the effects of a modified DASH diet rich in plant protein (two-thirds plant sources, 18% protein), a modified animal protein DASH diet (two-thirds animal sources, 18% protein), and a high animal protein diet (two-thirds animal sources, 27% protein) on indices of cardio-metabolic health in overweight adults [281]. All groups reduced blood pressure with no differences between any of the diets. However, the study did not meet recruitment goals and was not powered to detect between group differences (only powered to detect within-group changes), thus biasing between-group statistical analysis towards the null. Further, the significant weight loss of all participants was hypothesized by investigators to wash out between-group differences. Roussell et al. conducted a similar study investigating the same diets *without weight loss* [296]. Only the animal-rich high protein diet resulted in decreases in systolic blood pressure in comparison to the control diet. There were no significant improvements in blood pressure from the other diets. At first glance, this could appear to be evidence in favor of diets rich in animal protein. However, there were no improvements in similar DASH diets which differed only in protein source. It is more likely that the benefit in the high protein, animal rich diet came from the increased *quantity* of protein (27% vs 19% protein), as opposed to the *source* of protein. Results from two recent meta-analyses are in agreement with

this conclusion [226, 227]. Collectively, findings from experimental trials suggest that there is no difference between plant or animal sources on blood pressure.

Protein and blood pressure – conclusions from experimental research

Similar to the observational research conclusion, findings from experimental research collectively suggest a modest favorable effect of dietary protein on blood pressure. While there is some evidence that dietary protein exerts antihypertensive effects independent of concomitant carbohydrate reduction, a substitution effect cannot be ruled out. Dietary interventions increasing protein intake are most effective in reducing blood pressure in participants with higher baseline blood pressure. Conclusions from experimental research diverge most from observational research when protein source is considered. Similar to when comparing supplementation to dietary modification trials, the possibility of the non-protein components of food influencing blood pressure is a key consideration when investigating differential effects of plant and animal protein sources. The experimental design may be particularly useful in answering this research question as it can minimize the profound effect of confounding variables when comparing plant-based diets to omnivorous diets in observational settings. Individuals who consume more protein from plant sources than from animal sources are more likely to be nonsmokers, exercise more, consume less alcohol, and consume an overall healthier diet [286]. The greater control achieved in experimental designs likely minimizes these confounding variables which all have been associated with blood pressure reductions, thus curtailing the spurious beneficial effect of plant protein on blood pressure. The relative contributions of dietary protein source and quantity towards blood pressure regulation is still unclear, as there is evidence in support of the significance of both. However, currently there is a greater body of literature suggesting dietary protein quantity *irrespective of source* to be more important in blood pressure regulation.

1.7.7. Acute blood pressure responses to protein and exercise

Compared to studies assessing the chronic effects of greater consumption of dietary protein on blood pressure, there is relatively little research investigating the acute postprandial effects of protein on blood pressure. The findings of acute studies of dietary protein on postprandial blood pressure are mixed [228, 236, 313]. Importantly, these studies document the

postprandial blood pressure responses of individuals in resting states. However, individuals do not habitually consume meals and then remain completely sedentary in a controlled state, as seen in a clinical facility. Rather, they live, they work, they move, and hopefully, they exercise. Exercise, and moderate-intensity aerobic exercise in particular, is a frontline treatment for hypertension [314], with systolic blood pressure-lowering effects comparable to that of antihypertensive medication [315]. One of the major drawbacks of our current dietary guidelines is a lack of consideration towards physical activity, which may substantially alter nutrient – particularly protein – metabolism and requirements [316]. However, there remains a concern that acute bouts of exercise could cause cardiovascular events in those most likely to benefit from such physical activity [317]. Therefore, the study described in Chapter 4 was designed to determine if the suspected long-term benefits of increased dietary protein on blood pressure extend to attenuating the acute blood pressure increases during aerobic exercise.

1.8. Global conclusions and dissertation research purpose

Dysregulation of the balance between adipose tissue and skeletal muscle, either in the form of excess adiposity, ectopic adipose accumulation, or inadequate skeletal muscle mass, remains a prime concern. The first study (Study 1) packaged in this dissertation is primarily descriptive and unravels some of the intricacies of body composition, attempting to determine the relationship between different depots of intermuscular adipose tissue with indexes of cardiometabolic health. Recommendations are frequently made to consume a higher protein diet to improve body composition. Study 2 is designed to utilize a systematic review of published data to assess this point in a population underrepresented in protein research (women) at greater risk for inadequate protein consumption. The final study (Study 3) was conducted to expand the research focus of dietary protein beyond body composition-related outcomes to markers of cardiometabolic health using a randomized crossover trial to determine whether greater protein intake in a meal can improve acute vasoactive responses to exercise.

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CHAPTER 2. DIFFERENTIAL RELATIONSHIP BETWEEN INTERMUSCULAR ADIPOSE DEPOTS WITH INDICES OF CARDIOMETABOLIC HEALTH

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2.1. Abstract

Background. Globally, accumulation of intermuscular adipose tissue (IMAT) is positively associated with insulin resistance. Whether this association is observed consistently in different skeletal muscles and encompasses other markers of cardiometabolic health is not well known. *Objectives.* The purpose of this secondary analysis study was to investigate associations among thigh or calf IMAT stores and indices of cardiometabolic health in adults who are overweight and obese participating in dietary interventions. A subset of calf data were analyzed to assess relations between IMAT in the gastrocnemius (Type II fiber predominance) and soleus (Type I fiber predominance) with markers of cardiometabolic health. *Materials and Methods***.** Thigh and calf compositions were assessed via magnetic resonance imaging in 113 subjects (mean ± SD, age: 50 ± 16 y (range: 21-77 y), BMI: 31 ± 3 kg/m^2), 103 of which completed dietary interventions with or without energy restriction-induced weight loss. A subset of data (n=37) were analyzed for relations between muscle compartments (gastrocnemius and soleus) and cardiometabolic health. IMAT was regressed separately against fasting serum glucose

concentrations, insulin, homeostatic model assessment-insulin resistance (HOMA-IR), and lipids and lipoproteins. *Results.* In general, total thigh IMAT was predictive of markers of glucose control, while total calf IMAT was not. Specifically, baseline thigh IMAT was positively associated with fasting glucose, insulin, and HOMA-IR. IMAT content changes in any depot did not predict improvement in cardiometabolic health. *Conclusions.* The strength of the relationship between IMAT and glucose control-related indices of cardiometabolic health are dependent on IMAT location. Specifically, greater IMAT in the thigh is a better predictor of cardiometabolic risk than greater IMAT in the calf in adults who are overweight and obese.

2.2. Introduction

Obesity is implicated in the development of metabolic syndrome, a multifaceted disorder encompassing insulin resistance, hypertension, and dyslipidemia [1]. While the general deleterious effects of greater adiposity are well documented, the concept that the metabolic consequences associated with obesity may be more related to regional body fat distribution and ectopic fat deposition as opposed to absolute quantity has more recently emerged [2, 3]. Visceral adipose tissue (VAT), one such depot of ectopic fat, is strongly associated with metabolic syndrome in spite of VATs relatively small contribution to total adiposity [3-6]. Advances in imaging technology have enabled identification of intermuscular adipose tissue (IMAT; adipose tissue between muscle groups and beneath the fascia [7]), a unique ectopic adipose depot implicated in an array of pathological outcomes akin to VAT [8]. Until recently, IMAT has not garnered a level of attention commensurate to its potential impact on metabolic profile.

Intermuscular adipose tissue is associated with greater risk of all-cause mortality; each one standard deviation (SD) increase in IMAT (~6.8% greater IMAT) is associated with a 40% greater mortality risk over a 10-year period [9]. IMAT content is higher in individuals with obesity and type 2 diabetes [10-13]. While obesity and elevated body mass index (BMI) scores typically coincide with metabolic detriments, IMAT is independently linked with the metabolic syndrome in normal-weight and over-weight men [14]. This suggests that there are metabolic consequences of IMAT accumulation separate from consequences of obesity.

Mounting evidence implicates both relative and absolute IMAT quantity to be consistently associated with insulin resistance [8, 10, 15, 16] and inconsistently associated with worsened lipid-lipoprotein profile [6, 8, 17]. Determination of whole-body IMAT is a time-consuming and costly

endeavor [16]. Often, sections of the lower limbs are analyzed by magnetic resonance imaging (MRI) or computed tomography to quantify relative IMAT content. These sections of either the thigh [15, 17-21] or calf [12, 13, 22, 23] are interpreted as being representative of whole body muscle composition. However, IMAT infiltration into skeletal muscle may be muscle or muscle compartment specific [23, 24]. This finding presents a serious obstacle when interpreting data from literature on IMAT, which incorporates numerous different imaging techniques and extrapolates findings from different anatomical sites [25]. Additionally, difficulty exists in determining if IMAT directly affects metabolic function or is merely a marker of impairment [26]. This uncertainty arises from temporal issues such as IMAT showing strong associations with insulin resistance before interventions, yet failing to predict improvement in insulin sensitivity with reductions in IMAT [15, 27].

Given these shortcomings, the primary aim of the current research was to 1) investigate associations between thigh or calf IMAT stores and indices of cardiometabolic health with a special consideration of determining a potential differential predictive ability between anatomical sites analyzed. The secondary aims were to 2) analyze the relations between IMAT in the soleus (type I fiber predominance) or gastrocnemius (type II fiber predominance) and indices of cardiometabolic health and 3) investigate associations between longitudinal changes in each of the IMAT compartments with changes in cardiometabolic health parameters. We hypothesized that greater IMAT content would be associated with worsened indices of cardiometabolic health with no difference on the basis of 1) location (thigh vs. calf) or 2) fiber type predominance (soleus vs gastrocnemius). Further, we hypothesized that 3) IMAT reductions in any depot would not predict longitudinal improvement in indexes of cardiometabolic health.

2.3. Materials and methods

2.3.1. Subjects and experimental design

The current study involved retrospective analysis of baseline data from one cross-sectional study, and baseline and post-intervention data from three clinical studies (Table 2.1). The rationale for conducting this secondary analysis was to pool and analyse data from disparate research studies conducted by our research group to get a global view of how dietary interventions influence changes in IMAT, and the relationship with indices of cardiometabolic

health. As such, the clinical studies included dietary interventions with (n=2) or without (n=1) weight loss and with (n=1) or without (n=2) exercise. Study participants were overweight and obese males and females recruited from the greater Lafayette, IN area. Inclusion criteria consisted of: weight stable (±4.5 kg within the past 6 months); non-smoking; no acute illness; not clinically diagnosed with diabetes mellitus. Baseline data were used from 113 subjects (39 males, 74 females), with 10 subjects from a cross-sectional study supplementing 103 subjects from the clinical studies (pre- & post- intervention data from 93 subjects). Data were extracted from original research files from parent studies and compiled by means of double entry. The Purdue University Biomedical Institutional Review Board approved the study protocols and all subjects provided written, informed consent and received monetary compensation for their participation. Clinical trial profiles of the four original studies can be found under NCT01396915, NCT01692860, NCT02187965, and NCT02066948.

Table 2.1. Descriptions of the randomized controlled trials included in a secondary analysis on the relationship between intermuscular adipose tissue and indices of cardiometabolic health

Author, year	Sample size	Duration (wk)	Age (y)	Energy Restriction	Exercise Training
Campbell et al. (unpublished)	10	CS	70 ± 4	N/A	N/A
Hudson et al. (2017) [28]	38	16	34 ± 9	750 kcal/d ER	RT - 3x/wk
Wright et al. (unpublished)	19	12	70 ± 4	No ER	No Training
Zhou et al. (unpublished)	46	16	52 ± 8	750 kcal/d ER	No Training

[¥]Individual study details regarding study characteristics (duration, energy restriction component, exercise training component); CS, Cross-sectional; ER, energy restriction; RT, resistance training. Data are presented as means ± SD, where appropriate.

2.3.2. Antropometric measurements and body composition

Subjects' height (± 0.1 cm) and weight (± 0.1 kg) were measured using a wall-mounted stadiometer and a digital balance scale, respectively. These measurements were used to calculate BMI (kg/m^2).

2.3.3. Magnetic resonance imaging and image analysis

MRI image acquisition and analysis were described previously [29]. Briefly, overnight fasted subjects arrived at a MRI facility (InnerVision West, West Lafayette, IN) and were

scanned using a 3.0T Signa HDx whole body MRI machine (*General Electric*, Waukesha, WI). Prior to scanning, subjects were instructed to lie in the supine position on a MRI-safe bed for 1 hour to minimize effects of body position on the scanning outcomes [30]. Following the rest period, subjects were shifted to the MRI machine bed while remaining in the supine position and the MRI device captured images of the dominant leg. Twenty-seven image slices were obtained and they were analyzed using Medical Image Processing, Analysis, and Visualization (MIPAV) MRI analysis software (v 7.0, Center for Information Technology, *National Institutes of Health*, Bethesda, MD) beginning with the first slice after the appearance of the *rectus femoris*, proceeding with every third slice, and ending with the appearance of the *gluteus maximus*. Total tissue, muscle tissue, subcutaneous adipose tissue (SAT), and IMAT regions were identified and respective areas were calculated. Each slice chosen for analysis represented three slices in total: itself, the slice previous, and the slice following. Average IMAT area (IMATa; absolute IMAT quantity, cm^2), average muscle tissue area (MT; cm^2), average subcutaneous adipose tissue (cm^2), and average total cross-sectional area (CSA; cm^2) were determined. IMAT, SAT, and MT in the total thigh and total calf were standardized to CSA. The gastrocnemius and soleus were semi-automatically segmented and analyzed individually. Average gastrocnemius IMAT and soleus IMAT areas were standardized to average calf muscle area for analysis. MRI images from parent studies were reanalyzed with the aforementioned protocol to reconcile potential differences in imaging methodology among studies.

2.3.4. Blood collection and analysis

Following a 10-h overnight fast, blood samples were obtained from an antecubital vein and placed in tubes containing a clot activator to obtain serum or sodium heparin to obtain plasma. Serum tubes were held at room temperature for 30 minutes and then centrifuged at 4,000xg at 4°C for 15 minutes. Serum tubes were sent to a commercial analytical laboratory (MidAmerica Clinical Laboratories, Indianapolis, IN) for determination of concentrations of lipids and lipoproteins, including high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), total cholesterol (TC), and triglycerides (TG). Serum or plasma glucose concentrations were measured using a photometric assay (Chemistry Immuno Analyzer AU5700, Olympus, Center Valley, PA). Serum insulin concentrations were measured in duplicate using an electrohemiluminescence immunoassay method on the Elecsys 2010 analyzer

(Roche 108 Diagnostic Systems). The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as previously described [31].

2.3.5. Statistical analysis

A multiple linear regression model was used to assess the associations of IMAT in each depot and indices of cardiometabolic health. Specifically, thigh IMAT or calf IMAT (standardized to CSA) and soleus IMAT or gastrocnemius IMAT (standardized to MT) were regressed against glucose, insulin, HOMA-IR, TG, TC, LDL, HDL, and TC: HDL. All estimates were adjusted for age and sex. Longitudinal estimates were adjusted for age, sex, and baseline dependent variable. Multiple linear regression p-values are presented raw and adjusted for multiple testing using the False Discovery Rate procedure [32]. Independent two-tailed t-tests were used to compare baseline subject characteristic data of males and females, and paired two-tailed t-tests were used to determine time effects. All analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Data are presented as mean ± standard deviation (SD) and adjusted regression coefficients (β*) are reported (statistical significance accepted at $P<0.05$).

2.4. Results

2.4.1. Demographic, clinical, and muscle composition data

The characteristics of the 113 subjects (107 Caucasian, 4 African American, 2 Asian) are presented in Table 2.2. With the exception of HDL, all indices of cardiometabolic health improved over time. There were no significant sex differences for age, BMI, LDL, TC, TG, insulin, and HOMA-IR (Supplemental Table 1; Appendix A). Overall, thigh and calf CSA and total IMATa decreased over time due to the interventions (Table 2.2). Reductions in SAT (thigh: -20.94 ± 13.58 cm^2, calf: -3.31 ± 2.96 cm^2) were greater than reductions in MT (thigh: -3.49 ± 6.92 cm^2, calf: -2.43 ± 2.57 cm^2); indicating improved body composition. Relative decreases in IMAT were greater than decreases in CSA.

Table 2.2. Clinical and cardiometabolic characteristics of all subjects.

General Characteristics	***n = 113 (74 F, 39 M)***		
Age (yr)	50 ± 15		
Height (cm)	170 ± 10		
Weight (kg)	90.1 ± 13.3		
BMI (kg/m^2)	31.2 ± 2.9		
Cardiometabolic Health	***Pre-***	***Post-***	***Change***
Glucose (mmol/l)	5.16 ± 0.49	5.02 ± 8.8	**-0.12 ± 7.8***
Insulin (pmol/l)	84.73 ± 44.45	55.56 ± 28.47	**-27.78 ± 40.98***
HOMA-IR	2.85 ± 1.64	1.82 ± 1.01	**-0.97 ± 1.47***
Total Cholesterol (mmol/l)	10.46 ± 1.97	9.30 ± 1.82	**-0.99 ± 1.24***
Triglycerides (mmol/l)	6.79 ± 2.89	5.55 ± 2.31	**-1.39 ± 2.54***
HDL (mmol/l)	2.64 ± 0.84	2.50 ± 0.61	0.01 ± 0.36
LDL (mmol/l)	6.47 ± 1.74	5.68 ± 1.61	**-0.74 ± 0.96***
TC:HDL	0.24 ± 1.34	0.22 ± 1.06	**-0.03 ± 0.75***
Thigh IMAT			
CSA (cm^2)$^¥$	229.48 ± 47.09	212.24 ± 40.31	**-24.46 ± 16.10***
IMATa (cm^2)	11.12 ± 3.46	9.84 ± 3.10	**-1.46 ± 1.01***
IMAT	0.0498 ± 0.0164	0.0478 ± 0.0159	**-0.0015 ± 0.0044***
MT (cm^2)$^¥$	115.55 ± 32.14	115.11 ± 30.27	**-3.49 ± 6.92***
SAT (cm^2)	113.71 ± 49.18	97.13 ± 42.04	**-20.94 ± 13.58***
Calf IMAT			
CSA (cm^2)$^¥$	97.68 ± 15.31	92.47 ± 14.22	**-5.74 ± 4.64***
IMATa (cm^2)	6.49 ± 2.31	5.96 ± 2.29	**-0.63 ± 0.83***
IMAT	0.0670 ± 0.0229	0.0649 ± 0.0238	**-0.0027± 0.0073***
MT (cm^2)$^¥$	61.09 ± 13.26	58.67 ± 11.68	**-2.43 ± 2.67***
SAT (cm^2)	36.59 ± 15.29	33.44 ± 13.91	**-3.31 ± 2.96***

Data are mean ± SD; significance determined through paired T-Tests, P-values < .05 *
CSA, cross-sectional area of segment; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; IMAT, intermuscular adipose tissue (standardized to cross-sectional area) IMATa, intermuscular adipose tissue (absolute quantity); LDL, low-density lipoprotein; MT; muscle tissue; SAT, subcutaneous adipose tissue; TC, total cholesterol; TG, triglyceride
¥Bone area removed

2.4.2. Associations of thigh of calf IMAT with cardiometabolic health indexes

Baseline associations of thigh or calf IMAT with cardiometabolic health indexes

Greater relative thigh IMAT was associated with all measured markers of glucose control (Table 2.3). Specifically, thigh IMAT was associated with greater glucose concentrations, insulin, and HOMA: IR. Conversely, there were no relationships between calf IMAT and markers of glucose control.

While trends for greater relative thigh IMAT being associated with some, but not all measured lipid and lipoprotein outcomes were apparent, significance was lost with FDR adjustment for multiple testing (Table 2.3). There was no relationship between calf IMAT and lipid-lipoprotein profile.

Associations between changes in thigh or calf IMAT and changes in cardiometabolic health indexes

Thigh or calf IMAT reductions were not associated with changes in any index of cardiometabolic health (Table 2.3).

Table 2.3 Associations between thigh or calf intermuscular adipose tissue and indices of cardiometabolic health

Baseline Associations	**Thigh IMAT:CSA (n=108)**		**Calf IMAT:CSA (n=95)**	
	β [a] (95% CI)	***P*-value (FDR-Adjusted P)**	**β [a] (95% CI)**	***P*-value (FDR-Adjusted P)**
Glucose (mmol/l)	6.38 (1.17, 11.59)	**0.020* (0.041)**	-0.69 (-5.20, 3.81)	0.761 (0.854)
Insulin (pmol/l)	886.24 (312.43, 1460.06)	**0.009* (0.026)**	128.13 (-380.63, 636.90)	0.618 (0.854)
HOMA-IR	32.77 (11.64, 53.90)	**0.009* (0.026)**	1.74 (-17.04, 20.51)	0.854 (0.854)
Triglycerides (mmol/l)	47.33 (15.21, 79.44)	0.079 (0.349)	-2.92 (-33.41, 27.57)	0.849 (0.849)
Total Cholesterol (mmol/l)	19.67 (-3.19, 42.54)	0.155 (0.349)	11.14 (-9.18, 31.46)	0.279 (0.349)
LDL (mmol/l)	13.59 (-7.45, 34.62)	0.257 (0.349)	10.60 (-8.08, 29.29)	0.263 (0.349)
HDL (mmol/l)	-7.58 (-16.42, 1.26)	0.203 (0.349)	-2.66 (-8.97, 3.66)	0.405 (0.450)
TC:HDL	21.85 (7.46, 36.24)	0.033 (0.332)	9.84 (-3.62, 23.31)	0.150 (0.349)
Δ Associations	**Δ Thigh IMAT:CSA (n=94)**		**ΔCalf IMAT:CSA (n=90)**	
	β [a] (95% CI)	***P*-value (FDR-Adjusted P)**	**β [a] (95% CI)**	***P*-value (FDR-Adjusted P)**
ΔGlucose (mmol/l)	-8.22 (-20.73, 4.28)	0.197 (0.421)	4.95 (-8.94, 18.84)	0.480 (0.721)
ΔInsulin (pmol/l)	-437.86 (-1908.87, 1033.14)	0.883 (0.883)	968.92 (-359.38, 2297.22)	0.151 (0.421)
ΔHOMA-IR	-15.37 (-67.12, 36.37)	0.868 (0.883)	30.19 (-17.42, 77.80)	0.149 (0.421)
ΔTriglycerides (mmol/l)	-34.65 (-117.29, 47.98)	0.677 (0.752)	26.35 (-53.60, 106.31)	0.514 (0.752)
ΔTotal Cholesterol (mmol/l)	-7.30 (-48.43, 33.83)	0.560 (0.752)	14.15 (-26.22, 54.52)	0.488 (0.752)

Table 2.3 continued

ΔLDL (mmol/l)	6.00 (-26.71, 38.71)	0.949 (0.949)	16.90 (-14.62, 48.42)	0.289 (0.752)
ΔHDL (mmol/l)	-1.74 (-13.65, 10.18)	0.578 (0.752)	-5.10 (-16.82, 6.62)	0.390 (0.752)
ΔTC:HDL	-0.51 (-24.84, 23.83)	0.647 (0.752)	17.50 (-6.38, 41.39)	0.149 (0.752)

All estimates are adjusted for age and sex. Longitudinal analyses adjusted for age and baseline dependent variable.
[a]Estimates of adjusted regression coefficient between glucose, insulin, HOMA-IR, TC, TG, LDL, HDL, and TC:HDL with thigh and calf IMAT; P-values < .05 *
CI, confidence interval; HDL, high-density lipoprotein; HOMA:IR, homeostatic model assessment of insulin resistance; IMAT, intermuscular adipose tissue (standardized to cross-sectional area of segment); LDL, low-density lipoprotein; TC, total cholesterol; TG, triglyceride

2.4.3. Associations of calf muscle compartment IMAT with cardiometabolic health indexes

Demographic, clinical, and muscle composition results

Supplemental Table 3 (Appendix A) presents the characteristics of the 37 subjects in the calf muscle compartment analyses subgroup. Dietary interventions decreased IMAT within and muscle area of the soleus and gastrocnemius. IMAT represented ~8.7% of muscle area analyzed in the gastrocnemius pre-intervention, and was reduced to 7.8% of muscle area analyzed post-intervention. There was relatively greater IMAT content in the soleus, with 11.6% IMAT at pre-intervention, which was reduced to 10.7% post-intervention. Absolute, but not relative, reductions of IMAT in the soleus were greater than those in the gastrocnemius.

Baseline associations between gastrocnemius and soleus IMAT with cardiometabolic health indexes

Neither gastrocnemius nor soleus IMAT was associated with any index of cardiometabolic health (Supplemental Table 4; Appendix A).

Associations between changes in gastrocnemius and soleus IMAT with changes in cardiometabolic health indexes

Similar to total calf and thigh IMAT, changes in gastrocnemius and soleus IMAT did not predict improvement in markers of any index of cardiometabolic health (Supplemental Table 4; Appendix A).

2.5. Discussion

Contrary to our hypothesis that greater IMAT would be associated with worsened cardiometabolic health with no difference on the basis on location or fiber type predominance, our results suggest that 1) IMAT in the thigh was more predictive of cardiometabolic dysfunction than IMAT in the calf. Consistent with our hypothesis, there was no differential relationship between soleus and gastrocnemius IMAT and indexes of cardiometabolic health. Also consistent with our hypothesis and other research, 3) changes in IMAT did not reliably predict improvements in indices of cardiometabolic health [15, 19, 27, 33].

Understanding of adipose tissues has progressed from the notion that these tissues were inert storage depots to our current understanding of adipose as an important endocrine organ [34]. As part of this evolution, the concept that not all adipose tissue possesses the same biochemical attributes and confer similar metabolic risk was recognized [34-36]. Investigations of associations between body fat distribution and metabolic health include comparing subcutaneous adipose tissue and VAT [10, 14]. VAT now has a well-defined role in increasing cardiometabolic risk factors relative to subcutaneous adipose tissue [36]. In a similar manner, the comparison of different ectopic fat depots is not without precedent. There is some debate over whether IMAT contributes to metabolic disturbance in a similar fashion to VAT. Previously, the relative importance of VAT and IMAT on metabolic profile were compared [8, 16]. In some research, VAT appeared to possess stronger relations with indexes of cardiometabolic health [6, 20], while IMAT was a better predictor of cardiometabolic health in other research [8, 17]. Akin to comparisons conducted between VAT and IMAT, various IMAT depots were compared. Investigators compared thigh and calf muscle composition and concluded that thigh muscle quality (reduced IMAT) was the stronger contributor to physical function [37]. Similarly, we now conclude that thigh IMAT is the stronger contributor to cardiometabolic dysfunction. Our findings agree with other research that significant relations exist between IMAT in the thigh and glucose [8, 13, 21, 38], insulin [21, 39], and HOMA-IR [6, 39]. While mechanistic support is lacking and some research does not support the relation between IMAT and markers of glucose control [33, 40], our results support the preponderance of research suggesting that IMAT contributes to (or is a product of) disrupted glucose homeostasis [26].

While there is strong evidence supporting the relationship between greater IMAT content and impaired glucose homeostasis, there is considerably less support in favor of relations

between IMAT and cardiovascular disease risk factors. Relations between IMAT and TC [8], TG [6], and an inverse association with HDL [6] were reported. However, null findings concerning the relations between IMAT and TC [6, 18, 38], TG [8, 18, 38], LDL [6, 18], and HDL [8, 38] are more frequently reported. Consistent with this, we did not detect any associations between IMAT and lipid-lipoprotein profile in the overall sample. Discordance in the literature towards lipid findings may relate to differences in subject characteristics. One study found that loss of IMAT was associated with increases in HDL and LDL particle size and a subsequent decrease in cardiovascular disease risk in men, but not women [17]. Our findings are not consistent with these results, as we detected a stronger association between IMAT and lipid-lipoprotein profile in women. Specifically, our results indicate that IMAT in the thigh was associated with elevated TG, TC, LDL, and TC: HDL in women, but not in men. These results should be interpreted with caution, however, as there were almost twice as many women as men in the analysis. Thus, we cannot rule out potentially being underpowered to detect these associations in the male subgroup. Previously, investigators reported that the relationship between IMAT and total cholesterol was markedly stronger in Caucasians compared to African-Americans [8]. The impact of IMAT on lipid-lipoprotein profile may be underestimated due to the purposeful oversampling of African-Americans in some of the most influential IMAT research parent studies [8, 13, 17, 38, 41]. Our disparate findings regarding IMAT and cardiovascular risk factors may be at odds with a majority of the literature because our study population was predominantly Caucasian.

In regards to the longitudinal findings, it is important to note that our results were independent of overall adiposity changes as a result of the dietary interventions, as changes in body size were accounted for with the standardization of IMAT to CSA. With that said, changes in IMAT typically do not predict improvement in risk factors for cardiometabolic disease [15, 27]. Our findings in regards to the relations between changes in segmental IMAT and changes in cardiometabolic health indexes support this consensus.

The current observational research did not include means of delineating mechanisms of IMAT accumulation and its contribution to worsened cardiometabolic health. Myogenic cells possess the ability to differentiate into adipocytes [42], and the relative conversion rate is potentially modifiable through diet, exercise, and pharmacological means [43, 44]. Hyperglycemia and elevated concentrations of long-chain fatty acids increase adipogenic conversion from muscle stem cells [43, 44]. In a bi-directional fashion, just as disturbances to

metabolic health could lead to increases in IMAT, increases in IMAT can feed back and contribute to alterations in metabolic health. As muscle is the primary site for glucose metabolism, the close proximity of IMAT to muscle can act in a manner analogous to VAT and the liver by altering the local metabolic environment [8]. IMAT is thought to exert its deleterious effects primarily through impairing insulin action and glucose metabolism [45, 46]. IMAT can also contribute to development of insulin resistance by impairing blood flow to muscles [15], inducing a pro-inflammatory environment in muscle [47, 48], and increasing oxidative stress [49]. Due to the interconnections between insulin resistance, inflammation, and dyslipidemia, these potential IMAT-induced alterations can impact cardiovascular health parameters as well [1]. Despite these associations and supporting data, a fully realized model in which IMAT definitively causes worsened metabolic health is yet to be widely accepted.

Our use of MRI to quantify IMAT is a strength of the current research. MRI allows researchers to directly measure IMAT [50] and possesses greater sensitivity than computed tomography [51], which indirectly measures IMAT. Pooled analysis of three RCTs from our research group make this the largest investigation of intervention-induced changes in IMAT on cardiometabolic health indexes, to the authors' knowledge. This research is not without limitation. We recognize that secondary analysis of RCTs with dissimilar intervention features, particularly presence or absence of exercise training and energy restriction, are significant sources of heterogeneity and may limit the ability to detect meaningful associations. Therefore, we want to stress our objective to obtain a global view of how dietary interventions influence changes in IMAT; these findings are exploratory in nature. Further, the lack of clear mechanistic support hinders conclusions we are able to draw from the current research.

2.6. Conclusions

In conclusion, our findings suggest that relations between IMAT depots and indices of cardiometabolic health vary by body site. Specifically, greater IMAT in the thigh is a better predictor of cardiometabolic risk than greater IMAT in the calf. Consistent with other research, changes in thigh or calf IMAT do not reliably predict improvements in cardiometabolic health parameters.

2.7. Conflicts of Interest

R.E.B. and J.E.K. have no conflicts of interests to declare regarding publication of this paper. W.W.C received research funds from Pork Checkoff, American Egg Board-Egg Nutrition Center, National Dairy Council, and Beef Checkoff, and served on the National Dairy Council's Whey Protein Advisory Panel, during the time this research was being conducted.

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2.9. Author Contributions

R.E.B., J.E.K., and W.W.C. designed the research; R.E.B. conducted the research; R.E.B. analyzed the data; R.E.B and W.W.C. wrote the manuscript with editorial assistance from J.E.K, and had primary responsibility for the final content of the manuscript. All authors read and approved the final manuscript.

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CHAPTER 3. EFFECT OF WHEY PROTEIN SUPPLEMENTATION ON BODY COMPOSITION CHANGES IN WOMEN: A SYSTEMATIC REVIEW AND META-ANALYSIS

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3.1. Abstract

Context: The impact of whey protein supplementation on body composition changes in women. **Objective:** This systematic review and meta-analysis assessed the effects of whey protein supplementation with or without energy restriction and resistance training on changes in body mass, lean mass, and fat mass in women. **Data Sources:** Pubmed, Scopus, Cochrane, and CINAHL were searched using the keywords "whey protein," "body composition," and "lean mass." **Study Selection and Data Extraction:** Two researchers independently screened 1845 abstracts and extracted 276 articles. **Data Synthesis:** Thirteen randomized controlled trials with 28 groups met the inclusion criteria. **Conclusion:** Whey protein supplementation improves body composition by modestly increasing lean mass without influencing changes in fat mass. Body composition improvements from whey protein are more robust when combined with energy restriction.

Keywords: weight loss, exercise, resistance training, caloric restriction, body weight

3.2. Introduction

There is a preponderance of evidence implicating higher-protein diets as an effective means to improve body composition in various energy states and exercise training conditions[1-3]. Protein supplementation is one dietary strategy to help attain a higher total daily protein intake. Protein supplementation is promoted to help individuals improve their body composition,

especially when consumed in conjunction with weight loss and (or) exercise training. Whey protein (WP) may be a particularly effective form of protein supplementation to increase muscle protein synthesis [4, 5] due to its rapid absorption kinetics[6] and high concentration of branched-chain amino acid [7, 8]. Indeed, WP may be an optimal protein source to support lean body mass gains [8, 9].

Despite discordance among individual studies, recent systematic reviews and meta-analyses tend to indicate that protein supplementation favors modest increases in lean mass [10-12]. While subject training history [11] and age [3, 13] were investigated as potential mediators in the relationship between protein supplementation and changes in body composition, relatively little attention has been paid to potential sex differences in body composition responses. Notably, females are underrepresented in this line of research, as evinced by male-only populations constituting fifteen out of twenty-two studies in the most-cited protein supplementation meta-analysis [12]. Of practical concern, there is a public perception that WP supplementation will lead to excessive hypertrophy or "bulkiness" in women (Consumer Whey Protein Tracking Study, 2014). Therefore, the purpose of the present systematic review and meta-analysis of randomized control trials was to assess the effect of WP supplementation on body composition changes over time in adult women. It was hypothesized that globally, WP supplementation would moderately improve body composition, but would not cause excessive muscle hypertrophy. This investigation was conducted in a 2x2 factorial manner with *a priori* subgroup analyses to assess whether this effect is demonstrable during weight stability 1) with and 2) without resistance training (RT) and during diet-induced weight loss (WL) 3) with and 4) without RT.

3.3. Methods

This systematic review and meta-analysis was conducted in accordance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines [14]. The procedures for identification, screening, data extraction, and analysis were agreed upon in advance between all authors. The PICOS (population, intervention, comparison, outcome, and setting) criteria was used to define the research questions (**Table 3.1**).

3.3.1. Data sources

A systematic search of literature was conducted independently by the primary reviewer (RB) and secondary reviewer (JH) in January 2017, and is current to August 2017. Databases searched included PubMed, Cochrane, Scopus, and CINAHL. The combinations of keywords and specific search parameters can be found in Supplemental Table 1 in Appendix B. Additionally, manual searches and reference lists of previous protein supplementation reviews were used for identification of articles.

3.3.2. Inclusion and exclusion criteria

Published randomized controlled trials that included WP supplementation were considered for this systematic review and meta-analysis. Study population was limited to apparently healthy (not characterized as having a specific chronic disease), non-pregnant females ≥ 19 years old. In addition to studies with only female participants, studies with both male and female participants were included if data on primary outcome measures were available specifically for female participants in the manuscript (or if data were able to be obtained by contacting authors). Interventions had to be of parallel design of at least 6 weeks duration. The treatment group (WP supplementation) had to be contrasted with an isocaloric non-WP control. Multi-ingredient supplements were acceptable if the only difference between the treatment and control is WP (ex. WP+CHO+Calcium vs CHO+Calcium). WP concentrates, isolates, and hydrosylates were considered acceptable forms of supplementation, but the supplement cannot include other types of protein (e.g. Casein). Acceptable forms of body composition measurement include: dual-energy X-ray absorptiometry (DXA), air-displacement plethysmography, and hydrostatic weighing. Interventions involving very low calorie diets (< 800 kcal/d) were excluded. There was no lower-limit for publication date.

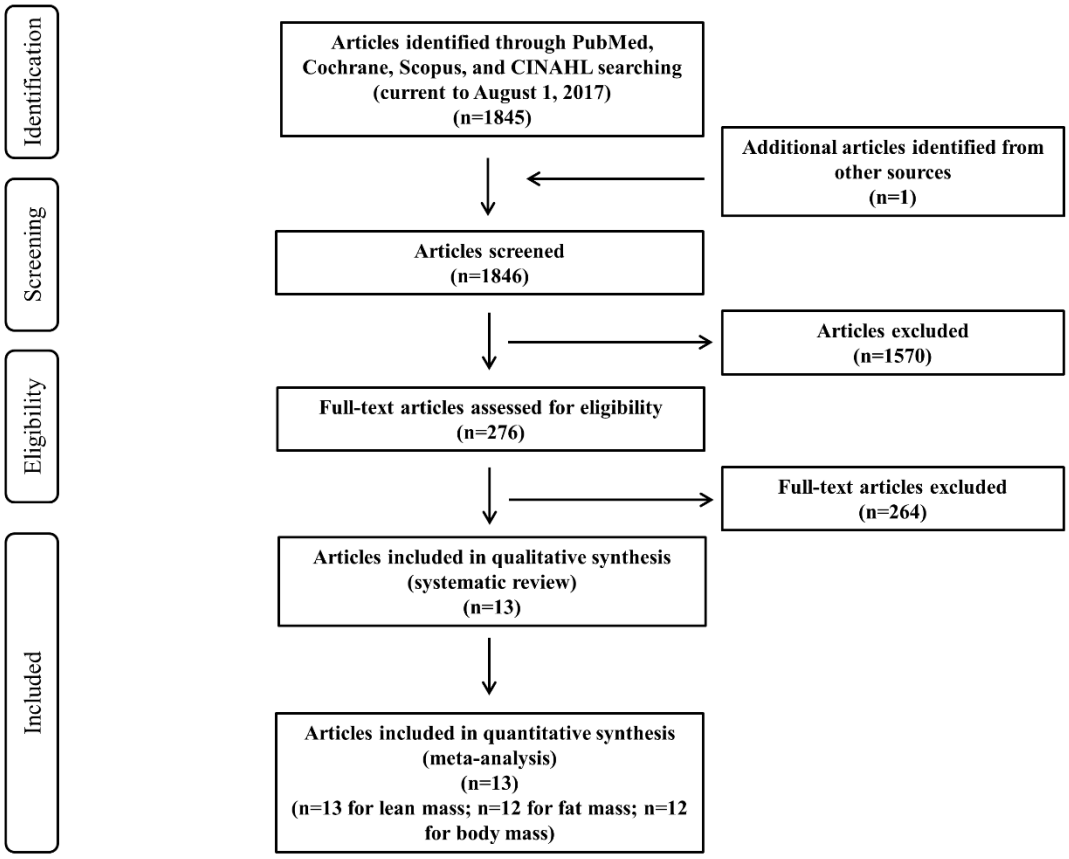

Figure 3.1 PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flow chart of literature selection process.

3.3.3. Article identification and data extraction

A multi-pass method was employed to identify relevant studies from the 1845 articles captured in the original search (Figure 3.1). The first pass involved independently screening titles and abstracts to determine if studies met inclusion criteria. If the abstract did not provide sufficient information to categorically exclude the article, the entire article was retrieved for review in the next pass. After each pass, a cross-check was performed and differences between reviewers were discussed and reconciled. 1569 studies were excluded from the first pass, leaving 276 articles for full-text review in the second pass. 238 articles were excluded following full-text review for the following reasons: subject group mean <19 y, study subjects characterized as having a chronic disease or severe injury, study used an unacceptable method of body composition assessment, primary outcomes not reported or only reported graphically (or if

reported, most often group means for men + women, but data only on women not available), leaving 38 articles for potential inclusion in qualitative and quantitative analysis. Notably, studies with both sexes were retained as part of these 38 articles.

Complete data for inclusion in the meta-analyses were available in four articles [15-18]. Corresponding authors of the remaining 34 articles were contacted via email to acquire unpublished data (most often to acquire female-only data). Authors of nine articles [19-27] provided data via email which permitted inclusion into the meta-analysis. Twenty-four articles were not included in the final analysis when additional information led to discovery of: casein included in WP supplement (n=1), original data lost (n=1), primary outcome data unavailable (n=22).

When individual studies included multiple groups that would classify as an intervention group (e.g. different WP doses), each would be treated as a distinct intervention. When studies included multiple groups that would classify as a comparator group (e.g. soy and carbohydrate separately compared to WP), only the carbohydrate control group was included. Change value (Δ) means and SDs for each primary outcome (lean mass, fat mass, body mass) were extracted when available. Otherwise, Δmean and ΔSD were calculated from pre- and post-intervention values when raw data were provided. When ΔSD was not available, the correlation cofactor (corr) was calculated from studies in which pre SD, post SD, and ΔSD were available, as described previously [28].

The following data were extracted from selected articles independently by both reviewers: Author's last name, publication year, title, body composition assessment method, sample size of each intervention, mean age, intervention duration, exercise characteristics and modality, intervention supplement characteristics, WP supplementation dose (g/d & g/kg/d), number of WP supplementation doses/d, amount of WP per supplementation dose, total protein intake (g/d & g/kg/d), energy deficit, techniques for dietary control and monitoring compliance, pre- and postintervention and net changes in body mass, lean mass, and fat mass, and term used to define *lean mass*.

Table 3.1. Description of the PICOS criteria used to define the research question

Parameter	Description
Population	Adult women, mean age ≥ 19
Intervention	a. Groups with purposeful diet-induced energy restriction undergoing resistance training protocol and whey protein supplementation b. Groups with purposeful diet-induced energy restriction and whey protein supplementation c. Groups in energy balance (without purposeful weight loss or weight gain) undergoing resistance training protocol and whey protein supplementation d. Groups in energy balance (without purposeful weight loss or weight gain) consuming a whey protein supplement
Comparison	a. Groups with purposeful diet-induced energy restriction undergoing resistance training protocol without whey protein supplementation b. Groups with purposeful diet-induced energy restriction without whey protein supplementation c. Groups in energy balance (without purposeful weight loss or weight gain) undergoing resistance training protocol without whey protein supplementation d. Groups in energy balance (without purposeful weight loss or weight gain) consuming a whey protein supplement
Outcome	Changes in whole-body composition, including body mass, lean mass, and fat mass
Setting	Randomized controlled trials
Research Question	What is the effect of whey protein supplementation on whole-body composition in women (with or without ER and with or without RT)?

Table 3.2. Risk of bias assessment of randomized controlled trials included in a meta-analysis on the effects of consuming a whey-protein supplement on body composition in women

	Selection bias		Performance bias	Detection bias	Dietary control		Body composition assessment method
Author, year	Randomization	Allocation concealment	Blinding of participants and study investigator	Blinding of outcome assessment	Dietary prescription	Dietary compliance	
Adechian et al. (2012)	Unclear	Unclear	Unclear	Unclear	Personalized menus to provide energy equal to the basal energy requirements; Provided supplement	-	DXA
Duff et al. (2014)	Low risk	Unclear	Low risk	Unclear	Provided supplement	Supplement log; Returned excess supplement	DXA
Gordon et al. (2008)	Unclear	Unclear	Unclear	Unclear	Diets developed to elicit 400 kcal/d energy deficit; Provided lunch, dinner, and snack meals; Provided supplement to high-pro group	-	DXA
Holm et al. (2008)	Unclear	Unclear	Low risk	Unclear	Provided supplement	Completed 4-d weighed food record at 0, 12, and 24 wk;	DXA
Keogh et al. (2008)	Low risk	Unclear	Low risk	Unclear	Provided supplement; advised to replace 2 meals per day with protein supplement	Compliance assessed by daily checklist and participant returning empty satchets	DXA
Kjølbæk et al. (2017)	Low risk	Low risk	Low risk	Low risk	Monthly dietary counseling sessions; Provided Supplement	Completed 3-d food records at baseline, 6 weeks, and upon completion	DXA
Martens et al. (2015)	Unclear	Unclear	Low risk	Unclear	Personalized menus/recipes to maintain body weight; Provided supplement	Dietary compliance monitored weeks 5 and 9 via interim visits to facility and interview	Bod Pod
Mojtahedi et al. (2011)	Low risk	Low risk	Low risk	Low risk	Diet education classes every 2 weeks; Diets developed to elicit 500 kcal/d energy deficit; Provided supplement	Completed 3-d food diaries at baseline, month 3, and post-intervention; used supplement containers collected and weighed	DXA
Stragier et al. (2016)	Unclear	Unclear	Unclear	Unclear	Provided supplement; instructed to maintain normal eating pattern	Completed food survey over 7 continuous days; Nitrogen balance determined	-
Sukumar et al. (2011)	Low risk	Unclear	Unclear	Unclear	Diet developed to elicit 500 kcal/d energy deficit; 36 weight-loss counseling sessions; Provided supplement	Completed food records for at least 1 week each month; FFQ and 24-h recall every 3 months; Protein intake compliance monitored via BUN	DXA
Taylor et al. (2016)	Low risk	Unclear	Unclear	Unclear	Maintain normal dietary intake; Provided supplement	Consumed supplement under supervision	DXA
Verreijen et al. (2015)	Low risk	Low risk	Low risk	Low risk	Dietary counseling session every 2 weeks; Diets developed to elicit 600 kcal/d energy deficit; Provided supplement	Completed 3-d food records at baseline and after 7 and 13 week of intervention and checked for completeness; Recorded supplement intake in a diary	DXA
Weinheimer et al. (2012)	Unclear	Unclear	Low risk	Unclear	Provided supplement	Completed 4-d food records at 0, 18, and 36 wk; measured UUR; completed daily supplement logs;	DXA

Abbreviations: BUN, blood urea nitrogen; DXA, dual-energy X-ray absorptiometry; FFQ, food frequency questionnaire; UUR, urine urea nitrogen.

3.3.4. Assessment of risk of bias

The risks of selection, performance, and detection biases were evaluated from included studies using a modified Cochrane tool for assessing risk of bias (Table 3.2)[1-13]. Both reviewers independently assessed risk of bias by scoring domains of selection bias, performance bias, and detection bias as 'high risk', 'low risk', or 'unclear risk' of bias.

3.3.5. Statistical analysis

Statistical analyses were conducted on Stata/SE 12 software (StataCorp LP, College Station, TX, USA), and results are reported as mean ± standard deviation (SD) or weighted mean differences (WMDs) and 95% confidence intervals (CIs). The overall effect sizes were calculated using the Stata 12 metaan function, using either the fixed-effects or random-effects option, depending on heterogeneity statistics. Heterogeneity was assessed using chi-square tests and the I^2 statistic. A significant chi-square test ($P < 0.05$) and an I^2 statistic of 50% or greater indicated heterogeneity in effect sizes among the studies and therefore warranted the use of a random-effects model. When the chi-square test was nonsignificant ($P \geq 0.05$) and the I^2 statistic was less than 50%, a fixed-effects model was used.

Sensitivity analyses were performed by removing each study one-by-one and repeating the analysis. Thirteen studies contributing 15 comparisons were included in the primary meta-analysis. Removal of any individual study, with the exception of Weinheimer et al.[13], did not influence results or degree of heterogeneity. Variation in point estimates attributable to heterogeneity (I^2) is largely a function of sample size in individual studies [14]. Removal of large studies (even those near the middle of the distribution of effect sizes, such as Weinheimer et al., **Figure 3.2** [1-13]) can increase within-study precision and reduce I^2 despite the removal potentially increasing heterogeneity[14]. Weinheimer et al. contributes ~33% weight of the primary meta-analysis. Given this weight (and markedly smaller CIs than other comparisons), any failure of confidence intervals overlapping would indicate an 'inconsistency' with other studies from metrics used to assess heterogeneity. We retained Weinheimer et al. in the primary meta-analysis given these considerations and the fact that eligibility criteria were sound and data were correct.

Each specific objective (effect of WP on body composition with or without ER and with or without RT) is presented as sub-group analyses. Secondary sub-group analyses include WP versus a carbohydrate control independent of ER and RT status; WP versus control with and without RT separately (independent of ER); WP versus control with and without ER separately (independent of RT).

3.4. Results

3.4.1. Study features and participant characteristics

Thirteen articles that met all inclusion criteria contributed 28 intervention groups (488 female participants) resulting in 15 WP versus control comparisons were included in this review. Two articles each contributed two intervention comparisons to a control [6, 13]. Descriptions of study features and participant characteristics are summarized in **Table 3.3** [1-13]. Concerning the original 2x2 factorial design; one comparison met criteria for ER + RT [12], six comparisons were classified as No ER + RT [2, 4, 9, 11, 13], five comparisons were ER + No RT [1, 3, 5, 8, 10], and three comparisons were No ER + No RT [6, 7]. Publication dates ranged from 2008 to 2017, and intervention durations ranged from 6 weeks to 12 months. Among the 28 intervention groups, mean ages ranged from 20 ± 2 years to 64 ± 3 years. WP was compared to a carbohydrate control (n=12 comparisons), bovine colostrum (n=1 comparison), skim milk powder (n=1 comparison), and casein (n=1 comparison). WP supplementation dosage ranged from at least 6 g protein/d [10] to 48g protein/d [11]. Daily total protein intakes were directly available from 6 studies (n=8 comparisons), and were calculated from 3 additional studies. Total protein intake averaged 1.25 ± 0.19 g/kg/d in WP groups, and 0.93 ± 0.17 g/kg/d in control groups ($p < 0.001$). Protein intake from dietary sources were 0.81 ± 0.17 g/kg/d among WP groups and 0.93 ± 0.18 g/kg/d among control groups ($p=0.125$). Individual study details regarding contributions of dietary and supplemental protein to total protein intake (g/kg/d and % total protein intake) are summarized in **Table 3.4** [1-13]. Fourteen of the 15 comparisons were assessed with body composition measured using DXA, while one used BOD POD [7].

3.4.2. Quality of selected articles

Of the 13 articles included in this review, three articles [6, 8, 12] were deemed at low risk and ten articles had unclear risk of selection and detection biases based on provision of specific information pertaining to randomization and allocation concealment, and specific methods for the blinding of the outcome assessment, respectively (**Table 3.2**). Seven articles [2, 4, 6-8, 12, 13] had low risk and the remaining six articles had unclear risk for performance bias based on delineation of specific methods of participant and investigator blinding. No article was at high risk for bias in any domain. All 13 articles indicated that research staff provided supplements to the participants. Methods of measurement and assurance of compliance were described in 11 of the 13 articles.

3.4.3. Results of meta-analysis

Results of primary meta-analysis. Overall (15 comparisons), WP supplementation favored positive lean mass changes (WMD 0.37 kg; 95%CI= 0.06 to 0.67) relative to a non-WP control (**Figure 3.2**). WP supplementation did not influence changes in fat mass (**Figure 3.3** [1-7, 9-13]; WMD -0.20 kg; 95%CI= -0.67 to 0.27) or body mass (WMD -0.12 kg; 95%CI= -0.90 to 0.65), relative to a non-WP control (see Figure S1 in supporting information online). Similar results occurred when WP was compared to carbohydrate controls (n=10) (See Figure S2 in supporting information online). WP supplementation favored positive lean mass changes (WMD 0.36 kg; 95%CI= 0.01 to 0.70), but did not influence changes in fat mass (WMD -0.22 kg; 95%CI= -0.75 to 0.31) or body mass (WMD -0.03 kg; 95%CI= -0.84 to 0.78).

3.4.3.1. Results of a priori subgroup analyses

Studies without energy restriction and with resistance training (n=5 articles, 6 comparisons). WP did not influences changes in any body composition variable (see Figure S3 in supporting information online). Lean mass (WMD 0.22 kg; 95%CI= -0.19 to 0.64), fat mass (WMD 0.08 kg; 95%CI= -0.46 to 0.62), and body mass (WMD -0.23 kg; 95%CI= -1.41 to 0.96) changes were not different between groups.

Studies without energy restriction and resistance training (n=2 articles, 3 comparisons). WP resulted in decreased fat mass (WMD -0.57 kg; 95%CI= -1.03 to 0.11) without influencing changes in lean mass (WMD 0.21 kg; 95%CI= -0.24 to 0.65) or body mass (WMD -0.12 kg; 95%CI= -1.23 to 0.99).

Studies with energy restriction and resistance training (n=1 article, 1 comparison). There were not enough data to conduct meta-analysis for this condition.

Studies with energy restriction and without resistance training (n=5 articles, 5 comparisons). WP supplementation resulted in the most robust positive change in lean mass (WMD 1.04 kg; 95%CI= 0.38 to 1.70) of all analyses (primary and subgroup) (Figure S4 in supporting information online). WP did not influence changes in fat mass (WMD 0.22 kg; 95%CI= -0.37 to 0.81) or body mass (WMD 0.48 kg; 95%CI= -0.51 to 1.47) (Figure S4 in supporting information online).

Results of secondary subgroup analyses.

The beneficial effect of WP on lean mass was lost when only including studies with RT in the analysis (n=6 articles, 7 comparisons; WMD 0.23 kg; 95%CI= -0.17 to 0.63) (**Figure 3.4**).[1-13] Results did not differ from the primary analysis when only including studies without RT (n=7 articles, 8 comparisons) (**Figure 3.4**). The beneficial effect of WP on lean mass was more robust when only including studies with an ER component (n=6 articles, 6 comparisons; WMD 0.90 kg; 95%CI= 0.31 to 1.49) (**Figure 3.5**)[1-13]. There was no effect of WP on lean mass in studies without ER (n=7 articles, 9 comparisons; WMD 0.22 kg; 95%CI= -0.12 to 0.57) (Figure 5).

Table 3.3. Descriptions of the randomized controlled trials included in a meta-analysis on the effects of whey protein supplementation versus a control on body composition changes in adult women[¥]

	Study Details			WP Group Details				Contrast Group Details			
Author, year	Duration (wk)	ER (+/-)	RT (+/-)	Type of supplement	n	Age (y)	Protein supplemented (g/d)	Type of supplement	n	Age (y)	Protein supplemented (g/d)
Adechian et al. (2012)	6	+	-	MSP (WP)	17	32 ± 6	-	Casein	15	34 ± 4	-
Duff et al. (2014)	8	-	+	WP	13	58 ± 6	38	Bovine Colostrum	12	62 ± 5	38
Gordon et al. (2008)	20	+	-	WP	9	57 ± 6	32	Dietary Compensation	15	59 ± 7	0
Holm et al. (2008)	8	-	+	WP + CHO + Calcium	13	55 ± 4	10	CHO + Calcium	16	55 ± 4	0
Keogh et al. (2008)	52	+	-	WP (GMP enriched)	11	50 ± 12	30	Skim Milk Powder	9	50 ± 12	30
Kjølbæk et al. (2017)	24	-	-	WP	32	42 ± 11	45	Maltodextrin	32	38 ± 11	0
Kjølbæk et al. (2017)	24	-	-	WP + 1000 mg Calcium	31	41 ± 11	45	Maltodextrin	32	38 ± 11	0
Martens et al. (2015)	12	-	-	WP with α-lactalbumin	8	24 ± 5	-	Maltodextrin	10	24 ± 5	0
Mojtahedi et al. (2011)	26	+	-	WP	13	65 ± 4	45	Maltodextrin	13	65 ± 5	0
Stragier et al. (2016)	24	-	+	WP hydrolysate + 0.6 g Leu	7	64 ± 3	40	Maltodextrin	7	64 ± 3	0
Sukumar et al. (2011)	52	+	-	WP	26	59 ± 4	-	Dietary Compensation	21	57 ± 5	0
Taylor et al. (2016)	8	-	+	WP	8	20 ± 2	48	Maltodextrin	8	21 ± 3	0
Verreijen et al. (2015)	13	+	+	WP + Leu + Vit D	16	64 ± 6	21	CHO	16	63 ± 6	0
Weinheimer et al. (2012)	36	-	+	WP	41	47 ± 8	20	Maltodextrin	40	50 ± 6	0
Weinheimer et al. (2012)	36	-	+	WP	29	50 ± 8	40	Maltodextrin	40	50 ± 6	0

[¥]Individual study details regarding study characteristics (duration, presence of energy restriction component, presence of resistance training component), whey protein group details (type of supplement, n, age, protein supplemented (g/d)), and contrast group details (type of supplement, n, age, protein supplemented (g/d)); CHO, carbohydrate; ER, energy restriction; MSP, milk soluble protein; RT, resistance training; WP, whey protein. Data are presented as means ± SD, where appropriate.

Table 3.4. Descriptions of the contribution of dietary and supplemented protein towards total protein intake in a meta-analysis on the effects of whey protein supplementation versus a control on body composition changes in adult women[¥]

	WP Group Details			Contrast Group Details		
Author, year	Total protein intake (g/kg/d)	Dietary protein (g/kg/d) & % contribution	Supplemented protein (g/kg/d) & % contribution	Total protein intake (g/kg/d)	Dietary protein (g/kg/d) & % contribution	Supplemented protein (g/kg/d) & % contribution
Adechian et al. (2012)	-	-	-	-	-	-
Duff et al. (2014)	-	-	-	-	-	-
Gordon et al. (2008)	1.3 ± 0.2	0.93 (71.5%)	0.37 (28.5%)	0.6 ± 0.1	0.6 (100%)	0.00 (0%)
Holm et al. (2008)	1.10	0.94 (85.5%)	0.16 (14.5%)	0.94	0.94 (100%)	0.00 (0%)
Keogh et al. (2008)	-	-	-	-	-	-
Kjølbæk et al. (2017)	1.41 ± 0.48	0.86 (61.0%)	0.55 (39.0%)	1.17 ± 0.32	1.17 (100%)	0.00 (0%)
Kjølbæk et al. (2017)	1.25 ± 0.44	0.72 (57.6%)	0.53 (42.4%)	1.17 ± 0.32	1.17 (100%)	0.00 (0%)
Martens et al. (2015)	-	-	-	-	-	-
Mojtahedi et al. (2011)	1.20 ± 0.14	0.63 (52.5%)	0.57 (47.5%)	0.86 ± 0.20	0.86 (100%)	0.00 (0%)
Stragier et al. (2016)	1.23 ± 0.34	0.55 (44.7)	0.68 (55.3%)	1.08 ± 0.30	1.08 (100%)	0.00 (0%)
Sukumar et al. (2011)	1.04	-	-	0.78	0.78 (100%)	0.00 (0%)
Taylor et al. (2016)	1.37	0.65 (47.4%)	0.72 (52.6%)	1.12	1.12 (100%)	0.00 (0%)
Verreijen et al. (2015)	1.11 ± 0.28	0.87 (78.4%)	0.24 (21.6%)	0.85 ± 0.24	0.85 (100%)	0.00 (0%)
Weinheimer et al. (2012)	1.13 ± 0.23	0.89 (78.8%)	0.24 (21.2%)	0.96 ± 0.28	0.96 (100%)	0.00 (0%)
Weinheimer et al. (2012)	1.64 ± 0.38	1.01 (61.6%)	0.63 (38.4%)	0.96 ± 0.28	0.96 (100%)	0.00 (0%)

[¥]Individual study details regarding absolute (in g/kg/d) and relative (in % of protein intake) contributions of dietary and supplemental protein towards total report protein intake (in g/kg/d; mean ± SD, when available); WP, whey protein.
Total protein intake is equal to dietary protein + supplemented protein. Data are presented as means ± SD, where appropriate.

References	Whey Protein Mean (kg)	Whey Protein SD (kg)	Whey Protein Participants (no.)	Control Mean (kg)	Control SD (kg)	Control Participants (no.)	Weight (%)	Mean difference (kg), random-effects (95% CI)
Adechian et al (2012)[24]	-3.0	1.6	17	-4.0	2.1	15	4.1	1.00 (-0.31 to 2.31)
Duff et al (2014)[25]	0.5	1.8	13	0.7	2.8	12	2.3	-0.20 (-2.06 to 1.66)
Gordon et al (2008)[18]	-2.1	1.8	9	-4.1	2	15	3.1	2.00 (0.45 to 3.55)
Holm et al (2008)[27]	0.8	1.2	13	0.5	1.3	16	6.7	0.30 (-0.61 to 1.21)
Keogh and Clifton (2008)[21]	-1.5	2.3	11	-2.4	2.1	9	2.2	0.90 (-1.03 to 2.83)
Kjølbæk et al (2017)[20] -Ca	1.9	1.5	31	1.7	1.2	38	9.5	0.20 (-0.45 to 0.85)
Kjølbæk et al (2017)[20]	1.9	1.4	32	1.7	1.2	38	9.9	0.20 (-0.42 to 0.82)
Martens et al (2015)[22]	0.3	4.1	8	-0.3	4.1	10	0.6	0.60 (-3.24 to 4.44)
Mojtahedi et al (2011)[17]	2.0	2.3	13	0.6	1.5	13	3.3	1.38 (-0.11 to 2.87)
Stragier et al (2016)[26]	1.0	0.4	7	1.2	0.5	7	12.4	-0.23 (-0.67 to 0.21)
Sukumar et al (2011)[16]	-1.2	2.5	26	-1.4	2.2	21	4.1	0.20 (-1.12 to 1.52)
Taylor et al (2016)[19]	1.6	1.0	8	1.4	1.3	8	5.0	0.20 (-0.94 to 1.34)
Verreijen et al (2015)[23]	0	1.8	16	-0.3	2.0	16	4.0	0.30 (-1.04 to 1.64)
Weinheimer et al (2012)[15]-20g	1.3	0.2	41	0.6	0.2	40	16.4	0.71 (0.62 to 0.80)
Weinheimer et al (2012)[15]-40g	0.7	0.3	29	0.6	0.2	40	16.3	0.13 (0.02 to 0.24)
Total							100	0.37 (0.06 to 0.67)

Heterogeneity: $Tau^2 = 0.145$; $Chi^2 = 80.13$, df = 14 (P = 0.000); $I^2 = 83\%$

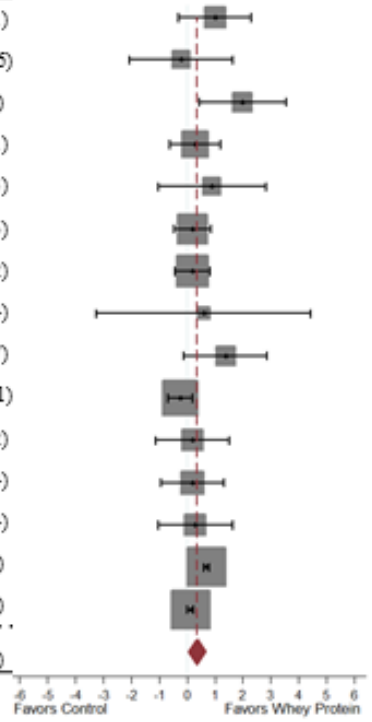

Figure 3.2. Effect of whey protein supplementation on changes in lean mass in women. A random-effects model was used for lean mass, since heterogeneity was observed in pooled data. *Abbreviations*: Ca, calcium; CI, confidence interval; SD, standard deviation

References	Whey Protein			Control			Weight (%)	Mean difference (kg), random-effects (95% CI)
	Mean (kg)	SD (kg)	Participants (no.)	Mean (kg)	SD (kg)	Participants (no.)		
Adechian et al (2012)[24]	-3.3	1.3	17	-3.4	1.9	15	7.3	0.10 (-1.04 to 1.24)
Duff et al (2014)[25]	-0.2	0.89	13	0.1	1.42	12	8.4	-0.30 (-1.24 to 0.64)
Gordon et al (2008)[18]	-6.3	3	9	-6.3	3	15	2.8	0.00 (-2.48 to 2.48)
Holm et al (2008)[27]	-0.5	1.9	13	-0.6	2.3	16	5.4	0.10 (-1.43 to 1.63)
Keogh and Clifton (2008)[21]	-7.8	6.5	11	-7.3	6.1	9	0.7	-0.50 (-6.04 to 5.04)
Kjølbæk et al (2017)[20] -Ca	-0.2	4.6	31	0.2	3.2	38	4.1	-0.40 (-2.31 to 1.51)
Kjølbæk et al (2017)[20]	-0.1	4.3	32	0.2	3.2	38	4.4	-0.30 (-2.10 to 1.50)
Martens et al (2015)[22]	-0.3	0.5	8	0.3	0.6	10	11.2	-0.60 (-1.09 to -0.11)
Stragier et al (2016)[26]	-0.8	1.66	7	1.37	1.3	7	5.3	-2.13 (-3.70 to -0.57)
Sukumar et al (2011)[16]	-4.2	1.6	26	-4.5	0.9	21	9.7	0.30 (-0.43 to 1.03)
Taylor et al (2016)[19]	-1.3	1.3	8	-0.7	1.4	8	6.3	-0.60 (-1.92 to 0.72)
Verreijen et al (2015)[23]	-3	0.79	16	-1.8	1.11	16	10.1	-1.20 (-1.87 to -0.53)
Weinheimer et al (2012)[15]-20g	-0.2	0.53	41	-0.95	0.53	40	12.3	0.77 (0.54 to 1.00)
Weinheimer et al (2012)[15]-40g	-0.4	0.75	29	-0.95	0.53	40	12.0	0.52 (0.20 to 0.84)
Total							100	-0.20 (-0.67 to 0.27)

Heterogeneity: Tau^2= 0.452; Chi^2 = 64.17, df = 13 (P = 0.000); I^2 = 80%

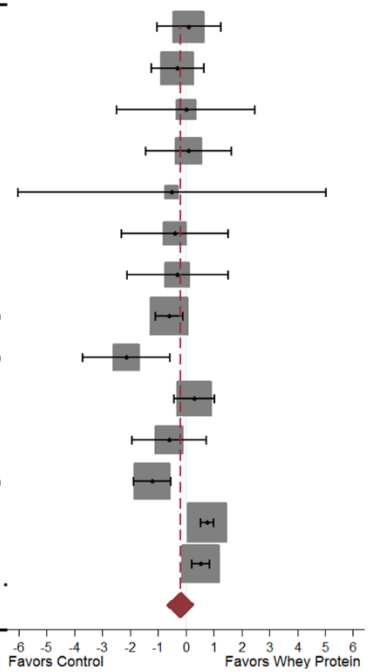

Figure 3.3. Effect of whey protein supplementation on changes in fat mass in women. A random-effects model was used for fat mass, since heterogeneity was observed in pooled data. *Abbreviations*: Ca, calcium; CI, confidence interval; SD, standard deviation

Results of sensitivity analyses.

One-by-one removal of 14 of the 15 comparisons did not significantly influence the results or the statistical model employed. Removal of the 20 g WP group from Weinheimer et al.[6], and removal of both Weinheimer et al. comparisons influenced the effect of WP on lean and fat mass. Specifically, the effect of WP on lean mass was either blunted (20g WP group removed: WMD 0.14 kg; 95%CI= 0.04 to 0.24) or ablated (both WP groups removed: WMD 0.19 kg; 95%CI= -0.07 to 0.44), while the effect of WP on fat mass was strengthened (both WP groups removed: WMD -0.48 kg 95%CI= -0.86 to -0.10). Furthermore, removal of the 20 g group, or both the 20 g and 40+ g groups permitted use of a fixed-effects model for lean mass changes, indicating reduced heterogeneity.

3.5. Discussion

The goal of this systematic review and meta-analysis was to assess the effect of WP supplementation on body composition changes over time in adult women. Overall findings presented herein suggest that WP supplementation favors modest increases in lean mass, while not influencing fat mass or total body mass, irrespective of the state of energy sufficiency and exercise training. This moderate increase in lean mass over time (0.37 kg) represents < 1% of total lean mass of study participants and therefore does not support the public perception that WP causes excessive hypertrophy or ‘bulkiness’ in adult women.

Some systematic reviews and meta-analyses of literature suggest that protein supplementation augments gains in lean mass [14-17], while others indicate a null effect [18, 19]. Potential discordance in findings could stem from reviews differing in inclusion criteria for age, training status, energy balance, and protein source. Despite these differences, one constant among all of these reviews is the inclusion of both sexes in analyses. There is a paucity of protein supplementation research in women, as discussed previously [16]. In line with this, females are underrepresented in protein supplementation meta-analyses; 68% of studies in the most-cited protein supplementation meta-analyses were comprised of only males [14]. Therefore, recommendations on the effectiveness of WP supplementation in women are of limited value. To address this concern, the present study was inclusive of only female participants in all of the

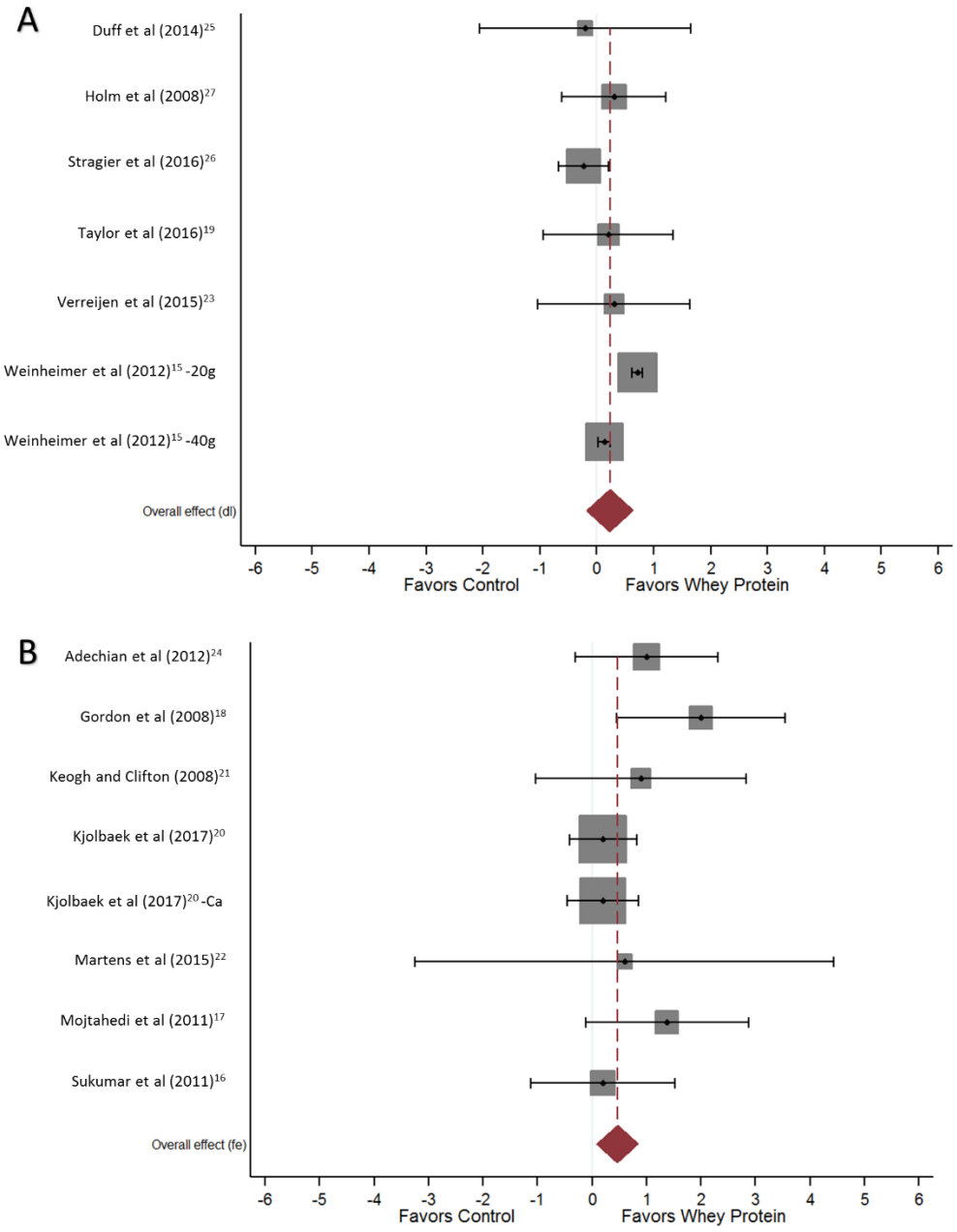

Figure 3.4. Effect of whey protein supplementation on lean mass changes in women with or without resistance training. (A) Results of a random-effects meta-analysis representing pooled mean differences with 95% CIs on lean mass in women participating in a resistance training protocol (WMD 0.23 kg; 95%CI= -0.17 to 0.63). (B) Results of a fixed-effects meta-analysis representing pooled mean differences with 95% CIs on lean mass in women not participating in resistance training (WMD 0.47 kg; 95%CI= 0.10 to 0.84). *Abbreviations*: Ca, calcium; CI, confidence interval; WMD, weighted mean difference

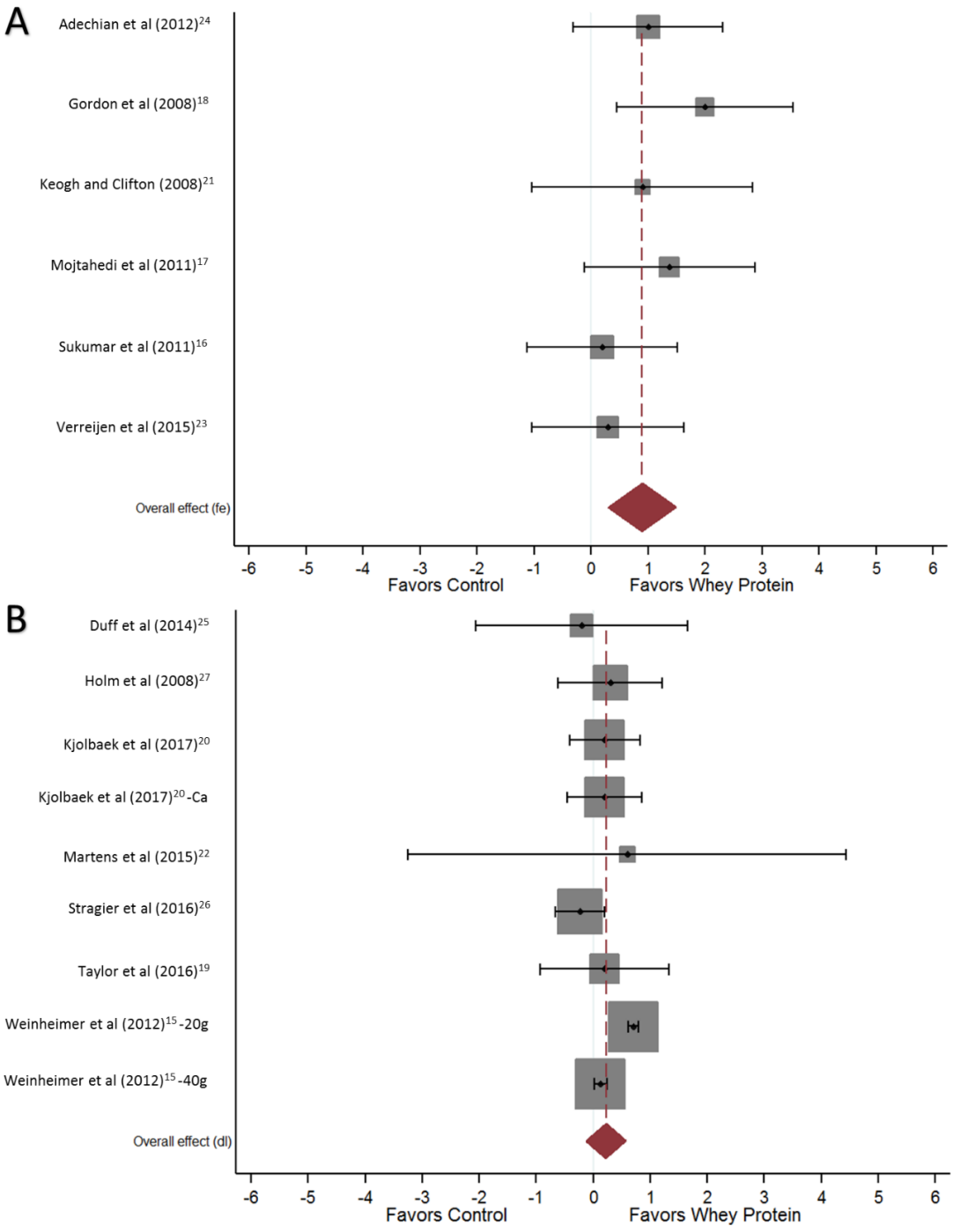

Figure 3.5. Effect of whey protein supplementation on lean mass changes in women with or without energy restriction. (A) Results of a fixed-effects meta-analysis representing pooled mean differences with 95% CIs on lean mass in women participating in studies with an energy restriction component (WMD 0.90 kg; 95%CI= 0.31 to 1.49). (B) Results of a random-effects meta-analysis representing pooled mean differences with 95% CIs on lean mass in women participating in studies without an energy restriction component (WMD 0.22 kg; 95%CI= -0.12 to 0.57). *Abbreviations*: Ca, calcium; CI, confidence interval; WMD, weighted mean difference

analyses. Overall, these data specifically in women are in agreement with the majority of meta-analyses inclusive of both sexes supporting a modest increase in lean mass as a result of WP supplementation. Another constant throughout previous systematic reviews and meta-analyses of protein supplementation literature is the inclusion of RT in featured studies [14-19]. In contrast, the present study features analyses with and without studies including RT in order to determine the separate and combined effects of WP supplementation and RT on body composition. One meta-analysis reported no overall effect of WP on lean mass in men and women, but sub-group analysis of only studies including a RT component suggested WP supplementation increased lean mass [18]. In contrast to these findings, WP did not augment gains in lean mass in studies of women who performed RT (n=6), but did result in increased lean mass relative to control in studies without RT (n=7). RT may be a potent enough anabolic stimulus that it washes out any potential effect of dietary protein manipulation on changes in lean mass [20, 21]. Therefore, the secondary subgroup analyses suggest that the beneficial effect of WP on lean mass in women is more robust in the absence of RT.

Pooled data on the effects of protein supplementation on fat mass and body mass are limited, compared to assessments of lean mass. One meta-analysis reported no effect of protein supplementation on fat mass [14], while another suggested protein supplementation reduces fat mass (without influencing body mass)[18]. The only meta-analysis with data on fat mass and body mass specific to WP concluded that WP supplementation significantly reduced fat mass and body mass [18]. The present findings in a female-only population are inconsistent with these results, as there was no detected effect of WP supplementation on changes in either fat mass or body mass. Likely the most influential factor on changes in fat mass is energy restriction [22]. The lack of ER stimulus in over half of the comparisons (n=9) in the primary analysis may have washed out or not permitted the potential fat mass-reducing effects of WP supplementation to manifest. However, secondary subgroup analysis including only studies with an ER component did not influence findings for fat mass or body mass. Presence or absence of ER most strongly influenced differential changes in lean mass from WP or control supplementation. There was no effect of WP on lean mass in analysis of studies without ER, while there was a pronounced positive difference in lean mass between WP and control in studies with ER. These findings are in line with the sentiment that higher protein intake may be of greater importance for promoting

positive changes in body composition during weight loss, relative to potential benefits of higher protein intake during weight maintenance [23].

A priori sub-group analyses more precisely assessed the separate or combined potential for ER and RT to modulate the effects of WP supplementation on body composition. There were not enough studies to permit meta-analysis on the effects of WP on body composition in groups who were participating in both ER and RT (n=1). In comparisons with RT but without ER (n=6), WP supplementation did not influence changes in lean mass, fat mass, or body mass. Likewise, in comparisons without both ER and RT (n=3), there was no effect of WP on any body composition outcome. In secondary subgroup analyses, the effects of WP on lean mass are amplified in studies with ER and blunted in studies with RT. This claim is further supported by subgroup analysis of studies featuring ER without RT (n=5) presenting with the most robust effects of WP supplementation on lean mass of all analyses (1.04 kg versus 0.37 kg in overall analysis). Therefore, these findings suggest WP supplementation may be less effective when energy needs are met, and more effective in conditions where increased dietary protein is purported to be of increased importance (ER).

3.6. Strengths and limitations

This review is subject to standard limitations of systematic reviews and meta-analyses such as publication bias and inconsistencies in experimental features of selected studies. To address this limitation, manual searches of relevant systematic reviews and meta-analyses were conducted. The original goal was to determine the effects of WP supplementation on body composition in women in 4 conditions in a 2x2 factorial manner (with and without RT, with and without ER). Due to a paucity of data in specific subgroups, firm conclusions about the effects of WP on body composition with respect to specific energy and training statuses cannot be reached. Another consideration is that total protein intake was greater in the WP groups when compared to control groups. Therefore, differential changes in body composition may be attributable to greater total protein intake [24] as opposed to specifically WP supplementation [6]. However, specific effects of WP on body composition cannot be disentangled from the overall effects of greater daily total protein intake (seen in WP groups) on body composition because only 3 comparisons included in this meta-analysis assessed WP versus another protein source; 12 comparisons were between WP and a carbohydrate control.

Only 5 of the studies included in this review were in female-only populations [2, 4, 8, 12, 13]. As body composition data in the remaining studies are typically presented in manuscripts with means and SDs for mixed-sex groups, acquisition of data was challenging. Therefore, there were more studies conducted with female participants as part of a mixed-sex populations than are reported herein. Future studies should include sex-specific data in manuscripts or supplemental tables. Additionally, a random-effects model was used in most analyses due to inherent heterogeneity in studies assessed. To address this shortcoming, sensitivity analyses were conducted to determine potential sources of heterogeneity. Removal of any single comparison did not significantly influence findings on body composition outcomes, including removal of one comparison [6], which permitted use of a fixed-effects model.

3.7. Conclusion

In summary, findings from this systematic review and meta-analysis indicate that WP supplementation improves body composition in adult women by modestly increasing lean mass without influencing changes in fat mass. This null effect on fat mass and <1% increase in lean mass is not in line with the public perception that WP causes excessive hypertrophy or 'bulkiness' in adult women. WP may be more beneficial for improving body composition when included as part of a weight loss program. While more research is needed to specifically assess the effects in varying states of energy sufficiency and exercise training, the overall findings support consumption of WP in women seeking to modestly improve body composition.

3.8. Acknowledgments

Author Contributions. R.E.B., J.L.H., and W.W.C. designed the research; R.E.B. and J.L.H conducted the research; R.E.B. analyzed the data; R.E.B, J.L.H., and W.W.C. wrote the manuscript and had primary responsibility for the final content of the manuscript. All authors read and approved the final manuscript.

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Declaration of interest. R.E.B. and J.L.H have no conflicts of interests to declare. W.W.C received research funds from the National Pork Board, American Egg Board-Egg Nutrition Center, National Dairy Council, and National Cattlemen's Beef Association, and served on the National Dairy Council's Whey Protein Advisory Panel, during the time this study was being conducted.

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CHAPTER 4. EFFECTS OF A HIGH PROTEIN MEAL ON BLOOD PRESSURE AND VASOACTIVE BIOMARKER RESPONSES TO ACUTE EXERCISE

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4.1. Abstract

Habitual higher dietary protein intake is associated with lower resting blood pressure (BP). However, few studies have assessed the impact of high-protein meals on acute BP and vasoactive biomarker responses to exercise. Thirty-one subjects completed this randomized, double-blind, cross-over acute feeding study. Pre-hypertensive subjects consumed either a higher-protein, lower-fat meal (HP; 30 g protein, 17 g fat) or a lower-protein, higher-fat meal (LP; 13 g protein, 25 g fat). One hundred sixty-five minutes after consuming the test meal, subjects exercised on a cycle ergometer at 70% VO_2 max for 30 minutes. Blood pressure was measured prior to the meal and periodically before, during, and after exercise during a 315-minute period. Blood samples were periodically collected to quantify plasma arginine, arginine metabolites (asymmetric dimethylarginine, symmetric dimethylarginine; ADMA, SDMA), endothelin-1, nitrates, and nitrites. Consuming meals with higher protein did not influence the BP responses to exercise, including systolic BP area under the curve or post-exercise return to baseline BP. While the HP meal resulted in greater postprandial plasma arginine concentrations,

ADMA, SDMA, endothelin-1, nitrates, and nitrites were not altered. In conclusion, consuming a higher-protein, lower-fat meal does not influence BP or vasoactive biomarker responses to exercise compared to a lower-protein, higher-fat meal.

4.2. Introduction

An estimated one billion individuals worldwide meet criteria for hypertension, with up to 7.1 million deaths per year attributable to the disease [1, 2]. Despite the consistently documented beneficial effects of regular exercise on blood pressure (BP) improvement and cardiovascular health, acute exercise may trigger cardiovascular events [3]. This association may be partially explained by the fact that an acute bout of exercise causes a rise in BP as a result of a series of hemodynamic and cardiac responses [4]. Particularly, systolic BP (SBP) rises with the increasing workload, while diastolic BP (DBP) typically remains the same [5]. The magnitude of the exercise-induced elevation in SBP is implicated with the development of chronic hypertension and cardiovascular events [6]. A recent meta-analysis showed that each 10mm Hg higher SBP response to exercise is associated with an annual 4% increased risk of cardiovascular events and mortality [7].

Dietary protein and skeletal muscle are inseparable; the utility of increased protein intake is often thought to stem from promotion of skeletal muscle mass accretion and function, in conjunction with exercise [8-10]. From a consumer standpoint, it is of interest to determine if the proposed beneficial effects of protein intake may extend beyond skeletal muscle. Chronically, greater consumption of dietary protein is associated with reduced resting BP [11-15]. If a higher protein diet results in chronically reduced BP, one may think that a higher protein meal would acutely decrease BP, as well. However, few studies have assessed the acute postprandial effects of dietary protein modulation on BP, and are generally not supportive of an acute protein-superiority effect [16-18]. To our knowledge, no studies have investigated the effects of meals with different amounts of protein on BP responses to exercise.

The purpose of this randomized, cross-over acute feeding and exercise study was to determine if consumption of a higher-protein, lower-fat meal (HP) would influence BP responses to exercise relative to a lower-protein, higher-fat meal (LP). We hypothesized that consuming the HP meal would attenuate the BP response to exercise and result in a more robust post-exercise hypotensive response. In an exploratory manner, we additionally sought to investigate potential

mechanisms by which meals containing higher dietary protein could attenuate BP responses to exercise by measuring relevant vasoactive biomarkers. Dietary proteins are generally insulinotrophic and may increase insulin-induced nitric oxide (NO) release [19]. Meals with greater protein contain more of the amino acid arginine, which is the precursor of NO production in endothelial cells. Additionally, bioactive peptides in protein-rich foods are shown to promote endothelium-dependent vasodilation and reduce BP through angiotensin converting enzyme-inhibitory dependent and independent pathways [20-23]. The potent vasoconstrictor endothelin-1 (ET-1), the potent vasodilator NO, as well as arginine and arginine metabolites (asymmetric dimethylarginine; ADMA, and symmetric dimethylarginine; SDMA), which are central in NO metabolism, emerged as prime targets of investigation. We hypothesized that the differential BP responses would occur concurrent with differential changes in vasoactive biomarkers indicative of greater vasodilation in response to the HP meal.

4.3. Materials and methods

4.3.1. Participants

Participants were recruited from the Greater Lafayette, IN, community through online newsletter, website, and bulletin board postings. Inclusion criteria were: male or female, age ≥ 21 years, normo- or pre-hypertensive (SBP 120-139 mm Hg or DBP 80-89 mm Hg) based on clinical standards when the study was conducted, body weight <300 lb (136 kg), BMI between 20 and 34.9 kg/m^2, fasting plasma glucose <6.1 mmol/L, total cholesterol <6.7 mmol/L, LDL cholesterol <4.1 mmol/L, TG <4.5 mmol/L, no pre-existing or history of cardiovascular, renal or liver disease, not currently or previously (past 6 months) consuming a weight-loss diet or other special/non-balanced diets, no weight loss or gain (±4.5 kg) within the past 6 months, and no physical impairments limiting or preventing exercise. Our primary inclusion criterion was BP in the high-normal range (SBP 120-139 mm Hg or DBP 80-89 mm Hg). Conceptually, we wanted to include individuals that could theoretically benefit from dietary means (pre-hypertensive individuals) but were not uncontrolled hypertensives (at greater risk during exercise) or taking medication for hypertension. Pre-study screenings included self-administered medical history, and measured fasting state height, weight, BP, and a blood sample (for determination of a complete blood count with differential, complete metabolic panel, and lipid panel). The study

physician reviewed all subject screening data to confirm eligibility. All participants provided written informed consent and received a monetary stipend. The research was conducted according to the Declaration of Helsinki. The study protocol and all study documents were approved for use by the Purdue University Biomedical Institutional Review Board. This study is registered at clinicaltrials.gov as NCT03073252.

4.3.2. Experimental design

This randomized cross-over study included one, 60-min pre-testing day and two, 330-min testing days separated by a minimum of 1 week (**Figure 4.1**). Subjects were randomized (using an online randomization plan generator; http://www.randomization.com/) to consume either the LP breakfast or the HP breakfast on the first testing day. Randomization and subject allocation were performed by a clinical laboratory manager that did not participate in data analysis or interpretation. Both the investigators and the participants were blinded to the test meal composition throughout the study. The investigators remained blinded throughout processing and data analysis; the randomization code was broken after all analyses were completed. Between testing days, participants were instructed to consume their self-chosen, unrestricted diets and to maintain their customary levels of physical activity. Forty-eight hours prior to each testing day, participants were instructed to not perform strenuous physical activity. Between 19:00 and 20:00 on the day before testing, each participant consumed a controlled, energy sufficient meal based on their estimated energy requirement [24]. Each subject's total energy requirement was estimated using the sex-specific equations for normal weight, overweight or obese adults with a low activity level [25]. Participants were also instructed to avoid caffeinated beverages the mornings of testing.

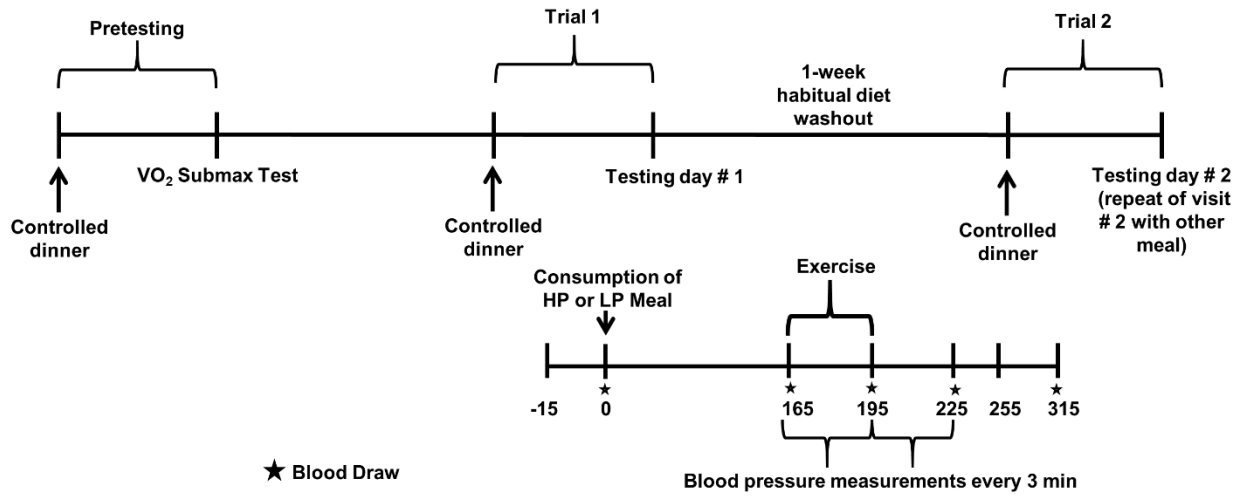

Figure 4.1. Experimental protocol for an acute feeding and exercise training cross-over study.

On the pre-testing day, participants arrived at the testing facility in a fasted state and completed the YMCA VO_2 submaximal exercise test on a cycle ergometer [26]. Heart and work rates were recorded at regular intervals and used to estimate the participant's 70% VO_2 max. Blood pressure and perceived exertion (6-20 scale; Borg Rating of Perceived Exertion [27]) were monitored periodically during the exercise session [28].

On each testing day, upon arrival in a fasting state urine samples were obtained to determine hydration status via refractometer-derived specific gravity (Palm Abbe; Misco Refractometer; Solon, OH, USA). Prior to consuming the test meal, a catheter was placed in an antecubital vein. Blood samples were obtained immediately prior to the meal (0 min, fasting), and then 30, 165 (pre-exercise), 195 (post-exercise), 225, and 315 minutes (post-exercise recovery) after initiation of the meal. Blood pressure was measured in quadruplicate (most deviant BP measurement discarded and remaining three averaged; exercise BP measurements in singlet) immediately prior to the meal (0 min) and periodically before, during, and after exercise over a 315-minute period (**Figure 4.1**). Pilot research from our lab group established 180 minutes to be the peak of postprandial plasma amino acid concentrations [29]. A 12-lead electrocardiogram was used to measure heart rate during exercise (Quinton Q710; Mortara Instrument Inc.; Milwaukee, WI, USA). Subjects began a 30-min bout of individually prescribed moderate-intensity exercise (70% VO_2 max) on a cycle ergometer (Monark Ergomedic 828E; Monark Exercise; Vansbro, Sweden) 165 minutes after meal consumption so peak plasma amino

acid concentrations would align with the midpoint of the exercise bout. Moderate-intensity exercise was chosen because it has been demonstrated that hypertensive responses to exercise at moderate intensities, but not high intensities, predict adverse cardiovascular outcomes independent of age, resting BP, and other relevant cardiovascular disease risk factors [7]. Blood pressure was measured every 3 min [30] during the exercise bout by a specialized automated BP monitor designed to overcome the noise, motion, and physical difficulties associated with exercise testing (SunTech Medical; Tango M2 monitor; Morrisville, NC, USA). Blood pressure measurements at 3-min intervals extended 30 min post-exercise.

4.3.3. Test meals

In a randomized cross-over manner, participants consumed the HP breakfast (30 g protein, 17 g fat) or LP breakfast (13 g protein, 25 g fat) 165 minutes before doing the exercise bout. Each breakfast meal was designed to provide 500 kcal (2,092 kJ) and was matched for fiber, sodium, potassium, and carbohydrate contents (**Table 4.1**). Egg-based protein primarily accounted for the difference in protein content between the meals. Fat was modulated to achieve isoenergetic HP and LP meals. We recognize that the changes in primary study outcomes may be attributable to either higher protein or lower fat intake. We chose to adjust protein and fat intakes between meals while keeping carbohydrate intake constant, since carbohydrate is shown to have an acute BP-lowering effect [17]. Participants consumed water ad libitum to promote hydration during the testing day.

4.3.4. Serum and plasma analyses

Blood samples were obtained from an antecubital vein and placed in tubes containing either a clot activator to obtain serum or sodium heparin to obtain plasma. Serum tubes were held at room temperature for 30 minutes and then centrifuged at 4,000 x g at 4 ºC for 15 minutes. Serum tubes were sent to MidAmerica Clinical Laboratories (Indianapolis, IN, USA) for determination of cardiometabolic health parameters. Plasma was sampled at 5 time points throughout the testing day and was quickly refrigerated, processed, and aliquoted into microtubes. Plasma was stored at 80 ºC until thawed for analyses.

Table 4.1. Study test meal composition

Nutritional Information	HP Meal	LP Meal
Food Composition	**105 g** eggs, whole, raw weight **85 g** eggs, white only, raw weight **5.5 g** butter, regular, salted **105 g** onion, white **70 g** hashed brown potatoes **0.3 g** salt, regular **10 g** green pepper, sweet, raw weight **85 g** tomato, canned, regular, plain **35 g** bread crumbs, regular, commercial **0.5 g** Metamucil fiber supplement	**54 g** eggs, whole, cooked **23 g** butter, regular, salted **85 g** onion, white **147 g** Hashed brown potatoes **0.5 g** salt, regular **10 g** green pepper, sweet, raw weight **95 g** tomato, canned, regular, plain **20 g** bread crumbs, regular, commercial
Macronutrients (g)		
Protein	30	13
Fat	17	25
SFA	6	14
MUFA	5	7
PUFA	2	2
Carbohydrate	52	54
Micronutrients (mg)		
Calcium	146	99
Potassium	624	582
Sodium	730	618
Magnesium	242	164
Total Energy Content (kJ)	2071	2079
Amino Acids (g)		
Leucine	2.5	1.0
Isoleucine	1.6	0.6
Valine	2.0	0.8
Arginine	1.8	0.8

Values are from Nutrition Data System for Research Software (NDSR 2012, Nutrition Coordinating Center, University of Minnesota). HP, higher-protein meal; LP, lower-protein meal.

An extension of the study permitted exploratory analyses of vasoactive biomarkers in a subset of the original sample. The 15 participants from the original sample (n=31) with the highest baseline BP were among those included in the analyses of vasoactive biomarkers.

Nitrites and nitrates were measured using a colorimetric kit in accordance to the manufacturer's instructions (R&D Systems, Inc.; Minneapolis, MN, USA). Briefly, plasma samples prepared using a two-fold dilution were quantified via colorimetric detection of enzymatic conversion of nitrate to nitrite as an azo dye product of the Griess Reaction. Endothelin-1 (ET-1) in plasma samples was determined using a commercially available quantikine ELISA kit according to the manufacturer's instructions (R&D Systems, Inc.; Minneapolis, MN, USA). Samples were read at 540 nm on a Versa Max microplate reader (Molecular Devices Corporation; Sunnyvale, CA, USA). All ET-1, nitrate, and nitrite samples were assayed in duplicate (CV=5.1%, 8.8%, and 6.6%, respectively).

Plasma arginine, ADMA, and SDMA were quantified via high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) as described [31, 32]. Briefly, L-arginine, ADMA, and SDMA were extracted from plasma using a protein precipitation method. Plasma (100 µL) was spiked with 500 ng of $^{13}C_6$-Arginine, 15 ng of d_6-SDMA, and 5 ng of d_6-ADMA (Toronto Research Chemicals; Ontario, Canada). Cold acetonitrile (0.5 mL) was added prior to the samples being vortexed for 3 minutes, then centrifuged at 13,000 x g for 10 minutes to precipitate the protein. The supernatant was recovered and used for analysis on the HPLC-MS/MS instrument. The analysis was done with an Agilent 1200 liquid chromatography system coupled to an Agilent 6470 QQQ mass spectrometer (Santa Clara, CA). Hydrophilic interaction chromatography using an Imkakt Intrada Amino Acid column (2.0 x 150 mm) was used for separation (Kyoto, Japan). Data were processed using Agilent Masshunter Quantitative analysis software (V.B.08).

4.3.5. Statistics

Due to the novelty of our study design, no published results were available for estimating the sample size with regards to meal composition and BP responses to exercise. As a result, sample size estimation was based on mean SBP responses to exercise in pre-hypertensive individuals (change of 53 ± 19mm Hg) [33]. Thirty-one individuals were recruited to provide 80% power to detect a 10mm Hg BP difference in response at P=0.05 (G*Power version 3.0.10, Kiel, Germany). The 10mm Hg differential BP response was chosen based on the finding that "a 10mm Hg increase in SBP during exercise is associated with 4% increase in cardiovascular events and mortality [7]". No results were available for estimating the sample size with regards

to consuming a higher protein meal on vasodilation and vasoconstriction-related biomarkers during exercise. Previous researchers reported greater decreases of nitrites/nitrates after acute ingestion of a normal protein meal compared to a whey protein-based high protein meal in healthy adults and overweight, middle-aged adults, respectively (-1.8 ± 0.6 vs. -1.3 ± 0.4 µmol/L; -3.0 ± 2.0 vs. -1.0 ± 2.0 µmol/L). Considering these results, an estimated 11 participants would provide 80% power at P=0.05 (two-tailed) to statistically support a comparable difference in response with consuming an egg-based high protein meal. Power calculations based on plasma arginine, ADMA, and SDMA concentrations were not conducted due to the lack of preliminary data.

Data from all participants who completed the study were included in the analysis of blood pressures, and blood samples from a subset of participants with higher fasting SBP were included in biochemical analyses of vasoactive biomarkers, as described below. The main effects of test meal (between-day) and time (within-day) on the dependent variables (SBP and DBP values, arginine, ADMA, SDMA, ET-1, nitrates, nitrites) were determined via analysis of variance (ANOVA) with doubly repeated measures for between-day and within-day testing. Post-hoc analyses were conducted using the Tukey method when a significant omnibus F-test was established from the ANOVA. Test meal-specific least square means (LSmeans) at each time point were compared using the difference of LSmeans. Incremental areas under the curve (iAUCs) for the dependent variables were calculated using the trapezoidal rule. Recovery SBP ratio was calculated as 3 min post-exercise SBP/average exercise SBP. All statistical analyses were performed with SAS statistical software version 9.4; SAS Institute, Cary, NC. Results are reported as LSmeans ± SEM, unless otherwise noted, and significance is set at $P<0.05$.

4.4. Results

4.4.1. Participant characteristics

A total of 34 participants signed consent forms and were randomized. Three participants withdrew from the study due to: scheduling difficulty (n=1), concerns about test meals (n=1), and fainting during blood sampling (n=1), leaving 31 study completers included in the analyses. Baseline anthropometric and cardiometabolic health parameters are provided in **Table 4.2**.

Table 4.2. Descriptive characteristics of the study population

Outcome Variable	Value
General Characteristics	
Sex (M/F)	18/13
Age (y)	33 ± 14
Height (cm)	175 ± 8
Weight (kg)	80.8 ± 12.9
BMI (kg/m²)	26.5 ± 3.8
Estimated VO_{2max} ($mL \cdot kg^{-1} \cdot min^{-1}$)	37.4 ± 8.2
Estimated VO_{2peak} ($L \cdot min^{-1}$)	3.0 ± 0.9
Systolic Blood Pressure (mm Hg)	127 ± 5
Diastolic Blood Pressure (mm Hg)	77 ± 6
Total Cholesterol (mmol/L)	4.3 ± 0.9
Triglycerides (mmol/L)	1.1 ± 0.6
HDL (mmol/L)	1.3 ± 0.3
LDL (mmol/L)	2.5 ± 0.8
Total Cholesterol:HDL	3.47 ± 1.07
Glucose (mmol/L)	5.1 ± 0.3
Vasoactive Biomarkers	
Nitrites (µmol/L)	0.46 ± 0.12
Nitrates (µmol/L)	20.13 ± 6.12
Endothelin-1 (µmol/L)	1.20 ± 0.24
Arginine (µmol/L)	104.1 ± 27.1
Asymmetric dimethylarginine (µmol/L)	0.6247 ± 0.1071
Symmetric dimethylarginine (µmol/L)	0.4187 ± 0.0737

Values are means ± SD. All measurements were made and samples collected while participants were in an overnight fasting state.

4.4.2. Blood pressure responses

Fasting SBP was not different between the HP and LP testing days (HP 120 ± 3, LP 120 ± 3 mm Hg; P=0.78). Similarly, postprandial SBP was not different between HP and LP meals at any point (**Figure 4.2A**), including the pre-exercise time point (HP 119 ± 3, LP 120 ± 3 mm Hg; P=0.92). Blood pressure responses during the exercise period for HP and LP meals are presented in **Figure 4.3**. Increases in SBP in response to exercise were not different between test meals (HP +48 ± 4, LP +48 ± 3 mm Hg; P=0.85). There was no differential response in SBP iAUC during exercise by test meal (**Figure 4.3B**). Similarly, there were no differences between HP and LP meals for SBP and DBP iAUC over the ~6 h test day (**Figure 4.2A** & **2B**).

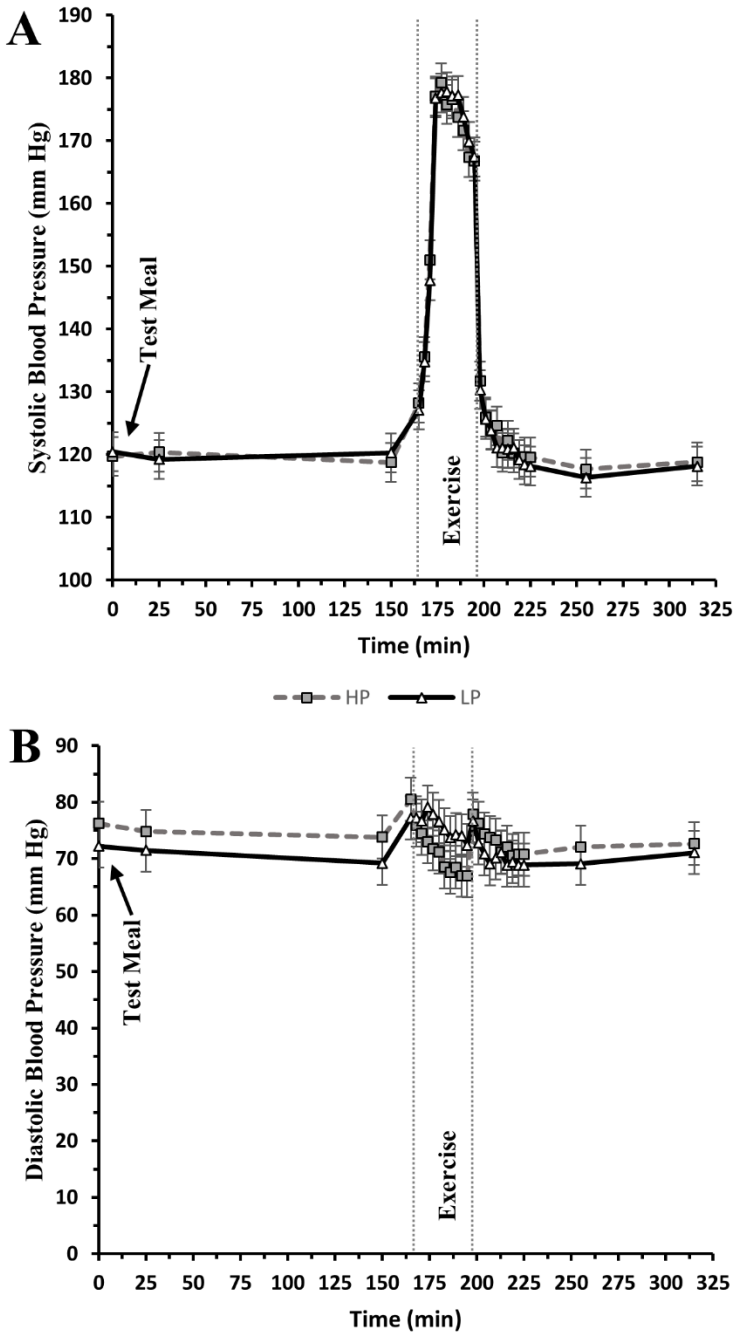

Figure 4.2. Systolic blood pressure (A) and diastolic blood pressure (B) before (0) and after ingestion of test meals (HP and LP). No significant difference between meals were detected at any time point. All values are LSmeans ± SE; n=31 (18 males, 13 females); significant difference between test meals denoted as ¥ ($P<0.05$).

Meal protein content did not affect peak (HP 190 ± 5, LP 190 ± 5 mm Hg; P=0.90) or average (HP 168 ± 4, LP 168 ± 4 mm Hg; P=0.91) exercise SBP. Recovery SBP ratio was not

influenced by test meal at both 3 min post-exercise (HP 0.80 ± 0.02, LP 0.78 ± 0.02; P=0.50) and 6 min post-exercise (HP 0.76 ± 0.02, LP 0.76 ± 0.02; P=0.67). Post-exercise SBP iAUC was not different between test meals (HP 102.3 ± 15.1, LP 67.0 ± 15.1 mm Hg*min; P=0.067). Likewise, there was no differential effect of test meal on BP responses to exercise in secondary analyses involving stratification of subjects by age, BP response to exercise (> or < Δ60 mm Hg SBP), or fasting, resting BP (> or < 130 mm Hg resting SBP).

4.4.3. Vasoactive biomarkers

The postprandial rise in plasma arginine was greater for HP versus LP (iAUC: HP 12 155 ± 1 146, LP 2 030 ± 1 146 µmol*min/L; P<0.0001; **Figure 4.4B**). The plasma arginine response to the HP meal was accompanied by a postprandial increase in ADMA iAUC, while ADMA decreased in the LP test meal (HP 11.0007 ± 6.1095, LP -6.5809 ± 6.1370 µmol*min/L; P=0.015; **Figure 4.4D**). Similar to ADMA, postprandial SDMA iAUC tended to be lower in the LP meal (HP 9.0021 ± 3.5561, LP -0.3544 ± 3.5561 µmol*min/L; P=0.068; **Figure 4.4F**). Postprandial ET-1 iAUC did not differ by test meal (HP -20.71 ± 16.13, LP 3.01 ± 16.13 µmol*min/L; P=0.117; **Figure 4.5B**). Postprandial nitrates (HP 562.12 ± 675.08, LP 1486.52 ± 675.08 µmol*min/L; P=0.235; **Figure 4.5D**) and nitrites iAUCs (HP 0.52 ± 0.01, LP 0.53 ± 0.01 µmol*min/L; P=0.236; **Figure 4.5F**) were not different between test meals. Likewise, exercise-dependent changes in any vasoactive biomarker (arginine, ADMA, SDMA, ET-1, nitrates, nitrites) did not vary by test meal (all P>0.05). ET-1 concentrations progressively rose after exercise in both HP and LP, with no difference between test meals (**Figure 4.5A**).

4.5. Discussion

To our knowledge, this is the first randomized cross-over study to assess the acute effects of consuming a higher-protein, lower-fat meal versus lower-protein, higher-fat meal on BP responses to exercise. Our experimental design allowed us to assess meals with different quantities of protein on BP responses in the general postprandial period (times 0 – 165 min), in the exercise period (165 – 195 min), and in the post-exercise period (195 – 315 min). Contrary to our primary hypothesis, greater intake of dietary protein at the breakfast meal (30 g vs 13 g) did not attenuate exercise-induced elevations in SBP. There was no effect of meal protein content on

general postprandial BP, peak exercise BP, post-exercise time to return to baseline BP or magnitude of the post-exercise hypotensive response. Vasoactive biomarker responses were not different between the HP and LP meals.

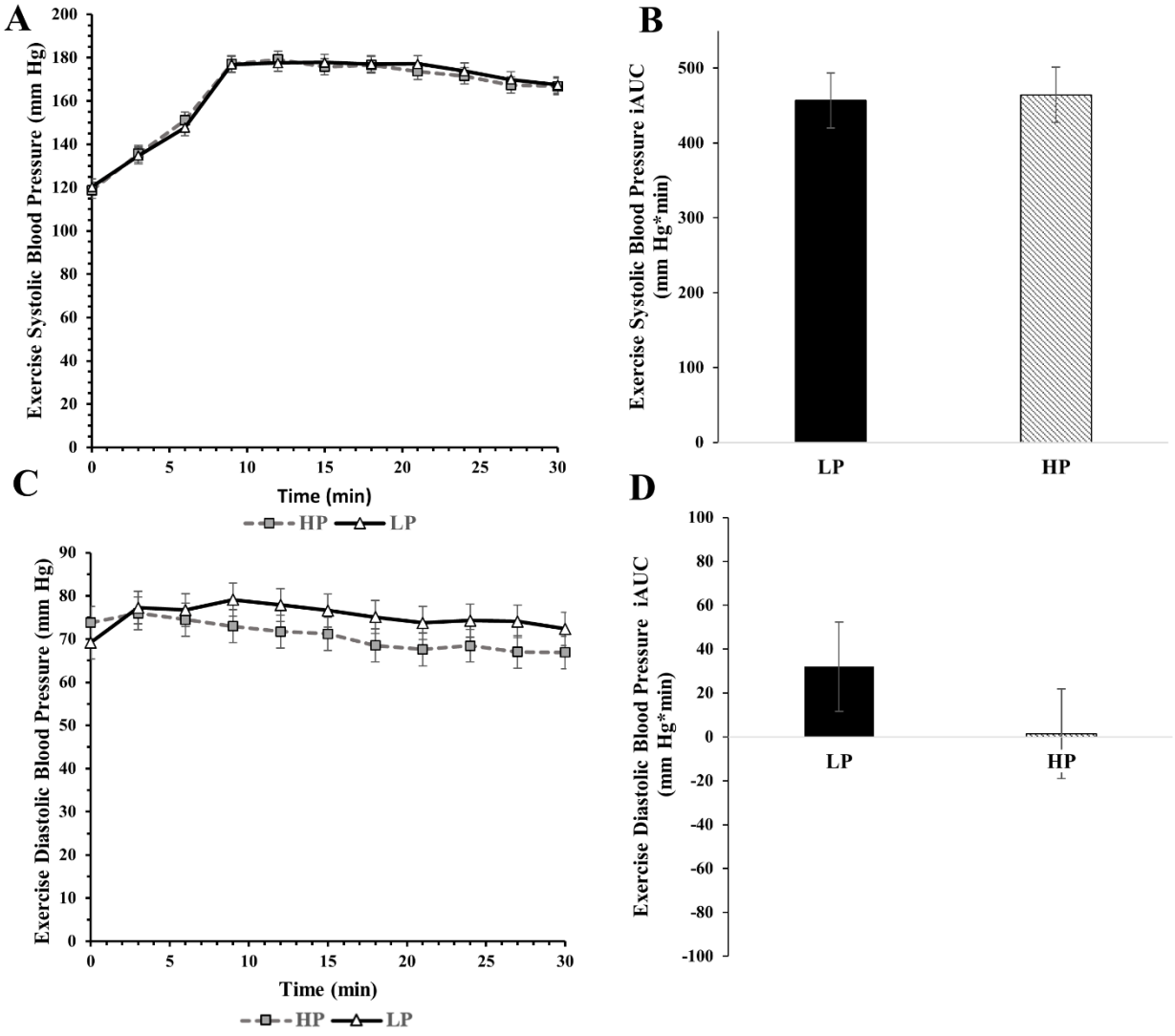

Figure 4.3. Systolic blood pressure (A) and diastolic blood pressure (B) responses during exercise that started 165 minutes after ingestion of test meals (testing minutes 165-195). Incremental areas under the curve (iAUC) of systolic blood pressure (C) and diastolic blood pressure (D) in response to exercise. There are no significant differences in blood pressure iAUC or at any time point. All values are LSmeans ± SE; n=31 (18 males, 13 females); significant difference between test meals denoted as ¥ ($P<0.05$).

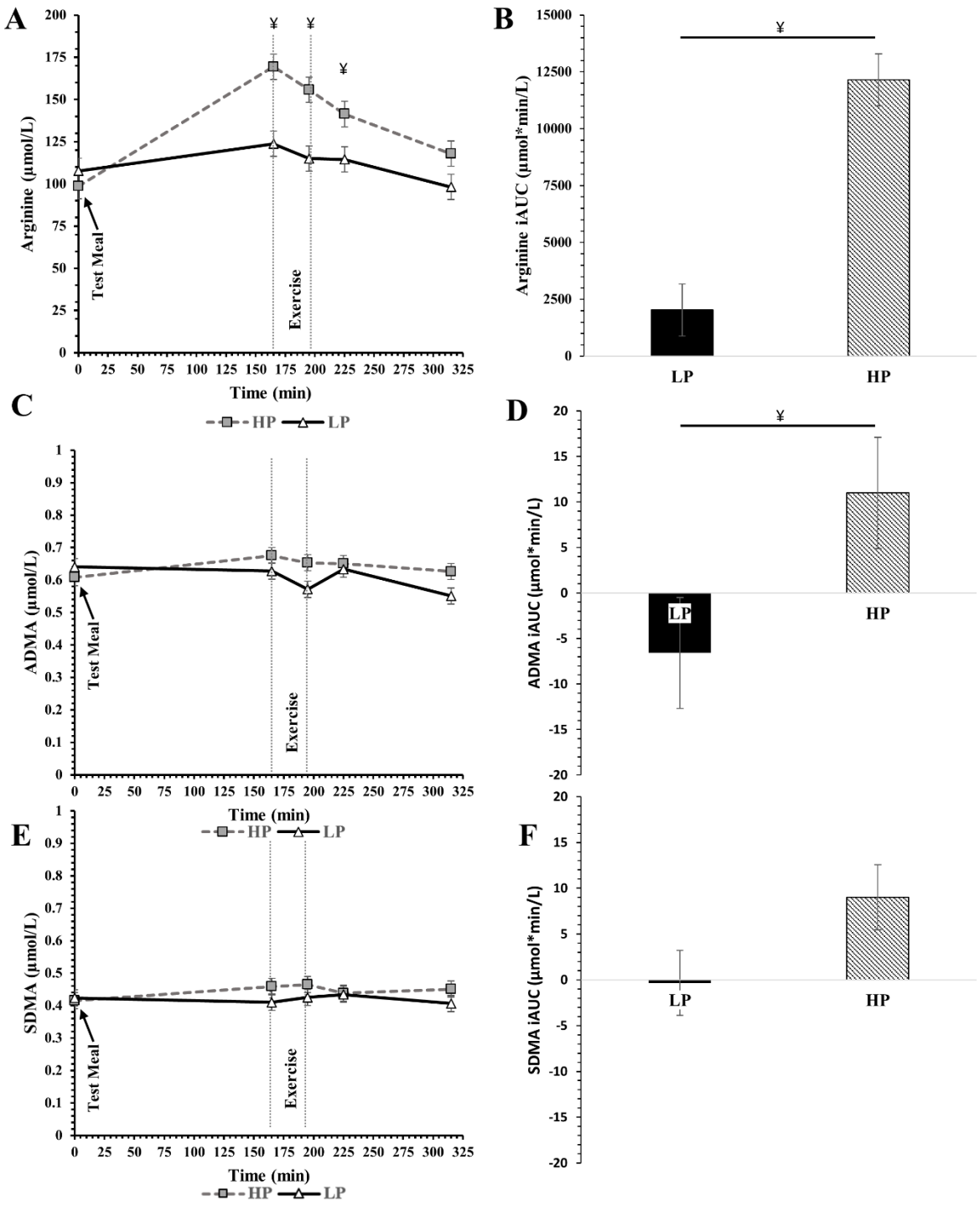

Figure 4.4. Plasma arginine concentrations (A) and incremental area under the curve (iAUC; B), plasma ADMA concentrations (C) and iAUC (D), and plasma SDMA concentrations (E) and iAUC (F) responses before (0) and after ingestion of test meals (HP and LP). Incremental areas under the curve (iAUC) of arginine (D), ADMA (E), and SDMA (F) for the entire postprandial period. L-arginine was significantly greater in the HP intervention at 150 min (P<0.001), 195 min (P<0.001), with a trend at 225 min (P=0.076). ADMA and SDMA were not significantly different at any time point after adjustment for multiple testing. Arginine iAUC and ADMA iAUC were greater in the HP intervention (p <0.001 and P=0.0151, respectively), with a trend for greater SDMA iAUC (P=0.067). All values are LSmeans ± SE; n=15; significant difference between test meals denoted as ¥ (P<0.05).

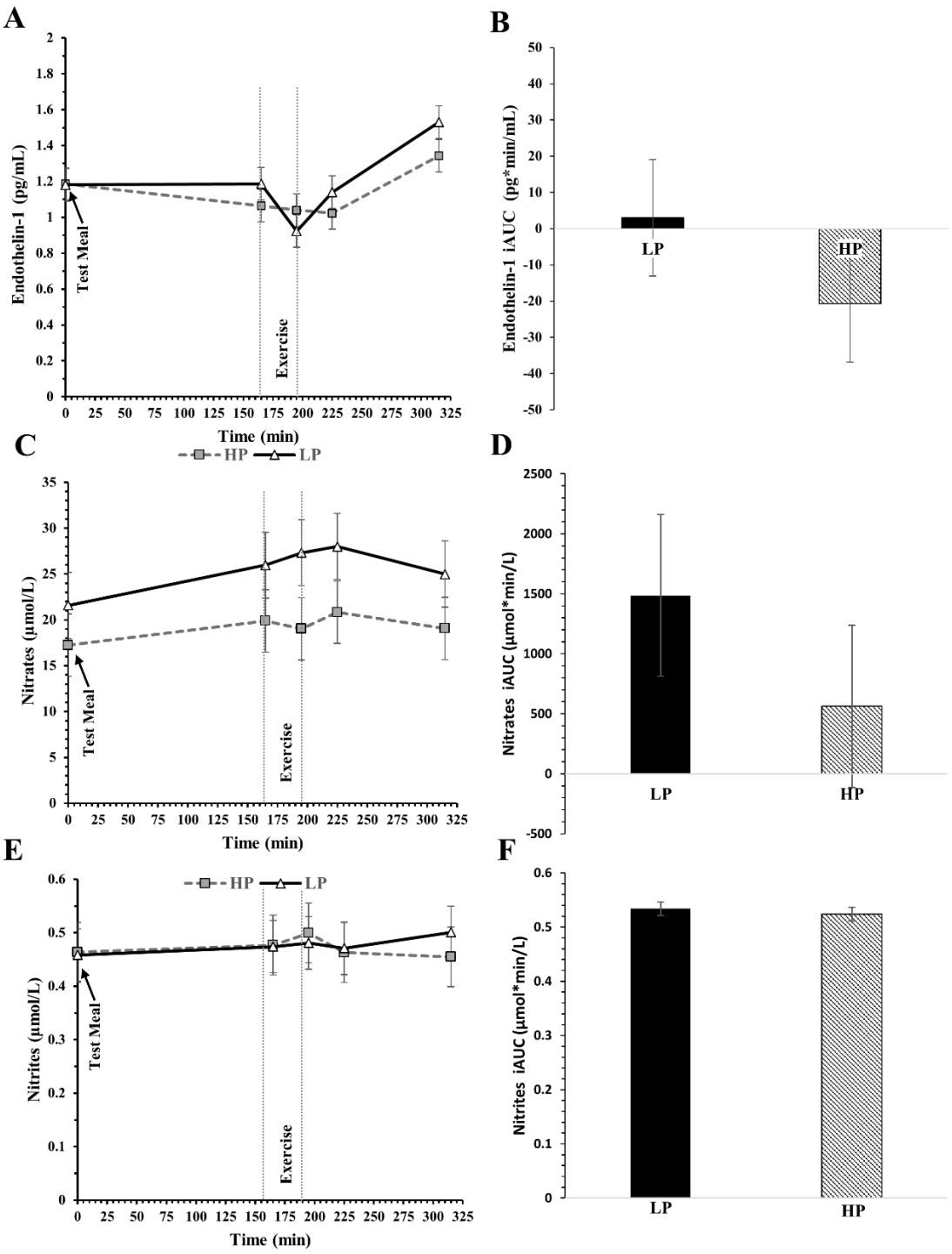

Figure 4.5. Plasma endothelin-1 concentrations (A) and incremental area under the curve (iAUC; B), plasma nitrate concentrations (C) and iAUC (D), and plasma nitrite concentrations (E) and iAUC (F) responses before (Time 0) and after ingestion of test meals (HP and LP). Endothelin-1, nitrates, and nitrites were not significantly different between interventions at any time point after adjustment for multiple testing. The HP meal resulted in a reduced endothelin-1 iAUC below baseline, but it was not significantly different from the LP intervention (P=0.117). There was no difference in nitrates and nitrites iAUCs. All values are LSmeans ± SE; n=11 (ET-1), n=7 (nitrates), n=11 (nitrites); significant difference between test meals denoted as ¥ (P<0.05).

Previous research has demonstrated that greater chronic protein intake can reduce fasting, resting BP [34-37]. However, protein intake does not consistently influence postprandial changes in BP [16-18, 38]. Our null findings support that greater protein intake does not affect the general postprandial BP response. Dietary protein-centered research on cardiometabolic health indices may be influenced by dietary carbohydrates and (or) fats when they are substituted to retain comparable energy intakes between diets or meals. Both chronic and acute studies typically assess the effects of higher protein intake on BP at the expense of carbohydrate [16, 17, 36, 39, 40]. Although carbohydrate intake acutely increases sympathetic activation (increasing BP), the concurrent insulin-mediated vasodilation can often overshoot this increased sympathetic activity, resulting in a net decrease in postprandial BP [41, 42]. Given this mechanistic support and experimental results suggesting that acute ingestion of a single high-carbohydrate meal can reduce BP to a greater extent than a high-protein meal [17], we elected to hold carbohydrate constant between meals and instead modulate dietary fat. Thus, our findings are more appropriately observed in the context of research that investigates differential effects of dietary protein and fat on cardiovascular health.

Results from the Omniheart trial indicate that diets higher in dietary protein and monounsaturated fat similarly reduce BP to a greater extent than diets with a higher carbohydrate content [35]. However, a substitution effect cannot be ruled out from these findings. Results from one study support a beneficial effect of protein on BP independent of carbohydrate substitution by demonstrating that 4 weeks of an energy-restricted high protein, low-fat diet is more effective at reducing BP than a low protein, high fat diet [34]. In an acute setting, a single high fat, high energy meal increases postprandial BP to a greater degree than a lower fat, lower energy (non-isoenergetic) meal [43]. The impact of fat content of an isoenergetic meal on vascular reactivity is more controversial. When energy intake was similar in test meals, a single high fat meal reduced endothelium-dependent vasodilation and increased SBP to a greater extent relative to a low fat, higher carbohydrate meal [44]. The current null results between the HP and LP meals support previous research showing that the macronutrient composition of a meal does not influence general postprandial BP responses [45-47].

Previous research has investigated the effects of dietary macronutrient composition in whole foods and liquid meals on vascular reactivity in response to physiological (exercise) and psychological stressors [40, 47-53]. Consistent with our findings, Rontoyanni et al. reported no

differential effects of protein- and carbohydrate-rich drinks on BP responses to exercise [40]. Jakulj et al. reported that participants consuming a high-fat meal presented with increased SBP, DBP, and total peripheral resistance in response to stress tasks when compared to the low-fat meal [48]. However, these findings were not replicated by the same research group in a subsequent study comparing high- and low-fat meals on vascular reactivity where protein and sodium content were matched between meals [52]. Our results are consistent with the latter observation, as we report that higher-fat meals elicit similar BP responses to lower-fat meals in participants exposed to a stressor, exercise. There are stark differences in study design between these studies and our research, including test meal contrasts [40, 47-53] (high-fat/high-protein vs. high-carbohydrate/high-protein) and experimental stressor [48, 49, 51, 52] (exercise vs. psychological). Our study was designed from a protein-centric perspective versus a fat-centric perspective [47-53]. Nonetheless, substitution must occur when comparing isoenergetic meals with varying macronutrient compositions. By holding carbohydrate constant, we equally assessed the influence of high/low protein meals and high/low fat meals, in a practical sense. Therefore, results from this study could equally support the concepts that, 1) dietary protein does not improve exercise-related BP responses, and 2) dietary fat does not negatively influence BP responses to exercise.

The physiology of the post-exercise recovery period may be distinct from during exercise, and may be predictive of cardiovascular health in the future (Luttrell et al. 2015). Specifically, post-exercise SBP (2 min SBP recovery ratio) is a robust prognosticator of future cardiovascular events, even when accounting for resting SBP (Kurl et al. 2001; Laukkanen et al. 2004). There is a paucity of research examining the differential effects of macronutrients on post-exercise hemodynamic recovery. In two separate studies contrasting protein-rich with carbohydrate rich drinks and high-carbohydrate with high-fat meals, Rontoyanni et al. report no differences in hemodynamic variable responses in the post-exercise recovery period [40, 47]. In line with these findings, we did not detect any difference in post-exercise SBP recovery from exercise (3 min SBP recovery ratio).

The HP meal contained 125% more arginine than the LP meal (1.8 vs. 0.8 g), which led to greater postprandial plasma arginine concentrations. As arginine is a key substrate in NO synthesis, we expected that the greater plasma arginine after the HP meal would increase NO production. Inconsistent with our hypothesis, greater plasma arginine after the HP meal did not

equate to increased concentrations of nitrites and nitrates (often summated as NOx; an indirect marker of NO synthesis). This result is not without precedent; investigators previously reported no difference in postprandial NOx concentrations between placebo and 6 g [54] and 10 g [55] supplemental doses of arginine. Indeed, while NO synthase is theoretically saturated at physiological levels of plasma arginine, dietary supplementation of arginine beyond physiological levels can be efficacious none-the-less in what is known as the 'arginine paradox' [56]. Supraphysiological doses of arginine, or elevated concentrations achieved from HP meals, could effectively increase NO synthesis by overcoming the competitive inhibition of ADMA [56, 57]. We report that ADMA concentrations increased above baseline in the HP test meal, while the LP meal resulted in the fall of ADMA concentrations below baseline. The absence of increased NOx (and reduced BP) in the presence of increased plasma arginine concentrations after the HP meal relative to the LP meal could be due to this competitive inhibition of NO synthesis by elevations in ADMA.

Aerobic exercise and resistance exercise training typically [58-60], but not always [61], result in chronic reductions in circulating concentrations of the potent vasoconstrictor ET-1. While NO is an inhibitor of ET-1, greater plasma arginine concentrations have not been shown to effectively decrease ET-1 [55]. The concentrations of ET-1 were shown to rise in response to intense exercise [62, 63], but minimal or no changes were observed during and immediately following moderate intensity exercise [64]. As we employed a moderate-intensity exercise protocol, our results are in agreement with these findings. Previous studies demonstrated that ET-1 elevations in response to exercise are delayed; ET-1 concentrations 30 minutes after exercise were greater than those immediately post-exercise, and return to baseline 60 minutes post-exercise [63, 64]. Our findings are inconsistent with this precise time course, as we report concentrations of ET-1 progressively rose and were the highest two hours post-exercise.

Strengths of this study include the high retention rate (31 of 34 participants, 91%, completed this study), the randomized controlled trial cross-over design, and the frequency of BP measurements obtained during exercise. One limitation to this study is the difficulty in ascribing the effects of trials to specific components of the test meals. Whether the effects (or lack of differential effects) should be attributed to the macronutrient profile of test meals (protein vs. fat) or food source-specific elements (egg content) is unclear with the study design. In a comparison of different protein sources on postprandial BP, Teunissen-Beekman et al. report that the egg

protein drink resulted in highest postprandial BP levels relative to pea and milk protein drinks [18]. As egg protein represents the majority of dietary protein in our test breakfast meals, our macronutrient comparison of high protein versus high fat meals on BP may be confounded by our selected protein source. This uncertainty is inherent to all research investigating physiological responses to whole-food meals, where factors other than macronutrient content are potentially meaningful variables. In a preceding paragraph, a case was made in which results from this study may be viewed in the context of a 1) protein-centric effect or a 2) fat-centric effect. A third consideration is that the effects of protein and fat are offsetting. Our experimental design does not permit an assessment of this possibility. We would require 6 groups (variations of high/low protein, fat, carbohydrate) to comprehensively parse out the effects of macronutrient composition on postprandial BP responses to exercise. Additionally, the heterogeneity in our sample may have influenced results. Our sample consisted of males and females with large age (age: 21 – 69 y) and fasting, resting BP (SBP: 120-139 mm Hg) ranges. Hypertensive individuals may respond to dietary protein-induced reductions in BP more robustly than normotensive individuals (He et al. 2005), but the effect of dietary protein on BP responses to exercise requires investigation. While limiting generalizability to the public, a more homogenous group of participants may present different results, particularly in a parameter with high variance such as BP. Crudely, secondary analyses of our data stratifying subjects by age, BP response to exercise, and fasting, resting BP suggest that the conclusion that protein content of a meal does not influence BP responses to exercise is likely generalizable among different segments of the adult population.

In conclusion, a higher-protein/lower-fat meal does not attenuate exercise-induced BP responses, enhance the post-exercise hypotensive response, or influence vasoactive biomarkers compared to a lower-protein/higher-fat meal. Our results build upon previous research suggesting that the beneficial effect of chronically elevated protein intake on BP is typically not observed in an acute setting by extending these findings to encompass BP responses to acute responses to exercise.

4.6. Acknowledgements

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W.W.C designed the research; R.E.B. conducted the research; R.E.B and B.T.R. analyzed the data, and R.E.B. wrote the paper with editorial assistance from W.W.C., B.T.R, and J.E.K. All authors share primary responsibility for final content. This work was funded through two American Egg Board-Egg Nutrition Center Young Investigator Research Awards; one to R.E.B. and one to J.E.K. The American Egg Board-Egg Nutrition Center had no role in study design, data collection, analysis and interpretation of data, and writing of this report. During the time this project was being conducted, W.W.C. received funds for unrelated projects from the National Pork Board, American Egg Board-Egg Nutrition Center, National Dairy Council, North Dakota Beef Commission, National Cattlemen's Beef Association, and Barilla Group, and served on the National Dairy Council's Whey Protein Advisory Panel. R.E.B conflicts: none. J.E.K conflicts: none. B.T.R. conflicts: none

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CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

On the surface, the findings of the research presented in this dissertation are quite disparate. The findings of the study described in Chapter 2 suggest that the relations between IMAT depots and indices of cardiometabolic health may differ depending upon the anatomic site analyzed; findings from the study described in Chapter 3 suggest that WP supplementation modestly improves lean mass without influencing fat mass; findings from the study described in Chapter 4 suggest that the beneficial effect of increased protein intake on BP do not extend to acute settings and do not improve BP responses to exercise. However, conceptual threads run through these projects which may serve to forward nutrition science, if properly recognized.

As the renowned general semanticist Wendell Johnson wrote over sixty years ago, "we see the world through our categories [1]." The importance of this framework of scientific thinking can be appreciated in all the studies presented herein, but it is perhaps best represented by research presented in Chapter 2. Not too long ago, all adipose tissues were blanketly categorized as one inert tissue used for energy storage. Our understanding has evolved to distinguish between numerous metabolically distinct varieties of adipose tissue based on several important grouping criteria. Borrowing from David Waters:

> *"Is leukemia a single disease or 15 different diseases? If one sees leukemia as a single disease, it is unlikely that one is very well-equipped to discover another form of leukemia. But if your categories enable you to see leukemia as 15 different diseases, why not discover a 16th ?"[2]*

Adopting this mindset has gainfully brought us to where we are today, with numerous useful and informative new categorizations of adipose tissue. Chapter 2 suggests that it may be useful to add yet another – a dimension of locality to the perhaps oversimplified categorization of IMAT. If we are comfortable in uncertainty and recognize that research is a *process*, we can earnestly pursue these new questions generated by the research, which almost always exceed the answers produced. Looking ahead, given the findings of Chapter 2, which suggest that IMAT accumulation in the thigh and calf are differentially associated with cardiometabolic health derangements, is it appropriate to group these studies together? Is IMAT in the thigh

representative of some breakdown in normal metabolism, or fundamentally different than IMAT in the calf in some way? There is considerable heterogeneity in research findings concerning IMAT and cardiometabolic health – how much of this is simply due to researchers looking in different places?

Chapter 2 can be foundational in directing future researchers to more deeply consider the potential for anatomical site-specific effects of different variables, and to more carefully weigh the appropriateness of locality-based proxy measures in place of broader measures. The single largest knowledge gap in the IMAT literature is the degree in which IMAT is predictive of future cardiometabolic dysfunction. Namely, relations between IMAT and indices of cardiometabolic health are present at baseline, suggesting an association, but not longitudinally, suggesting the lack of a causal role. Therefore, the current research is not able to settle the debate on whether IMAT is a cause or consequence of cardiometabolic dysfunction, but it is suggestive of the latter. Clinical research alone will not be able to fully answer this question. Instead, integrated efforts ranging from cellular models delineating the origins of IMAT to randomized controlled trials determining treatment models are warranted. Regarding treatment, some research findings are supportive of exercise reducing IMAT accumulation to a greater extent than diet alone [3]. A drawback of the current research was that only one of the four studies included in the retrospective analysis included exercise in the intervention. Inclusion of only one study that is expected to preferentially reduce IMAT limits our ability to comment on the predictive capacity of IMAT reductions improving cardiometabolic health. The dissimilar intervention characteristics may have even contributed further variance, washing out any potential relations between IMAT and cardiometabolic health. Future research should include conducting adequately powered longitudinal interventions investigating the effects of diet, exercise, and diet + exercise to categorically determine the separate and combined effects of both modalities on IMAT accumulation in the thigh and its relationship with cardiometabolic health. This type of prospective research is critical to determine if relations at baseline between IMAT in an *a priori*-defined depot extend to predict improvement in indices of cardiometabolic health in response to the accepted lifestyle interventions. This type of design could provide robust evidence supporting or rejecting a causal role of IMAT on cardiometabolic health, thereby improving clarity in the future investigative priority of inquiries surrounding IMAT.

Findings from Chapter 3 suggest that protein supplementation improves body composition in women by modestly improving lean mass without influencing fat mass. These findings are in agreement with other syntheses of literature showing a small benefit of higher-protein diets on body composition, achieved through either dietary or supplementary means [4]. Originally, this research was designed to determine the effects of protein supplementation in four different conditions of varying energy and training states. A major limitation of the current research was the inability to come to firm conclusions regarding the effects of protein supplementation in each of the specific subgroups due to limited data. Specifically, our systematic search of the literature yielded only three studies in women in energy balance and not resistance training, and only one study in women in energy restriction and resistance training. It is particularly interesting that there are limited data in individuals who are not dieting and not training, as this represents most of our population. Future research needs to address this knowledge gap in order to provide more targeted guidance for large parts of the population. Perhaps the most interesting consistent finding of these two meta-analyses (Appendix E) are the profound modulating effects of resistance training and energy restriction on the relationship between dietary protein quantity and body composition. In brief, the beneficial effects of increased protein intake are the most robust in concert with stressors such as energy restriction, while the differential effect of higher- versus lower-protein intake is muted without these stressors. This has potentially important implications in guiding future research and policy, underscoring the importance of combined nutrition and physical activity recommendations and investigation of protein requirements in the context of different physical activity and energy states.

The study described in Chapter 4 combines two aspects – nutrition and physical activity – and suggests that consuming a higher-protein meal does not improve BP responses to exercise. Importantly, this study documents the modulating effect of dietary protein on BP responses to a *challenge* or *stressor*, namely moderate-intensity exercise. Too often, nutrition research is conducted in highly controlled settings where the primary outcomes are responses during basal and/or steady states. This can be viewed as a major limitation for much of the clinical research conducted, as these resting and highly controlled periods are not typical of the demands placed on individuals in real-life settings. Chapter 4 embodies the concept of utilizing stressors to expose hidden heterogeneity [5]. Blood pressure assessment is a good vehicle for this concept

because greater cardiovascular risk can be "unmasked" from poor exercise BP responses even in individuals with apparently healthy resting BPs [6]. The findings presented in Chapter 4 suggest that higher-protein meals do not attenuate blood pressure responses to exercise. The research was originally designed to reduce cardiovascular risk by attenuating the 'hypertensive response to exercise', or an exaggerated blood pressure response to moderate-intensity exercise. Estimates of the prevalence of individuals displaying a hypertensive response to exercise range from 3 – 18% [7, 8]. One limitation of the current research was that while we recruited prehypertensive individuals at greater cardiovascular risk, practical reasons limited our ability to specifically recruit individuals displaying a hypertensive response to exercise. Therefore, it is possible that there was no differential effect because there was no pathology to correct, and that reducing blood pressure in individuals who do not display an exaggerated blood pressure response may not be a relevant endpoint. Therefore, specific research investigating the potential of diet to modulate the BP response to exercise in individuals truly at risk with hypertensive responses to exercise is warranted.

The combination of nutrition and physical activity aspects in this study is also important, as it represents the type of holistic research approach that will doubtless be featured prominently in the next generation of nutrition research. While we owe a lot to bottom-up reductionist approaches which have expanded our understanding of nutrition, we may be reaching a point of diminishing returns [9]. Future research should adopt a more holistic approach and place greater emphasis on understanding relationships between diet and health at each level of granularity – from eating patterns to whole foods, all the way to the nutrients we are so often preoccupied with [10].

Chapter 4 also exemplifies the importance of cognizance towards categorization. The study results are being ascribed to the potential BP-lowering effects to a macronutrient – dietary protein. However, we could just as easily attribute the BP-lowering effects (lack of effects – in this case) to the whole-food source of the relatively higher protein meal – eggs. In fact, postprandial BP responses to different protein sources were compared in one recent study, and egg protein elicited the *highest* BP response, relative to sources such as pea protein and whey protein [11]. Unlike adding a dimension of locality to advance the categorization of IMAT, this is not a new observation. Nutrition science researchers have been grappling with the issue of

naïve substitution – or erroneously conflating the effects of a food with that of a nutrient or seeing one form of a nutrient as equivalent to another [12] – for far too long.

We need sufficient particularity with our category usage, but does this mean we should not generalize? Of course not. Grouping is just as enlightening as it is limiting. After all, we would have no new knowledge if we were only describing narrow, isolated observations in a void – connected to nothing and therefore illuminating nothing. Consider this a call to be more vigilant with our categorizations and be ready to consider whether we are viewing our problem through the proper lens. Does that mean we should view Chapter 4 through a whole-food lens, and only say that 'meals with more eggs don't improve BP responses'? Not necessarily. Protein is a perfectly suitable grouping category – there are mechanistic data supporting means by which the constituent amino acids can improve BP responses [13]. However, the absolute and relative abundance of these amino acids, the co-ingested nutrients, and the food form (to name a few) are highly relevant contributing factors that are lost with the categorization 'higher versus lower protein'. There is no single inherently 'correct' categorization or degree of granularity; future researchers are just encouraged to inhabit a mindset of considering and properly contextualizing the originally presented categorization, but always striving to look at the question from a new angle.

In the broadest sense, this dissertation may be considered a commentary on the merits of adopting a generalist approach in a world of ever-increasing specialization. The three studies packaged herein utilize different techniques (retrospective analysis, systematic review & meta-analysis, acute cross-over trial) and report on different outcomes (cardiometabolic health, body composition). However, conceptual frameworks unite these works and can augment each other in a cross-disciplinary fashion. David Epstein cautioned that, "Overspecialization can lead to collective tragedy even when every individual takes the most reasonable course of action." [14] Dietary protein very clearly influences skeletal muscle, and therefore it is very reasonable to concentrate one's training in the pursuit of questions that begin and end in skeletal muscle. Our most profound advances may not reside in the same old paradigms, however. Perhaps to best advance our understanding *within* the nutrition sciences, we need to borrow from *without*.

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APPENDIX A. STUDY 1 SUPPLEMENTAL MATERIALS

Supplemental Table 1. Clinical and cardiometabolic characteristics of all subjects by sex.

Outcome Variable	**Female** ***(n = 74)***			**Male** ***(n = 39)***			**P-Value** *Group*
Age (yr)	50 ± 15			50 ± 17			0.966
Height (cm)	164.54 ± 6.80			178.61 ± 7.36			<0.001
Weight (kg)	84.9 ± 10.4			99.9 ± 12.8			<0.001
BMI (kg/m^2)	31.10 ± 2.90			31.28 ± 2.99			0.770
Cardiometabolic Health	*Pre-*	*Post-*	*Change*	*Pre-*	*Post-*	*Change*	*Group* time*
Glucose (mmol/l)	5.04 ± 0.46[a]	4.95 ± 0.50	-0.06 ± 0.44	5.38 ± 0.48[a]	5.16 ± 0.44	**-0.22 ± 0.40***	0.084
Insulin (pmol/l)	81.95 ± 39.59	56.25 ± 28.47	**-23.61 ± 41.67***	90.98 ± 51.39	54.87 ± 29.17	**-35.42 ± 39.59***	0.203
HOMA-IR	2.66 ± 1.41	1.80 ± 0.96	**-0.78 ± 1.45***	3.19 ± 1.97	1.86 ± 1.10	**-1.32 ± 1.46***	0.092
Total Cholesterol (mmol/l)	10.56 ± 1.77	9.47 ± 1.87	**-0.93 ± 1.09***	10.26 ± 2.29	8.98 ± 1.71	**-1.11 ± 1.49***	0.516
Triglycerides (mmol/l)	6.28 ± 5.73[a]	5.36 ± 2.01	**-1.03 ± 1.91***	7.74 ± 3.51[a]	5.90 ± 2.76	**-2.03 ± 3.36***	0.106
HDL (mmol/l)	2.86 ± 0.86[a]	2.64 ± 0.54	-0.03 ± 0.36	2.23 ± 0.64[a]	2.25 ± 0.66	0.09 ± 0.37	0.121
LDL (mmol/l)	6.44 ± 1.60	5.75 ± 1.67	**-0.69 ± 0.99***	6.54 ± 1.99	5.56 ± 1.51	**-0.84 ± 0.99***	0.463
TC:HDL	3.96 ± 1.20[a]	3.71 ± 0.96	**-0.38 ± 0.66***	4.90 ± 1.39[a]	4.22 ± 1.17	**-0.72 ± 0.87***	0.053
Thigh IMAT							
CSA (cm²)[¥]	233.93 ± 47.06	214.84 ± 39.80	**-26.41 ± 16.86***	221.3 ±46.64	207.58± 41.39	**-20.91 ± 14.16***	0.115
IMATa (cm²)	10.75 ± 3.51[a]	9.46 ± 2.57	**-1.30 ± 0.96***	12.37 ± 4.57[a]	10.92 ± 3.37	**-2.27 ± 2.99***	0.076
IMAT	0.0469 ± 0.015[a]	0.0448 ± 0.013	-0.0004 ± 0.004	0.0574 ± 0.022[a]	0.0537 ± 0.018	**-0.006± 0.01***	**0.026***
MT (cm²)[¥]	97.99 ± 18.09[a]	98.71 ± 19.23	**-1.97 ± 5.49***	147.90 ± 26.81[a]	144.54 ± 23.41	**-6.27 ± 8.35***	**0.011***
SAT (cm²)	135.28 ± 42.30[a]	116.13 ± 34.66	**-24.36 ± 14.32***	73.40 ± 33.38[a]	63.04 ± 31.38	**-14.63 ± 9.39***	**<0.001***
Calf IMAT							
CSA (cm²)[¥]	97.82± 15.51	92.28 ± 14.14	**-5.614 ± 4.363***	97.42 ± 15.15	92.83 ± 14.60	**-5.99 ± 5.22***	0.719
IMATa (cm²)	5.80 ± 1.79[a]	5.29 ± 1.66	**-0.51 ± 0.53***	7.80 ± 2.62[a]	7.21 ± 2.77	**-0.867 ± 1.205***	0.134
IMAT	0.0599 ± 0.02[a]	0.0578 ± 0.02	**-0.0021± 0.005***	0.0802 ± 0.025[a]	0.0781 ± 0.029	**-0.0039 ± 0.01***	0.382
MT (cm²)[¥]	54.22 ± 8.71[a]	52.74 ± 8.40	**-1.71 ± 1.86***	74.00 ± 10.41[a]	69.78 ± 8.41	**-3.87 ± 3.42***	**0.003***

SAT (cm²)	43.60 ± 12.93 [a]	38.89 ± 12.72	**-3.90 ± 3.06***	23.42 ± 9.70 [a]	23.05 ± 9.56	**-2.12 ± 2.37***	**0.006***

Data are mean ± SD; significance determined through paired and independent T-Tests, P-values < .05 *
CSA, cross-sectional area of segment; HDL, high-density lipoprotein; HOMA:IR, homeostatic model assessment of insulin resistance; IMAT, intermuscular adipose tissue (standardized to cross-sectional area) IMATa, intermuscular adipose tissue (absolute quantity); LDL, low-density lipoprotein; MT; muscle tissue; SAT, subcutaneous adipose tissue; TC, total cholesterol; TG, triglyceride
[a]Differences between males and females (P < 0.05)
¥Bone area removed

Supplemental Table 2. Associations between thigh or calf IMAT with indices of cardiometabolic health by sex

	Female (n=74)				**Male (n=39)**			
Baseline Associations	**Thigh IMAT:CSA**		**Calf IMAT:CSA**		**Thigh IMAT:CSA**		**Calf IMAT:CSA**	
	β [a] (95% CI)	***P* value**	**β [a] (95% CI)**	***P* value**	**β [a] (95% CI)**	***P* value**	**β [a] (95% CI)**	***P* v**
Glucose (mmol/l)	6.84 (-0.72, 14.41)	**0.033***	1.30 (-5.02, 7.62)	0.546	4.51 (-3.39, 12.41)	**0.004***	-4.04 (-10.41, 2.32)	0.
Insulin (pmol/l)	908.41 (13.89, 1802.92)	0.054	137.51(-514.62, 789.65)	0.797	925.77(-24.31,1876.54)	0.083	70.14(-810.48, 950.08)	0.
HOMA-IR	31.02 (-1.00, 63.04)	0.063	3.43 (-19.80, 26.66)	0.868	33.46 (-2.74, 69.66)	0.045	-2.40 (-35.65, 30.85)	0.
Triglycerides (mmol/l)	42.27 (4.57, 79.98)	**0.042***	17.53 (-18.34, 53.40)	0.341	72.16 (9.78, 134.54)	0.307	-23.46 (-80.96, 34.05)	0.
Total Cholesterol (mmol/l)	37.74 (12.09, 63.39)	**<0.001***	27.79 (4.03, 51.56)	**0.009***	18.78 (-25.14, 62.71)	0.705	-1.19 (-37.14, 34.76)	0.
LDL (mmol/l)	34.79 (10.21, 59.37)	**0.002***	30.20 (8.29, 52.28)	**0.004***	2.48 (-37.37, 43.43)	0.903	-6.86 (-39.42, 25.71)	0.
HDL (mmol/l)	-5.27 (-18.61, 8.07)	0.989	-5.89 (-14.61, 2.83)	0.269	-9.39 (-21.41, 2.62)	0.766	-0.27 (-1.57, 1.02)	0.
TC:HDL	26.81 (8.30, 45.32)	**0.011***	24.43 (8.05, 40.81)	**0.004***	26.82 (1.12, 52.52)	0.841	-4.91 (-28.21, 18.40)	0.
Longitudinal Associations	**ΔThigh IMAT:CSA**		**ΔCalf IMAT:CSA**		**ΔThigh IMAT:CSA**		**ΔCalf IMAT:CSA**	
	β [a] (95% CI)	***P* value**	**β [a] (95% CI)**	***P* value**	**β [a] (95% CI)**	***P* value**	**β [a] (95% CI)**	***P* v**
ΔGlucose (mmol/l)	8.29 (-23.36, 39.94)	0.884	14.80 (-9.72, 39.31)	0.058	-13.09 (-30.90, 4.72)	0.335	1.85 (-15.27, 18.96)	0.
ΔInsulin (pmol/l)	-3388.47 (-7313.09, 536.15)	0.260	1080.64 (-1287.60, 3448.19)	0.338	96.54 (-1643.88, 1836.26)	0.910	679.92 (-118.15, 2477.28)	0.
ΔHOMA-IR	-108.01 (-243.15, 27.13)	0.115	42.54 (-40.28, 125.35)	0.263	-0.54 (-66.10, 65.01)	0.621	20.84 (-47.17, 88.85)	0.
ΔTriglycerides (mmol/l)	-151.37 (-313.61, 10.88)	0.275	21.64 (-80.96, 124.24)	0.623	26.08 (-116.51, 168.68)	0.634	-0.21 (148.96, 148.56)	0.
ΔTotal Cholesterol (mmol/l)	-57.17 (-154.62, 40.28)	0.434	9.79 (-52.23, 71.82)	0.676	0.58 (-62.28, 63.44)	0.869	4.28 (-6.52, 70.64)	0.
ΔLDL (mmol/l)	-28.38 (-114.42, 57.66)	0.601	14.71 (-39.19, 68.62)	0.550	19.09 (-26.52, 64.69)	0.399	14.48 (-30.84, 59.81)	0.
ΔHDL (mmol/l)	3.17 (-27.57, 33.89)	0.843	-9.50 (-29.34, 10.34)	0.399	-12.86 (-29.04, 3.33)	0.906	-2.84 (-19.89, 14.21)	0.
ΔTC:HDL	-50.41 (-102.88, 2.07)	0.059	18.15 (-17.24, 53.53)	0.244	29.64 (-9.95, 69.22)	0.711	13.05 (-27.22, 53.31)	0.

All estimates are adjusted for age. Longitudinal analyses adjusted for age and baseline dependent variable.
[a]Estimates of adjusted regression coefficient between glucose, insulin, HOMA-IR, TG, TC, LDL, HDL, and TC:HDL with thigh and calf IMAT; P-values < .05 *
CI, confidence interval; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; IMAT, intermuscular adipose tissue (standardized to cross-sectional area of segment); LDL, low-density lipoprotein; TC, total cholesterol; TG, triglyceride

Supplemental Table 3. Clinical and cardiometabolic profile of calf composition subgroup by sex.

Outcome Variable	Women	Men	P-Value
General Characteristics	*(n = 24)*	*(n = 13)*	
Age (yr)	36 ± 9	31 ± 9	0.146
Height (cm)	167.8 ± 6.1	178.2 ± 5.3	**<0.001***
Weight (kg)	87.0 ± 9.8	99.9 ± 9.9	**<0.001***
BMI (kg/m²)	30.8 ± 2.3	31.5 ± 2.6	0.456
Cardiometabolic Health			
Glucose (mmol/l)	5.00 ± 0.44	5.11 ± 0.28	0.426
Insulin (pmol/l)	97.23 ± 41.67	83.34 ± 55.56	0.364
HOMA-IR	3.05 ± 1.31	2.61 ± 1.70	0.405
Total Cholesterol (mmol/l)	10.33 ± 1.44	11.22 ± 2.44	0.230
Triglycerides (mmol/l)	6.39 ± 2.22	8.40 ± 3.94	0.101
HDL (mmol/l)	2.72 ± 0.56	2.06 ± 0.39	**<0.001***
LDL (mmol/l)	6.33 ± 1.39	7.50 ± 2.11	0.049
TC:HDL	3.98 ± 1.12	5.62 ± 1.38	**<0.001***

Data are mean ± SD; significance determined through Independent T-Tests, P-values < .05 *
HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglyceride

Supplemental Table 4. Associations between Gastrocnemius and Soleus IMAT with Indices of Cardiometabolic Health.

Baseline Associations	**Gastrocnemius IMAT (n=37)**		**Soleus IMAT (n=37)**	
	β [a] (95% CI)	***P*-value (FDR-Adjusted P)**	**β [a] (95% CI)**	***P*-value (FDR-Adjusted P)**
Glucose (mmol/l)	-3.18 (-7.42, 1.05)	0.136 (0.407)	-1.39 (-3.18, 0.41)	0.125 (0.407)
Insulin (pmol/l)	111.81 (-413.92, 637.55)	0.666 (0.962)	29.17 (-179.18, 236.82)	0.777 (0.962)
HOMA-IR	1.01 (-16.58, 18.59)	0.908 (0.962)	-0.16(-7.11, 6.79)	0.962 (0.962)
Triglycerides (mmol/l)	-10.97 (-41.37, 19.42)	0.468 (0.932)	-4.10 (-17.04, 8.84)	0.524 (0.932)
Total Cholesterol (mmol/l)	-8.62 (-27.77, 10.52)	0.366 (0.932)	.35 (-7.88, 8.58)	0.932 (0.932)
LDL (mmol/l)	-6.91 (-24.19, 10.38)	0.422 (0.932)	1.44 (-5.96, 8.84)	0.694 (0.932)
HDL (mmol/l)	0.52 (-4.63, 5.67)	0.840 (0.932)	-0.27 (-2.46, 1.92)	0.804 (0.932)
TC:HDL	-4.39 (-17.37, 8.58)	0.496 (0.932)	0.55 (-4.99, 6.10)	0.840 (0.932)
Δ Associations	**Δ Gastrocnemius IMAT (n=37)**		**Δ Soleus IMAT (n=37)**	
	β [a] (95% CI)	***P*-value (FDR-Adjusted P)**	**β [a] (95% CI)**	***P*-value (FDR-Adjusted P)**
ΔGlucose (mmol/l)	-3.18 (-20.37, 13.99)	0.707 (0.942)	-3.82 (-12.69, 5.06)	0.386 (0.942)
ΔInsulin (pmol/l)	-125.01 (-2304.35, 2053.64)	0.906 (0.942)	276.41 (-861.87, 1415.39)	0.619 (0.942)
ΔHOMA-IR	-2.61 (-75.60, 70.38)	0.942 (0.942)	7.01 (-31.09, 45.11)	0.706 (0.942)
ΔTriglycerides (mmol/l)	28.16 (-79.59, 135.92)	0.597 (0.663)	31.49 (-20.73, 83.72)	0.227 (0.383)
ΔTotal Cholesterol (mmol/l)	29.27 (-23.80, 82.33)	0.268 (0.383)	32.54 (8.28, 56.81)	0.010 (0.104)
ΔLDL (mmol/l)	25.01 (-16.81, 66.82)	0.231 (0.383)	22.99 (3.07, 42.92)	0.025 (0.127)
ΔHDL (mmol/l)	-1.06 (-16.29, 14.18)	0.888 (0.888)	3.37 (-4.16, 10.90)	0.367 (0.459)
ΔTC:HDL	22.41 (-4.12, 48.95)	0.095 (0.932)	12.85 (-0.16, 25.85)	0.053 (0.175)

All estimates are adjusted for age and sex.

[a]Estimates of adjusted regression coefficient between glucose, insulin, HOMA-IR, TG, TC, LDL, HDL, and TC:HDL with thigh and calf IMAT; P-values < .05 *

CI, confidence interval; HDL, high-density lipoprotein; HOMA:IR, homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglyceride

APPENDIX B. STUDY 2 SUPPLEMENTAL MATERIALS

ONLINE SUPPLEMENTARY MATERIALS

Supplementary Table 1 Search strategy

Database	Search criteria
PubMed	((Dietary Protein[MeSH]) AND (Body composition[Mesh] OR "Weight Loss"[Mesh] OR "Body Weight Maintenance"[Mesh] OR "Weight Gain"[Mesh] OR Strength, Muscle[MeSH] OR Muscle, Skeletal[Mesh])) **Limitations:** 1. Humans 2. English 3. Adults: 19+ years 4. Female
Cochrane Reviews	("Dietary protein" or "Protein Supplementation" or "protein supplement" or "whey protein") and ("lean mass" or "fat mass" or "Muscle mass" or "body composition") **Limit:** Trials
Scopus	("Dietary protein" OR "protein intake" OR "protein supplement" OR "high protein") AND ("body composition" OR "lean mass" OR "fat mass" OR "fat free mass") AND ("Weight loss" OR "energy restriction" OR "resistance training" OR "weight maintenance") AND NOT (animals OR rats OR mice OR cells OR children OR adolescent) AND (LIMIT-TO(SUBJAREA," BIOC") OR EXCLUDE(SUBJAREA,"MEDI OR LIMIT-TO SUBJAREA ")) AND (LIMIT-TO(DOCTYPE,"ar")) AND (EXCLUDE(EXACTSRCTITLE,"Aquaculture OR EXCLUDE EXACTSRCTITLE ") OR EXCLUDE(EXACTSRCTITLE," Journal of the World Aquaculture Society") OR EXCLUDE(EXACTSRCTITLE,"Aquaculture Nutrition")) AND (LIMIT-TO(LANGUAGE,"English")) AND (EXCLUDE(SUBJAREA,"NURS")) AND (EXCLUDE(SUBJAREA,"HEAL") OR EXCLUDE(SUBJAREA,"EART")) AND (EXCLUDE(SUBJAREA,"IMMU") OR EXCLUDE(SUBJAREA,"NEUR")) AND (EXCLUDE(SUBJAREA,"ARTS") OR EXCLUDE(SUBJAREA,"CENG")) AND (EXCLUDE(SUBJAREA,"PHAR") OR EXCLUDE(SUBJAREA,"SOCI")) **Limits:** Document Type (Articles, Articles in Press) 1. Source Type (Journals) 2. Subject Area (Medicine, Biochemistry, Genetics and Molecular Biology, Agricultural and biological sciences) 3. Language (English)
CINAHL	((MM "Dietary Proteins+") OR (MM "Diet, High Protein+") OR "dietary protein" OR "protein intake" OR "protein supplement" OR "high protein" OR "whey protein") AND ((MM "Weight Loss+") OR (MM "Weight Reduction Programs+") OR (MM "Restricted Diet+") OR (MM "Body Weight Changes") OR "weight maintenance" OR "energy restriction" OR "low calorie" OR "weight loss" OR (MM "Body Weight Changes+") OR "weight reduction" OR "fat loss" OR "energy intake" OR weight OR restriction OR energy

OR reduction) AND ((MM “Exercise+”) OR (MM “Therapeutic Exercise+”) OR (MM “Resistance Training+”) OR (MM “Weight Lifting+”) OR “exercise intervention” OR “body composition” OR muscle OR “fat mass” OR “lean mass” OR fat free mass”)
Limit: Academic Journals

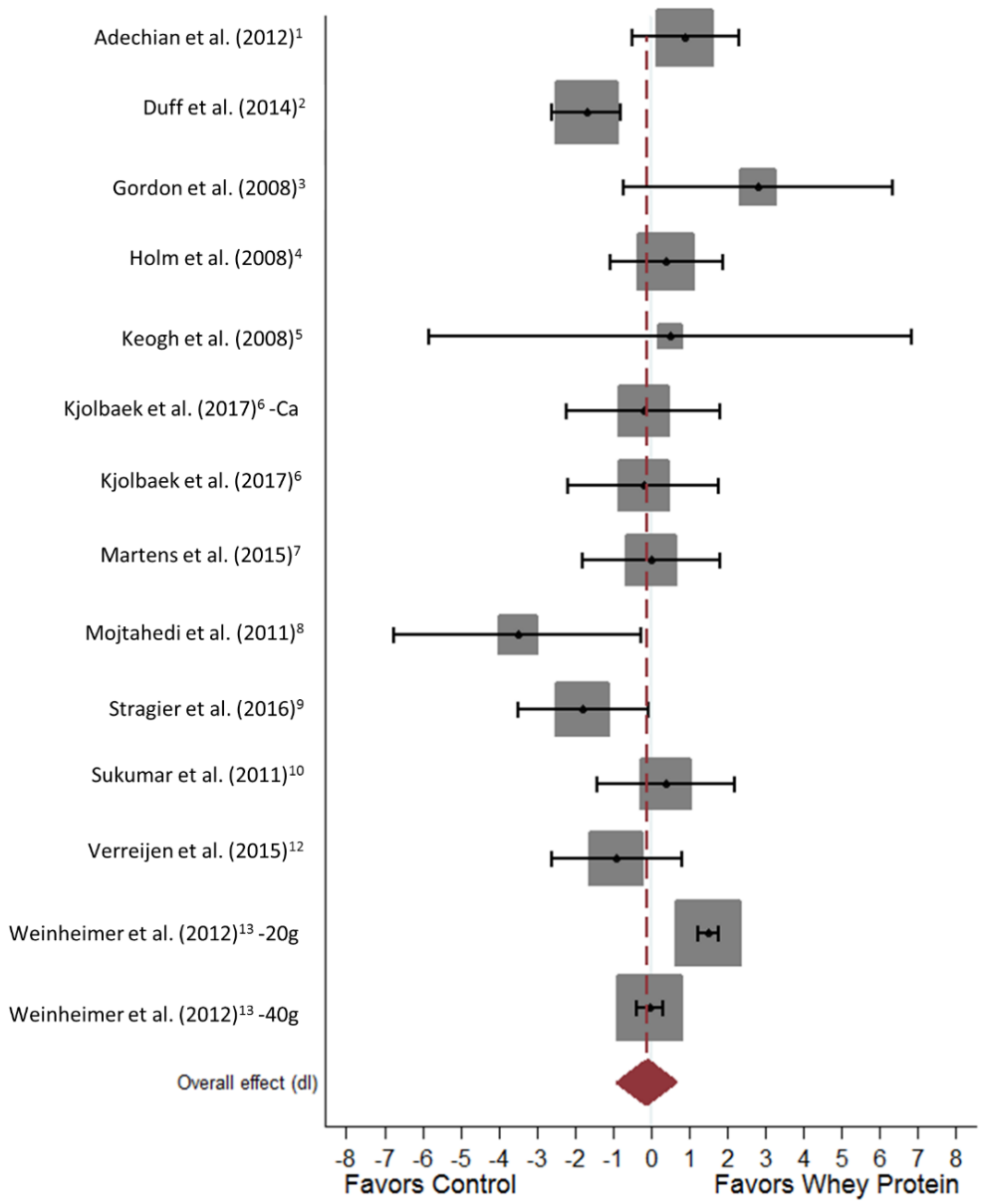


Figure S1 **Effect of whey protein supplementation on changes in body mass in women.** A random-effects model was used for lean mass, since heterogeneity was observed in pooled data. *Abbreviations*: Ca, calcium; CI, confidence interval; SD, standard deviation

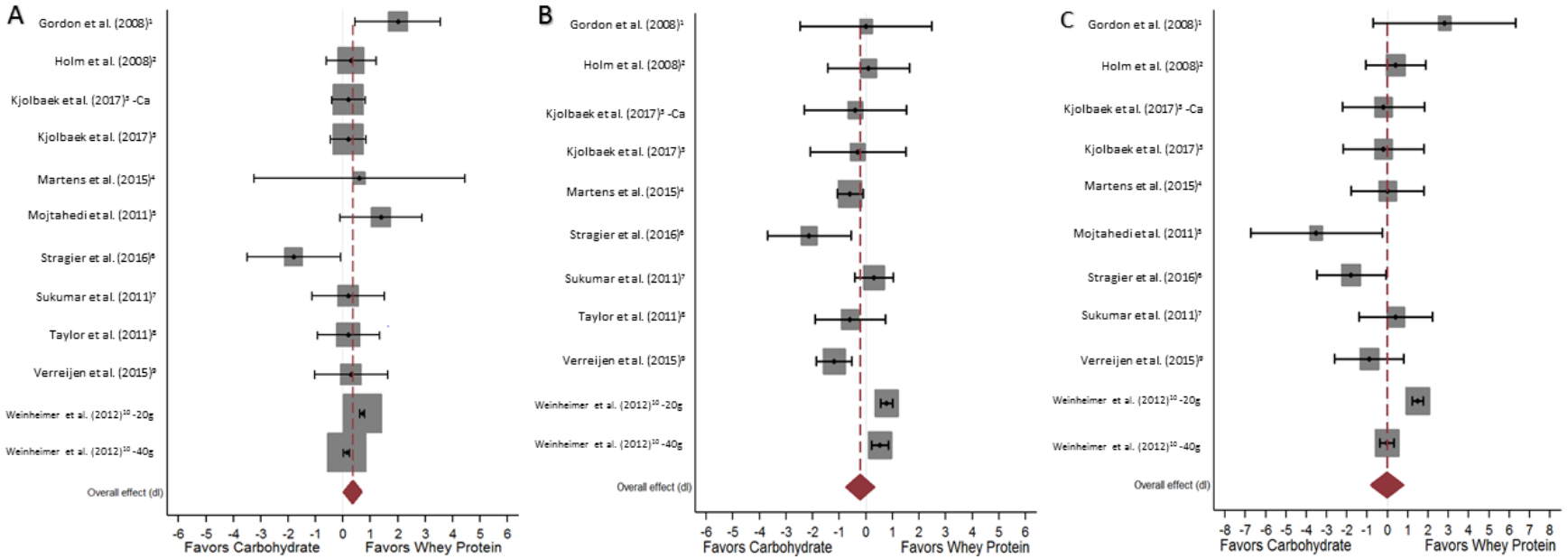


Figure S2 **Effect of whey protein supplementation versus carbohydrate control on changes in lean mass, fat mass, and body mass in women.** (A) Results of a random-effects meta-analysis representing pooled mean differences with 95% CIs on lean mass (WMD 0.36 kg; 95%CI= 0.01 to 0.70). (B) Results of a random-effects meta-analysis representing pooled mean differences with 95% CIs on fat mass (WMD -0.22 kg; 95%CI= -0.75 to 0.31). (C) Results of a random-effects meta-analysis representing pooled mean differences with 95% CIs on body mass (WMD -0.03 kg; 95%CI= -0.84 to 0.78). *Abbreviations*: Ca, calcium; CI, confidence interval; WMD, weighted mean difference

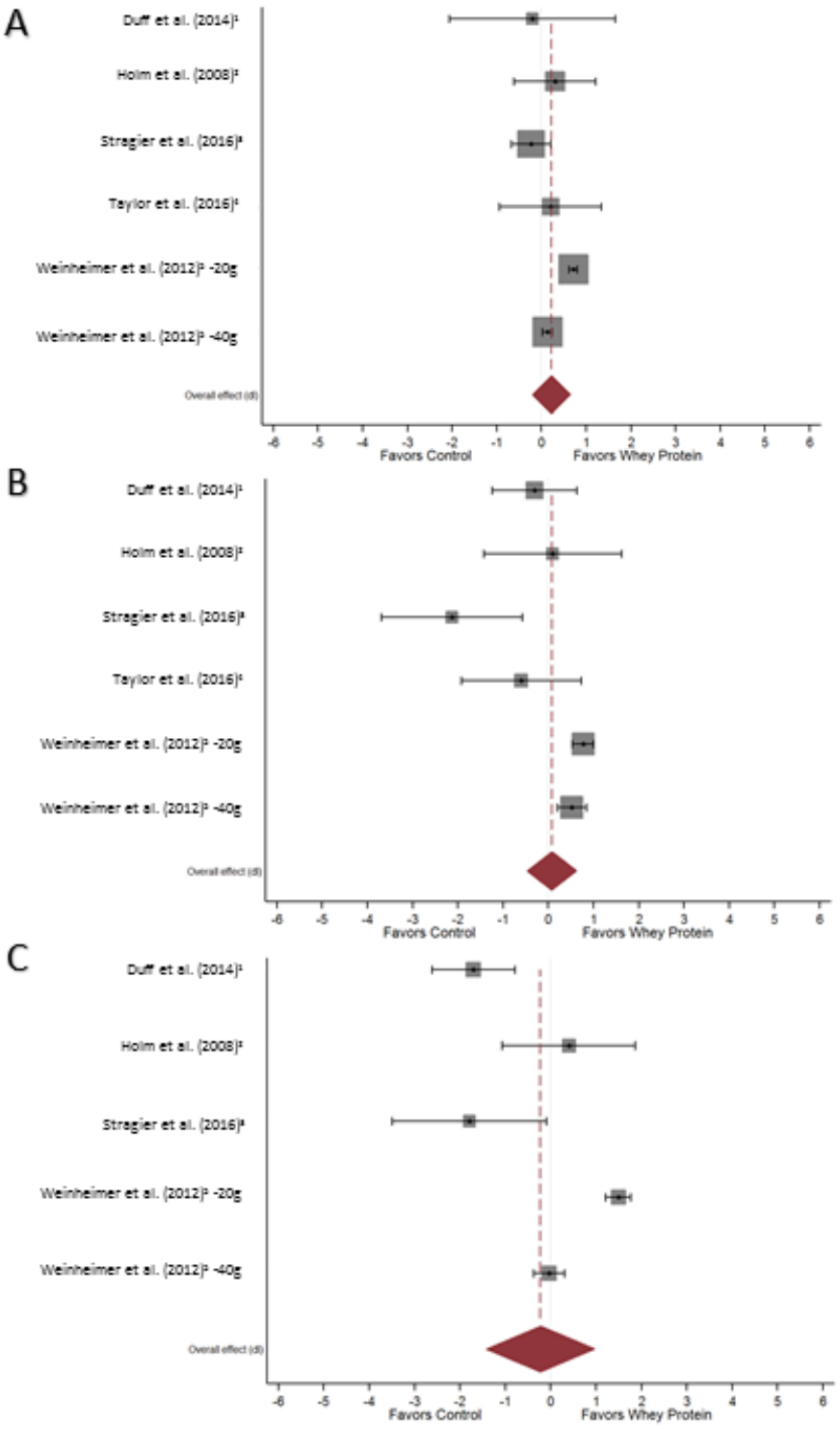


Figure S3 **Effect of whey protein supplementation on changes in lean mass, fat mass, and body mass in women without energy restriction and with resistance training.** (A) Results of a random-effects meta-analysis representing pooled mean differences with 95% CIs on lean mass (WMD 0.22 kg; 95%CI= -0.19 to 0.64). (B) Results of a random-effects meta-analysis representing pooled mean differences with 95% CIs on fat mass (WMD 0.08 kg; 95%CI= -0.46 to 0.62). (C) Results of a random-effects meta-analysis representing pooled mean differences with 95% CIs on body mass (WMD -0.23 kg; 95%CI= -1.41 to 0.96). *Abbreviations*: Ca, calcium; CI, confidence interval; WMD, weighted mean difference

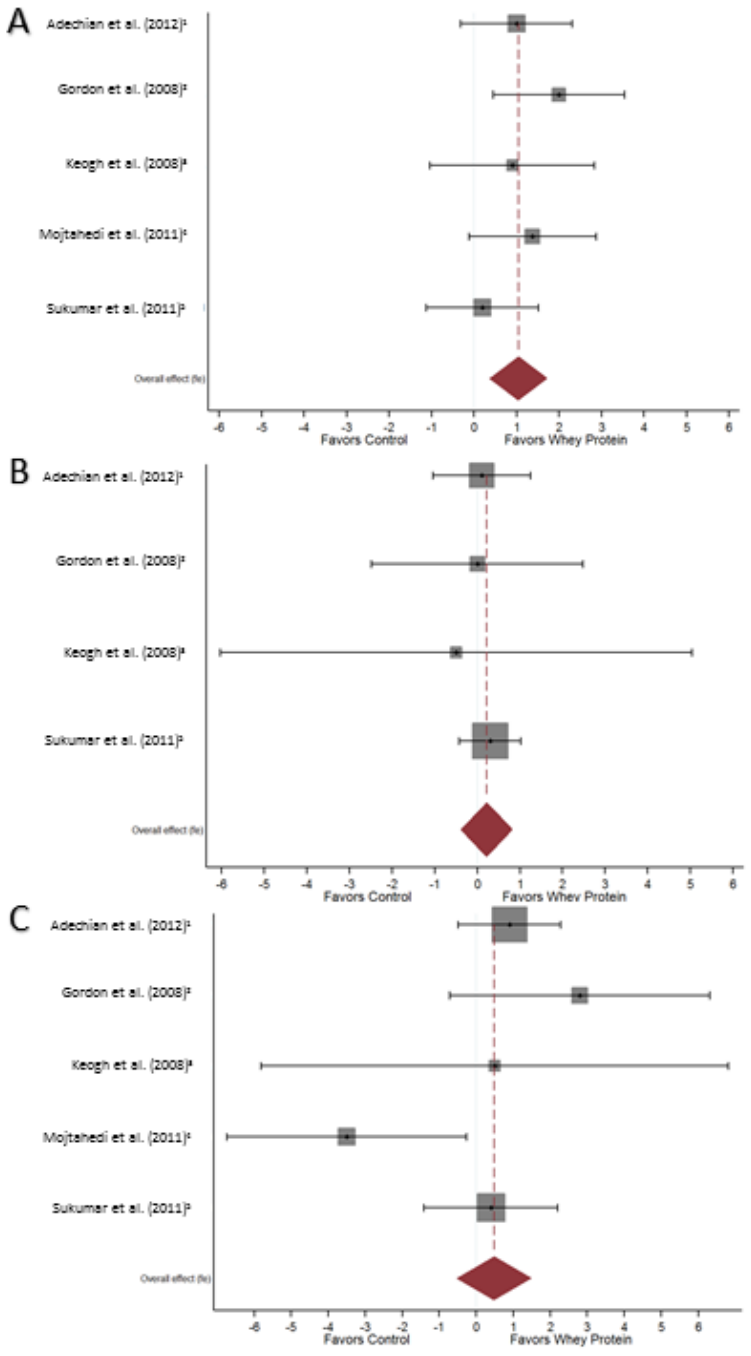


Figure S4 **Effect of whey protein supplementation on changes in lean mass, fat mass, and body mass in women with energy restriction and without resistance training.** (A) Results of a random-effects meta-analysis representing pooled mean differences with 95% CIs on lean mass (WMD 1.04 kg; 95%CI= 0.38 to 1.70). (B) Results of a random-effects meta-analysis representing pooled mean differences with 95% CIs on fat mass (WMD 0.22 kg; 95%CI= -0.37 to 0.81). (C) Results of a random-effects meta-analysis representing pooled mean differences with 95% CIs on body mass (WMD 0.48 kg; 95%CI= -0.51 to 1.47). *Abbreviations*: Ca, calcium; CI, confidence interval; WMD, weighted mean differenc

APPENDIX C. STUDY 3 SUPPLEMENTARY MATERIALS

SCREENING CONSENT FORM

Purdue IRB Protocol #: 1607017949 - Expires on: 03-OCT-2017

RESEARCH PARTICIPANT SCREENING CONSENT FORM

The Acute Effect of Egg-Based High Protein Meal
on Hypertensive Response to Exercise

Professor Wayne W. Campbell, Ph.D.
Department of Nutrition Science
Purdue University

What is the purpose of this screening process?

The primary purpose of this study is to explore the effect of high versus normal egg-based protein meals on acute exercise-induced elevated blood pressure.

What will I do if I choose to be in this screening process?

The following procedures will be completed during the screening:

Blood Sample
You will have a blood sample taken from a vein in your arm by a person trained to collect blood (phlebotomist). This blood sample will be taken in the early morning before you have had anything to eat (after fasting overnight for 12 hours). The sample will be used to measure various indicators of overall health. The total amount of blood drawn will not exceed 10 milliliters (0.5 ounces or ~3 teaspoons).

Body Weight and Body Height
Your weight will be measured using a platform scale and your height will be measured using a wall mounted ruler. Your Body mass index (kg/m^2) will be calculated from height and weight.

Blood Pressure
Your blood pressure will be measured using an automated blood pressure monitor. You will rest for 15 minutes and your blood pressure will be measured while you are sitting and while you are lying down.

Pregnancy Test
If you are a female, you will be asked to take a pregnancy test at screening.

Review Study Design
Discuss and answer any questions you may have regarding the study

How long will I be in the screening process?

You will complete one day of screening procedures that last a total of ~1 hour.

What are the possible risks or discomforts?

The potential risks you may encounter include pain and the development of a small bruise and/or infection at the puncture site on the arm where blood is drawn. You may also feel

lightheaded and there is a slight risk of fainting. There are no known risks when completing questionnaires or having your body weight, body height, and blood pressure measured.

Are there any potential benefits?

There are no direct benefits for participating in this screening process. You may benefit from the information given to you concerning your general overall health status from your blood sample and blood pressure measurements.

What alternatives are available?

There are no alternatives available.

Will I receive payment or other incentive?

You will not be paid for completing this screening process.

What happens if I become injured or ill because I took part in this screening process?

If you feel you have been injured due to participation in this screening process, please contact Professor Wayne Campbell or the Human Research Protection Program as listed below in this consent form.
Purdue University will not provide medical treatment or financial compensation if you are injured or become ill as a result of participating in this research project. This does not waive any of your legal rights nor release any claim you might have based on negligence.

Will information about me and my participation be kept confidential?

The project's research records may be reviewed by the Purdue University Institutional Review Board, the Purdue Office for Human Research Protection, by departments at Purdue University responsible for regulatory and research oversight, and by the screening process sponsor/funding agency. If you do not qualify to participate in the screening process, your information collected during the screening process will be immediately destroyed. Any information submitted via the Campbell Lab Website will be confidential and will only be viewed by the screening process coordinators. Original paper copies of all identifiable data will be kept indefinitely in locked storage cabinets and rooms which are only accessible by Prof. Campbell, and his research staff, and selected members of his department's information technology resources staff. All data will be de-identified prior to statistical analyses. There is a risk of breach of subject confidentiality but safeguards are in place to minimize this risk as outlined above.

What are my rights if I take part in this screening process?

Your participation in this screening process is voluntary. You may choose not to participate or, if you do agree to participate, you can withdraw your participation at any time without penalty or loss of benefits to which you are otherwise entitled.

Who can I contact if I have questions about the screening process?

If you have questions, comments or concerns about this research project, you can talk to one of the researchers. Please contact Professor Wayne Campbell, campbeww@purdue.edu 765-494-8236.

If you have questions about your rights while taking part in this screening process or have concerns about the treatment of research participants, please call the Human Research Protection Program at (765) 494-5942, email (irb@purdue.edu) or write to:

Human Research Protection Program - Purdue University
Ernest C. Young Hall, Room 1032
155 S. Grant St.
West Lafayette, IN 47907-2114

Please check only one of the two boxes below

☐ You agree to allow the use of your data and/or specimens collected during this screening evaluation to be used for future research that is unrelated to this screening process.

_____ _____
Screening-Participant's Signature Date

☐ You request your data and/or specimens collected during this screening evaluation to NOT be used for any future research that is unrelated to this screening process.

_____ _____
Screening-Participant's Signature Date

Documentation of Informed Consent

I have had the opportunity to read this consent form and have the research screening process explained. I have had the opportunity to ask questions about the research screening process, and my questions have been answered. I am prepared to participate in the research screening process described above. I will be offered a copy of this consent form after I sign it.

_____

_____
Participant's Signature Date

_____
Participant's Name

_____

_____
Researcher's Signature Date

STUDY CONSENT FORM

Purdue IRB Protocol #: 1607017949 - Expires on: 03-OCT-2017

RESEARCH PARTICIPANT STUDY CONSENT FORM

The Acute Effect of Egg Based High Protein Meal on Hypertensive Response to Exercise

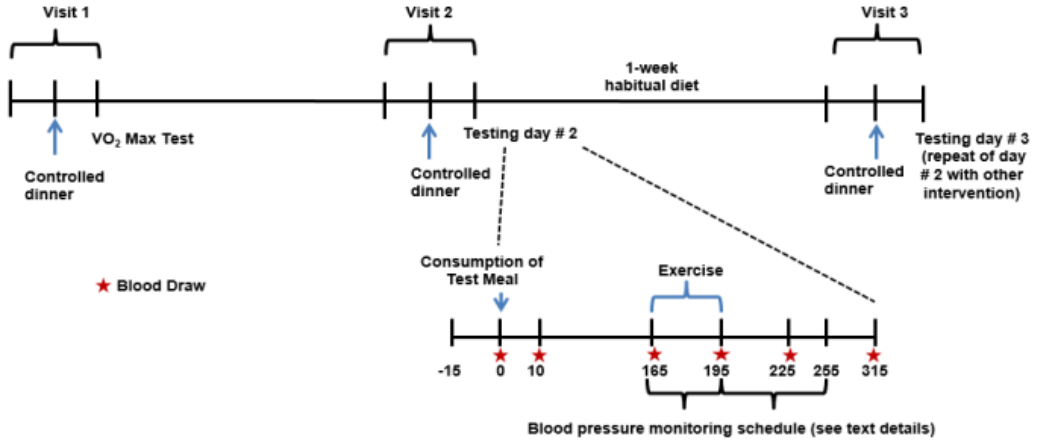
Professor Wayne W. Campbell, Ph.D.
Department of Nutrition Science
Purdue University

What is the purpose of this study?

The primary purpose of this study is to explore the effect of high versus normal egg-based protein meals on acute exercise-induced hypertension.

What will I do if I choose to be in this study?

An overview of the protocol to be used is shown in figure below.



At the start of the study, you will be given a complete schedule (calendar) of events to follow.

Diet

You will be provided with a dinner the day before each testing day (Δ). You will be asked to consume all of the foods and beverages provided and record when the items are eaten on a food checklist. All foods will be prepared, portioned, and provided to you by research staff in the Department of Nutrition Science Metabolic Kitchen. We will ask you not eat or drink any foods or caloric beverages other than that provided to you by the research staff after dinner. You will pick up your food 2 days before each testing day.

Pregnancy Test

If you are a female, you will take a pregnancy test at the beginning of each test day.

Test Day Procedures

On each of the 3 test days: You will be asked to not eat or drink any foods or caloric beverages after 9 pm the night before testing (10-h fasting with water allowed). You will arrive at the Purdue Clinical Research Center in the morning. You will be asked to complete a survey describing your physical state of well-being.

Acute Meal (Testing Day 2 and 3):
Following 15 min sitting period, you will eat a high protein or normal protein egg-based breakfast burrito. After consuming your burrito, we will immediately begin monitoring your blood pressure.

Blood Pressure Monitoring (Testing Day 2 and 3): We will be using a Suntech Medical Tango M2 monitor. This monitor is a validated device used in research settings to accurately and reliably measure blood pressure during exercise stress tests. This device will automatically take a blood pressure readings before exercise, every 3 minutes during exercise and for 1 hour after exercise testing.

VO_2 Submax Testing (**Testing Day 1, 2 and 3**):
You will eat a breakfast meal and then perform the YMCA VO_2 Submax testing method on a stationary bicycle. Test day 1, your heart and work rates will be recorded at regular intervals and used to determine your 70% VO_2 max and required work rate for testing days 2 and 3. Perceived exertion (0-10 scale) will be monitored throughout the exercise session and 15 min following the exercise bout to assess your overall physical state.

Blood Draw (Testing Day 2 and 3): On test mornings during the study, you will come to Purdue Clinical Research Center (Stone Hall) after an overnight fast (at least 10 hours without food). Just prior to your meal, you will have a catheter placed into your arm by a trained phlebotomist. You will have blood samples taken from a catheter in your arm by a trained clinical research technician before and after your meal, before you begin your exercise and post exercise time points 0, 30, 120 (100ml total).

How long will I be in the study?
Number of days: less than or equal to 45 days depending on your availability.
This study will consist of 3 test days with a minimum of 1 week break in between test days

What are the possible risks or discomforts?
You may experience stomach discomfort or altered bowel function associated with consuming a non-habitual menu.

You may experience light headedness, muscle tightness, soreness and fatigue, pulled muscles, and rarely joint and bone injury during or after the VO2 submax test. By following the proper warm-up and cool-down procedures these risks are minimal.

The manual blood pressure cuffs may cause some chaffing and/or bruising. With the inflation of the blood pressure cuff, you may also feel pressure and some discomfort on your arm.

The potential risks you may encounter include discomfort and the development of a small bruise and/or infection at the puncture site on the arm where the blood is drawn or at the site of the finger prick. You may also feel lightheaded and there is a slight risk of fainting. On test days, a small amount of saline (approximately
½ teaspoon) will be used after each blood draw to keep the blood draw site clear and clean. This amount of saline is small enough that it should present no hazard to your physical well-being.

Are there any potential benefits?

There are no direct benefits for participating in this study. You may perceive a benefit from knowing your blood profile from your blood draw at screening.

What alternatives are available?

There are no alternatives available.

Will I receive payment or other incentive?

You will receive a payment of $200 for completing the entire study. If you decide to withdraw from the study before completing both testing days, you will not be compensated. If you are found not to be following the diet to the satisfaction of the study dietitian, you will be removed from the study.

Are there costs to me for participation?

There will be no cost to you to participate in this study.

What happens if I become injured or ill because I took part in this study?

If you feel you have been injured due to participation in this study, please contact Professor Wayne Campbell or the Human Research Protection Program as listed below in this consent form.
Purdue University will not provide medical treatment or financial compensation if you are injured or become ill as a result of participating in this research project. This does not waive any of your legal rights nor release any claim you might have based on negligence.

Will information about me and my participation be kept confidential?

The project's research records may be reviewed by the Purdue University Institutional Review Board, the Purdue Office for Human Research Protection, by departments at Purdue University responsible for regulatory and research oversight, and by the study sponsor/funding agency. If you do not qualify to participate in the study, your information collected during the screening process will be immediately destroyed. Any information submitted via the Campbell Lab Website will be confidential and will only be viewed by the study coordinators. Original paper copies of all identifiable data will be kept indefinitely in locked storage cabinets and rooms which are only accessible by Prof. Campbell, and his research staff, and selected members of his department's information technology resources staff. All data will be de-identified prior to statistical analyses. There is a risk of breach of subject confidentiality but safeguards are in place to minimize this risk as outlined above.

What are my rights if I take part in this study?

Your participation in this study is voluntary. You may choose not to participate or, if you agree to participate, you can withdraw your participation at any time without penalty or loss of benefits to which you are otherwise entitled.

Who can I contact if I have questions about the study?

If you have questions, comments or concerns about this research project, you can talk to one of the researchers. Please contact Professor Wayne Campbell, campbeww@purdue.edu 765-494-8236.

If you have questions about your rights while taking part in the study or have concerns about the treatment of research participants, please call the Human Research Protection Program at (765) 494-5942, email (irb@purdue.edu) or write to:

Human Research Protection Program - Purdue University
Ernest C. Young Hall, Room 1032
155 S. Grant St.,
West Lafayette, IN 47907-2114

Please check only one of the two boxes below

☐ You agree to allow the use of your data and/or specimens collected during this evaluation to be used for future research that is unrelated to this study.

_____ _____
Study-Participant's Signature Date

☐ You request your data and/or specimens collected during this evaluation to NOT be used for any future research that is unrelated to this study.

_____ _____
Study-Participant's Signature Date

Documentation of Informed Consent

I have had the opportunity to read this consent form and have the research study explained. I have had the opportunity to ask questions about the research study, and my questions have been answered. I am prepared to participate in the research study described above. I will be offered a copy of this consent form after I sign it.

_____ _____
Participant's Signature Date

_____
Participant's Name

_____ _____
Researcher's Signature Date

TESTING DAY FLOW CHART

1

S-47 Testing Procedures

Subject Screening I.D. # S-47-____

Date: _____ **Time:**_____

Test Day 2

Actual Time	*Predicted Time*	*Estimated Time*	
_____			**-30 min** Consumed provided meal the evening before testing day Nothing else except water after 8pm _____
			One person retrieves meal and reheats/preps tray (water + utensils)
			-30 min Study Consent reviewed and signed _____
_____			**-25 min** Females Pregnancy Test_____
_____			**-20 min** Weight (kg) _____
_____			**-20 min** Shave/place Leads _____
			Subject goes to the bed
_____			**-15 min** Insert line (Robin)_____
_____			**-5 min** Blood Pressure *Subject sits for 15 minutes at 90 degree angle* **(Sitting** - Systolic/Diastolic _____/____) (Sitting - Systolic/Diastolic _____/_____)
_____			**-2 min** Fasting blood draw _____ ***3ml Tiger Top, 4 ml Lavender Top; one send out and three saves***
_____	_____		***0* min** *Eat meal - 15 min*
_____	_____		**+25 min** Blood Pressure **(Sitting** - Systolic/Diastolic _____/____) (Sitting - Systolic/Diastolic _____/_____)
_____	_____		***+30* min** *Blood Draw* ***1-6ml lavender top***
			Subject may read up to +140 during this time. Absolutely no sleeping
_____	_____		***+150* min** Blood Pressure **(Sitting** - Systolic/Diastolic _____/____) (Sitting - Systolic/Diastolic _____/_____)

Cont. onto next page 2

2

_____ _____ ***+155* min** *Blood Draw*
1-6ml lavender top

Subject moved to bicycle – leads placed and machine calibrated

_____ _____ ***+165* min** Blood Pressure- Immediately before exercise bout
(Sitting - Systolic/Diastolic _____/____) (Sitting - Systolic/Diastolic _____/_____)

Exercise for ½ hour
Blood Pressure EVERY 3 MINUTES

Time	*BP*		*BP*		*BP*		*BP*		*BP*		*BP*		*BP*		*BP*		*BP*		*BP*	
Actl																				
Pred																				

_____ _____ ***+195* min** *Blood Draw*
1-6ml lavender top

Subject moved back to the bed

Blood Pressure EVERY 3 MINUTES

Time	*BP*		*BP*		*BP*		*BP*		*BP*		*BP*		*BP*		*BP*		*BP*		*BP*	
Actl																				
Pred																				

Actual Time	***Predicted Time***	***Estimated Time***

_____ _____ ***+225* min** *Blood Draw*
1-6ml lavender top

_____ _____ ***+255* min** Blood Pressure
(Sitting - Systolic/Diastolic _____/____) (Sitting - Systolic/Diastolic _____/_____)

Actual Time	***Predicted Time***	***Estimated Time***

_____ _____ ***Subject may read up to +300 during this time. Absolutely no sleeping***

_____ _____ ***+312*** Blood Pressure
Subject sits for 15 minutes at 90 degree angle
(Sitting - Systolic/Diastolic _____/____) (Sitting - Systolic/Diastolic _____/_____)

_____ _____ ***+315*** *Blood Draw*
1-6ml lavender top

Label tubes and microtubes in advance of testing days	Set up Tray Tables with Catheter Kits, Safety Tubes, Gauze, Blood Tubes, Alcohol Wipes, Saline Syrines
Breakfast Utensils and Meal Should Be Ready In the G93 Black Refrigerator	Rack of Labeled Microtubes should be covered and placed in the processing area

APPENDIX D. PROTEIN SUPPLEMENTS WITH VS BETWEEN MEALS

Emerging Science

Effects of protein supplements consumed with meals, versus between meals, on resistance training–induced body composition changes in adults: a systematic review

Joshua L. Hudson, Robert E. Bergia III, and Wayne W. Campbell

Context: *The impact of timing the consumption of protein supplements in relation to meals on resistance training–induced changes in body composition has not been evaluated systematically.* ***Objective:*** *The aim of this systematic review was to assess the effect of consuming protein supplements with meals, vs between meals, on resistance training–induced body composition changes in adults.* ***Data Sources:*** *Studies published up to 2017 were identified with the PubMed, Scopus, Cochrane, and CINAHL databases.* ***Data Extraction:*** *Two researchers independently screened 2077 abstracts for eligible randomized controlled trials of parallel design that prescribed a protein supplement and measured changes in body composition for a period of 6 weeks or more.* ***Results:*** *In total, 34 randomized controlled trials with 59 intervention groups were included and qualitatively assessed. Of the intervention groups designated as consuming protein supplements with meals ($n = 16$) vs between meals ($n = 43$), 56% vs 72% showed an increase in body mass, 94% vs 90% showed an increase in lean mass, 87% vs 59% showed a reduction in fat mass, and 100% vs 84% showed an increase in the ratio of lean mass to fat mass over time, respectively.* ***Conclusions:*** *Concurrently with resistance training, consuming protein supplements with meals, rather than between meals, may more effectively promote weight control and reduce fat mass without influencing improvements in lean mass.*

INTRODUCTION

It is well established that consuming dietary protein proximate to resistance-type exercise sessions promotes a positive net protein balance during postexercise recovery.[1–4] Two meta-analyses demonstrated that consuming protein supplements concurrently with prolonged resistance exercise training increased lean mass compared with consuming a nonprotein supplement control.[5,6] These reviews did not consider the timing of protein supplementation with respect to meals. The effect of consuming protein supplements with meals, vs between meals, on resistance training–induced changes on body composition has not been reviewed.

Protein supplements are available in ready-to-drink, powdered, and solid form and are marketed to augment different outcomes such as weight gain, weight loss, and weight management. However, for each outcome, the promoted timing of protein intake varies. Protein supplements designed to augment weight gain or support weight stability are promoted for

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Key words: exercise, meal frequency, obesity, snacking, weight loss.

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consumption between meals.[7–10] For weight loss, protein supplements are often recommended for ingestion either with a meal or as a meal replacement.[11,12] There are scientific rationales that support consuming a protein supplement either with a meal or between meals to differentially influence body composition responses. Consuming a protein supplement between meals may decrease compensatory eating behaviors, thereby increasing energy intakes and body weight.[13] Conversely, consuming a protein supplement twice daily with meals led to complete energetic compensation in adults who performed resistance training, although body composition was not affected.[14] Consequently, the timing of protein supplementation may be of particular importance, depending on the desired body weight and body composition outcome. The aim of this systematic review of literature was to investigate whether the existing research studies support consuming protein supplements between meals, vs with meals, to differentially change body composition in adults who initiate resistance training regimens.

METHODS

The current systematic review followed the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. The description of the PICOS (population, intervention, comparison, outcome, and study design) criteria used to define the research question is presented in Table 1.

Data sources

A systematic search of the literature was conducted in April 2016 using the PubMed, Cochrane Reviews, Scopus, and CINAHL (Cumulative Index of Nursing and Allied Health) databases and is current to May 2017. Search terms, keywords, and phrases were selected to include appropriate articles on protein supplementation, lean mass, and resistance training (see Table S1 in the Supporting Information online).

Inclusion criteria

Inclusion criteria were as follows: randomized controlled trial with parallel design; intervention duration ≥6 weeks; group mean age ≥19 years; male or female participants; pre- or postmenopausal females; apparently healthy humans with no intentional/prescribed diet-induced energy restriction or surplus; concurrent resistance training with or without aerobic training; prescribed a protein supplement while indicating the timing of ingestion; use of an acceptable method of body composition assessment; and English language publication. Protein supplements (whey, casein, soy, bovine colostrum, and rice) were acceptable if they were isolates, concentrates, or hydrolysates consumed alone or in combination with other nutrients (creatine, amino acids, and carbohydrate) and protein sources. Dual-energy X-ray absorptiometry, air-displacement plethysmography, and hydrostatic weighing were deemed acceptable methods for detecting changes in lean mass on the basis of their high reliability and validity.[15–19] Measurement of total body potassium or doubly labeled water was also acceptable; however, none of the vetted articles used these methods. Articles that used skin folds and bioelectrical impedance were excluded because of unreliable estimations of lean mass.[20,21]

Table 1 **PICOS criteria for inclusion and exclusion of studies**

Parameter	Inclusion criterion
Population	Apparently healthy adults, mean age of group ≥19 years
Intervention	Groups that consumed protein supplements between meals
Comparison or control	Groups that consumed protein supplements with meals
Outcome	Changes in lean mass
Study design	Studies ≥6 weeks in length
Research question	What is the effect of consuming a protein supplement with meals, vs between meals, on changes in body composition in adults performing resistance training?

Article selection and data extraction

Collectively, database searches yielded 2074 articles (PubMed, 1207; Cochrane, 243; Scopus, 157; CINAHL, 468). After screening abstracts, 264 articles, including 3 other articles identified from other sources, were independently read and reviewed by 2 authors (J.L.H. and R.E.B.). A total of 230 were excluded for the following reasons: full text was not accessible to the authors, article did not report on protein supplement–related research; mean age of intervention group was less than 19 years; participants were in energy restriction; lean mass was not reported or was reported only graphically and numerical data were not accessible; researchers used an unacceptable method of body composition assessment; or participants were characterized as having a chronic disease or having severe injury. Four of 7 authors contacted for additional information responded and provided data included in this systematic review. Thirty-four articles were selected for inclusion in this systematic review (Figure 1).[14,22–54]

The following information was extracted independently by J.L.H. and R.E.B. from the selected articles using an electronic form: first author's last name;

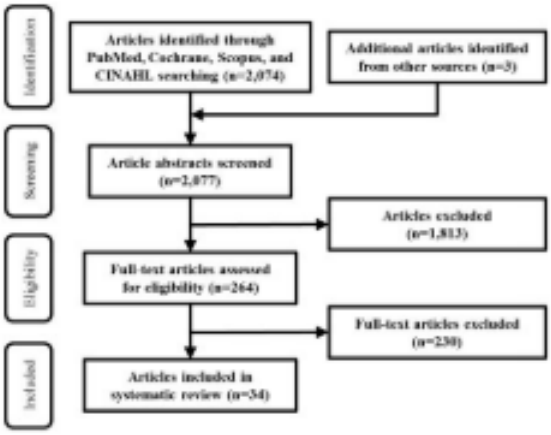

Figure 1 **Flow diagram of the literature search process.**

publication year; sample sizes of each intervention group; sex of participants; mean age of group; duration of intervention; prescribed timing of protein supplement intake; protein source; frequency of resistance training; method of body composition assessment; and pre- and postintervention and net changes in body mass, lean mass, and fat mass. Incongruous information extracted between the 2 authors was reexamined and discussed until concurrence was achieved.

Twenty-nine studies included in this review measured body composition using dual energy X-ray absorptiometry. Two more utilized air displacement plethysmography, and 3 used hydrostatic weighing to measure body composition (see Table S2 in the Supporting Information online). There were some discrepancies in how lean mass was reported, with 21 articles using the term "lean mass," 6 articles using "lean tissue mass," 6 articles using "fat-free mass," and 1 article using "bone-free fat-free mass." For this review, these terms were considered synonymous, and "lean mass" is used consistently. Articles that included bone mineral content within lean mass were included in the analyses because bone mineral content only accounts for approximately 5% of total lean mass[55]; moreover, bone turnover (remodeling) is very slow, requiring a minimum of 4 to 6 months.[56]

Critical appraisal

The risks of selection, performance, and detection biases were evaluated from selected articles using a modified Cochrane tool (see Table S2 in the Supporting Information online).[57] Details of the methods for assessment of dietary control and body composition are also included in Table S2 in the Supporting Information online.

Calculations

With-meal ingestion of protein (PRO-WITH; n = 9 studies and 16 groups[14,22–29]) is defined as consumption of a dietary protein-rich supplement immediately after a meal (n = 4), with a meal (n = 9), or as a high-protein meal replacement (n = 3) (see Table S3 in the Supporting Information online). Between-meal ingestion of protein (PRO-BET; n = 25 studies and 43 groups[30–54]) is defined as consumption of a dietary protein supplement predominantly either proximate to resistance training (n = 31) or during a generic time period (ie, before bed [n = 1], before breakfast and before bed [n = 1], between breakfast and lunch and before bed [n = 1], mid-morning and before bed [n = 3], mid-morning and evening [n = 2], morning and evening [n = 1], or upon waking and before bed [n = 3]) (see Table S3 in the Supporting Information online). Studies that included groups whose prescribed supplement timing fit into both the "with" and "between" categories were classified on the basis of the predominant timing of supplementation (n = 3 studies and 6 groups[24,25,29]). For example, a group consuming a dietary protein supplement with breakfast on non–resistance training days and a dietary protein supplement post resistance training would be placed in the PRO-WITH category if they trained 3 or fewer days per week.

The number of articles that reported changes in body mass, lean mass, and fat mass varied, since some articles did not report results for all parameters. Some articles also did not report changes from baseline. For these articles, the absolute change and the percentage of change were calculated as the difference between pre- and post-intervention values. The results from each group are presented qualitatively as categorical variables that either increased or decreased from baseline.

A modified form of a previously published coding system to classify strength of evidence of associations with physical activity in children and adolescents was used to summarize the effect of consuming protein supplements with meals, vs between meals, on body mass, lean mass, and fat mass (Table 2).[58] The coding system was used to provide discrete cutoff points to indicate whether the totality of the research included in the current review consistently or inconsistently affected the outcomes of interest. If 34% to 66% of groups experienced a change from baseline, the result was categorized as an inconsistent effect (designated as "↔"; Figure 2). When 67% to 100% of the groups experienced a change from baseline, the result was categorized as either a consistent positive (↑, increase from baseline) or a consistent negative (↓, decrease from baseline) effect. By default, if 67% to 100% of groups reported a change

from baseline for a specific outcome, 0% to 33% of groups reported a change in the opposite direction. Similarly, if a result was categorized as having an inconsistent effect, by default, the effect was inconsistent in both the positive and the negative directions. Therefore, only the inconsistent and consistent results are reported.

RESULTS

Trial features and participant characteristics

Thirty-four articles that included 59 intervention groups met all inclusion criteria. Descriptions of each article (Trial length and protein supplement timing) and the characteristics of participants (age and sex) are shown in Table S3 in the Supporting Information online. Forty-three of the groups were classified as PRO-BET and 16 were classified as PRO-WITH. This resulted in a total of 608 participants included in the PRO-BET category (age < 50 years, n = 30 groups; age > 50 years, n = 13 groups; mean age, 55 years; median age, 25 years; range, 19–74 years) and 373 participants in the PRO-WITH category (age < 50 years, $n = 3$ groups; age > 50 years, n = 13 groups; mean age, 55 years; median age, 25 years; range, 23–81 years). Twenty-seven groups in the PRO-BET category were less than 12 weeks in duration and 16 were between 12 and 16 weeks in duration. All 16 of the PRO-WITH groups were 12 to 36 weeks in duration, with 7 being 12 to 16 weeks long.

Table 2 **Rules for classifying the strength of evidence for the outcomes assessed**[a]

Percentage of groups in which evidence supported the outcome	Summary code	Meaning of code
34–66	↔	Inconsistent effect
67–100	↑	Consistent positive effect
	↓	Consistent negative effect

[a]Codes represent summary of effect. Inconsistent effect (↔): 34%–66% of groups reported either an increase or a decrease from baseline; consistent positive (increase) (↑) or negative (decrease) (↓) effect: 67%–100% of groups reported a change in that direction from baseline.

Quality of selected articles

Six articles were deemed at low risk of selection bias, since they provided specified methods of randomization and/or allocation concealment in the original articles, while the other selected articles did not clearly report the randomization and allocation concealment methods (see Table S2 in the Supporting Information online). Twenty-seven of the 34 articles included details on

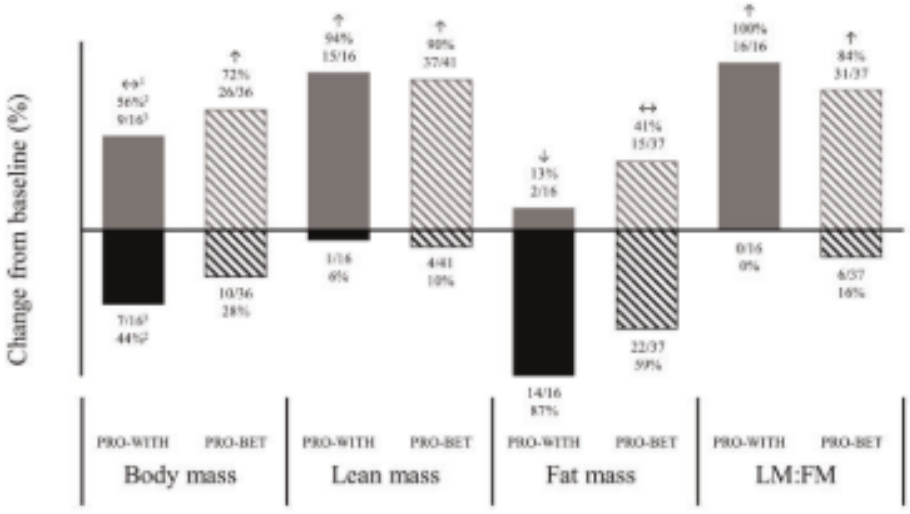

Figure 2 **Changes in body mass, fat mass, lean mass, and ratio of lean mass to fat mass in groups consuming protein supplements with meals vs between meals.** [1]A modified form of a previously published coding system was used to summarize the effects of consuming protein supplements with meals vs between meals.[58] [2]Represents the percentage of groups that reported either an increase or a decrease from baseline. [3]Represents the number of groups that reported either an increase or a decrease from baseline out of the total number of groups assessed for each outcome. *Abbreviations and symbols:* PRO-BET, groups ingesting protein supplements between meals; PRO-WITH, groups ingesting protein supplements with meals; FM, fat mass; LM, lean mass; ↔, inconsistent effect: 34%–66% of groups reported either an increase or decrease from baseline; ↑, consistent positive effect (increase from baseline): 67% to 100% of groups reported a change in that direction from baseline; ↓, consistent negative effect (decrease from baseline): 67% to 100% of groups reported a change in that direction from baseline.

whether participants and investigator(s) were blinded during the intervention or were blinded until data collection was completed. Thirty-one articles indicated that the protein supplements used in the article were provided to the participants.

Results of systematically searched assessment

A quantitative meta-analysis could not be performed because the systematic search of literature did not identify any randomized controlled trial that directly compared the effects of consuming protein supplements with meals, vs between meals, on body composition changes with resistance training. Qualitatively, consuming protein supplements between meals had a consistent effect on increasing body mass (increase in 72% of groups), whereas consuming protein supplements with meals had an inconsistent effect (increase in 56% of groups) (Figure 2).[59] When protein supplements were consumed with meals and between meals, there was a consistent positive effect on lean mass (increase in 90% of PRO-BET and 94% of PRO-WITH groups). Consuming protein supplements between meals had an inconsistent effect on fat mass (decrease in 59% of groups), whereas consuming protein supplements with meals had a consistent negative effect on fat mass (decrease in 87% of groups). With regard to changes in the ratio of lean mass to fat mass, consuming protein supplements between meals and with meals had a consistent positive effect on the ratio (increase in 84% of PRO-BET and 100% of PRO-WITH groups).

A secondary quasi-sensitivity analysis was conducted using results from groups whose interventions were 12 to 16 weeks in duration to better control for any potential temporal effects of the intervention on the outcomes (see Table S4 in the Supporting Information online). The results are comparable to the analyses in which all groups were included. Consuming protein supplements between meals (n = 16 groups) had consistent positive effects on body mass, lean mass, and the ratio of lean mass to fat mass; there was an inconsistent effect on fat mass. Consuming protein supplements with meals (n = 7 groups) had a consistent negative effect on body mass and fat mass and a consistent positive effect on lean mass and the ratio of lean mass to fat mass.

Further quasi-sensitivity analyses were conducted with younger adults (mean group age, 19–50 years); older adults (mean group age, > 50 years); groups consuming protein supplement doses estimated to achieve maximal muscle protein synthesis (doses of ≥0.24 and 0.40 g/kg in younger and middle-aged adults and in older adults, respectively[59]); matching dosing ranges (10–75 g/d); matching total protein intake ranges (0.9–1.6 g/kg/d); matching dosing and total protein intake ranges; and by excluding studies when a protein supplement also contained creatine (see Table S4 in the Supporting Information online). Creatine supplementation was found to increase total body mass and lean mass in older adults concurrently engaged in resistance training in 2 previous meta-analyses[60,61] and was assessed for any mediating effect of the results reported in the current review. The qualitative results from the quasi-sensitivity analyses are comparable with the those from the original analyses, although this cannot be statistically confirmed. The directional changes from baseline are reported here, and specific magnitudes of changes for each outcome are shown in Table S5 of the Supporting Information online.

DISCUSSION

The objective of this systematically searched assessment of literature was to assess whether consuming protein supplements with meals, vs between meals, differentially affected changes in body composition in adults concurrently performing resistance training. Qualitatively, results suggest that consuming protein supplements between meals may promote increases in body mass, and consuming protein supplements with meals may promote reductions in fat mass. Consistent improvements in lean mass and in the ratio of lean mass to fat mass may be achieved when consuming protein supplements either between meals or with meals in combination with resistance training.

Consuming protein supplements between meals consistently increased body mass, whereas consuming protein supplements with meals had an inconsistent result. One inclusion criterion for this systematic review was the absence of a controlled diet aimed at regulating energy intake. Groups were effectively "free-feeding" adults. With this in mind, protein supplements consumed between meals could be considered a snacking occasion. Although the definition of snacking is not clear, it is generally agreed that consuming energy-containing foods or beverages outside a primary eating occasion (ie, breakfast, lunch, dinner) is an acceptable designation.[62] In one 2-week trial, mandatory snacking promoted weight gain in free-living adults.[13] This effect was shown to be exacerbated when the snacks were consumed as beverages,[63,64] the predominant form of protein supplement among the groups included in this review. Consuming protein supplements between meals or as a snack may also increase eating frequency, which may promote higher body weight.[65–67] In contrast, protein supplements consumed with meals or as meal replacements renders them meal components. They may displace some of the energy that otherwise would

have been consumed at that meal time. Results from 1 randomized controlled trial showed that participants who consumed an approximately 200-kcal whey protein–rich supplement twice daily (with breakfast and lunch meals) for 36 weeks had complete energetic compensation in the diet and maintained their body weight.[14] Collectively, results from the current systematic review fit within the existing observational and randomized control trial literature, demonstrating that protein supplementation between meals may promote greater increases in body mass than protein supplementation with meals.

Consuming protein supplements either between meals or with meals in combination with resistance exercise training consistently increased lean mass. This finding suggests that resistance training is a more potent anabolic stimulus than the timing of protein supplementation in relation to meals. Consuming protein supplements while concurrently resistance training creates a positive net protein balance.[1–4] Two meta-analyses showed that participants who consumed protein supplements while resistance training had greater lean mass accretion than participants consuming a nonprotein placebo.[5,68] Results from the current study support previous findings that increases in lean mass can be attained through a combination of resistance training and consumption of protein supplements.[5,68]

Consuming protein supplements with meals consistently decreased fat mass, whereas consuming protein supplements between meals had inconsistent effects on fat mass. Consuming protein supplements with meals may lead to partial meal replacement that would displace the energy that would be consumed otherwise. The within-meal effects of protein supplementation are consistent with previous observations that adults may fully compensate for the additional energy from protein supplements that are consumed with meals.[14] The decrease in fat mass fits within the results of this systematic review, which showed a consistent increase in lean mass and an inconsistent change in body mass. Since lean mass consistently increased and body mass change was inconsistent, it follows that fat mass would decrease.

Strengths and limitations

This review is subject to the standard limitations of systematic reviews, such as publication bias and keyword formation that omit publications that would fit within the search parameters. However, in addition to systematically searching PubMed, CINAHL, Cochrane Reviews, and Scopus, 2 authors independently assessed articles within relevant meta-analyses for inclusion in this review to mitigate these limitations. Specific limitations to this study include a disproportionate over-representation of older adults in the PRO-WITH category compared with the PRO-BET category, variations in trial duration, and differences in supplementation quantity and total protein intake. To address these potential confounding factors, quasi-sensitivity analyses were conducted to investigate any potential influence on the outcomes (see Table S4 in the Supporting Information online). There do not appear to be differences in the proportion of groups within each outcome; however, the inability to perform statistical analyses on the subgroups prohibits conclusive statements about their influence.

The inclusion and exclusion criteria for this systematic review were designed to capture studies that adequately documented protein supplementation in relation to meal consumption. Incidentally, more PRO-BET groups consumed protein supplements proximate to resistance training sessions (before or after sessions) than PRO-WITH groups. Protein supplementation around resistance training in order to supply the skeletal muscle with the necessary precursor amino acids to promote skeletal muscle growth has often been a topic of interest. However, the influence of protein supplementation timing could not be adequately reviewed or speculated on here because there may be relevant research not captured during this review process. Four studies included in this review did test the effect of protein supplementation timing proximate to, vs not proximate to, resistance training.[33,35,40,43] Three studies reported that the timing of protein supplementation did not differentially affect changes in lean mass,[33,40,43] while 1 reported that protein supplementation before and after resistance training promoted greater gains in lean mass than when the same protein supplement was consumed in the morning and evening.[35] Supplements containing mixtures of protein and creatine may be another potential limitation.[60,61] A quasi-sensitivity analysis excluding groups that consumed protein supplements containing creatine did not alter the reported results. Collectively, these quasi-sensitivity analyses should be interpreted with caution because of the low number of groups within each category. There is also the possibility that the effects reported in the current review may not be specific to protein supplementation but rather to the energy contents of the supplements. Further research that includes nonprotein supplements is needed.

CONCLUSION

The results from this systematic review provide novel information for individuals who choose to consume a protein supplement as part of their dietary pattern to

promote body mass gain or improve body composition through fat mass reduction. Regardless of the timing of protein supplement intake in relation to meals, lean mass is likely to increase in response to resistance training. However, consuming protein supplements with meals, rather than between meals, may be a more effective dietary strategy to improve resistance training–induced changes in body composition by reducing fat mass, which may be relevant for adults looking to improve their health status. Conversely, consuming protein supplements between meals may be more effective at increasing overall body mass.

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Supporting Information

The following Supporting Information is available through the online version of this article at the publisher's website.

Table S1 **Search terms**

Table S2 **Risk-of-bias assessment of randomized controlled trials included in a systematically searched qualitative assessment on the effects of consuming a protein-rich supplement with meals vs between meals on body composition changes in resistance training adults**

Table S3 **Descriptions of the randomized controlled trials included in a systematically searched qualitative assessment on the effects of consuming a protein-rich supplement with meals vs between meals on body composition changes in resistance training adults**

Table S4 **Qualitative assessment on the directional effect of consuming a protein supplement with meals vs between meals on body composition changes in resistance training adults**

Table S5 **Qualitative assessment on the magnitude of effect of consuming a protein supplement with meals vs between meals on body composition changes in resistance training adults**

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APPENDIX E. PROTEIN RDA ON LEAN MASS

Protein Intake Greater than the RDA Differentially Influences Whole-Body Lean Mass Responses to Purposeful Catabolic and Anabolic Stressors: A Systematic Review and Meta-analysis

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ABSTRACT

Under stressful conditions such as energy restriction (ER) and physical activity, the RDA for protein of 0.8 g · kg^{-1} · d^{-1} may no longer be an appropriate recommendation. Under catabolic or anabolic conditions, higher protein intakes are proposed to attenuate the loss or increase the gain of whole-body lean mass, respectively. No known published meta-analysis compares protein intakes greater than the RDA with intakes at the RDA. Therefore, we conducted a systematic review and meta-analysis to assess the effects of protein intakes greater than the RDA, compared with at the RDA, on changes in whole-body lean mass. Three researchers independently screened 1520 articles published through August 2018 using the PubMed, Scopus, CINAHL, and Cochrane databases, with additional articles identified in published systematic review articles. Randomized, controlled, parallel studies ≥6 wk long with apparently healthy adults (≥19 y) were eligible for inclusion. Data from 18 studies resulting in 22 comparisons of lean mass changes were included in the final overall analysis. Among all comparisons, protein intakes greater than the RDA benefitted changes in lean mass relative to consuming the RDA [weighted mean difference (95% CI): 0.32 (0.01, 0.64) kg, $n = 22$ comparisons]. In the subgroup analyses, protein intakes greater than the RDA attenuated lean mass loss after ER [0.36 (0.06, 0.67) kg, $n = 14$], increased lean mass after resistance training (RT) [0.77 (0.23, 1.31) kg, $n = 3$], but did not differentially affect changes in lean mass [0.08 (−0.59, 0.75) kg, $n = 7$] under nonstressed conditions (no ER + no RT). Protein intakes greater than the RDA beneficially influenced changes in lean mass when adults were purposefully stressed by the catabolic stressor of dietary ER with and without the anabolic stressor of RT. The RDA for protein is adequate to support lean mass in adults during nonstressed states. This review was registered at www.crd.york.ac.uk/prospero as CRD 42018106532. *Adv Nutr* 2019;00:1–11.

Keywords: fat-free mass, exercise, adults, body composition, health, weight loss

Introduction

The protein RDA (0.8 g · kg^{-1} · d^{-1}) represents the relative quantity of high-quality protein needed to maintain nitrogen balance in 97.5% of apparently healthy males and females aged 19 y and older (1). However, the protein RDA is not necessarily an appropriate guideline to follow when considering the "optimal" protein (nitrogen) intake to promote morphological, physiological, and health-related changes in skeletal muscle, a component of lean mass. In part, the RDA is not intended to be used to estimate the protein needs for solely lean mass tissues, but rather the whole body. Furthermore, the DRIs do not address energy status [i.e., energy restriction (ER) (1)—which promotes lean mass catabolism] as a potential modifier of protein needs (2). Physical activity levels are acknowledged as a potential modifier; however, insufficient evidence was available to recommend different protein needs (1). Although acute and short-term methods [e.g., nitrogen balance and stable isotope kinetics (3, 4)] are predominantly used to estimate protein requirements and allowances, morphological, physiological, and other health-related outcomes associated with lean mass are highly pertinent.

All human cells require amino acids to function normally, but lean mass, particularly the skeletal muscle component,

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Abbreviations used: ER, energy restriction; RT, resistance training; WMD, weighted mean difference.

TABLE 1 The PICOS criteria for defining the research question[1]

Parameter	Description
Population	Adults, group mean age ≥19 y
Intervention	Groups consuming greater than the protein RDA (>0.85 g · kg^{-1} · d^{-1})
Comparison	Groups consuming the protein RDA (0.8 ± 0.05 g · kg^{-1} · d^{-1})
Outcome	Changes in lean or fat-free mass
Setting	Randomized controlled trials
Research question	What is the effect of consuming greater than the protein RDA compared with the RDA on changes in lean mass in adults?

[1]The PICOS criteria are taken from Moher et al. (19).

may be uniquely adaptable to fluctuations in protein intake and stress—anabolic and catabolic (5). Promoting lean mass through either growth, preservation, or loss attenuation ostensibly also promotes higher resting energy expenditures (1), functional movement maintenance (6–8), and healthy glucose control (9–11). Daily protein intakes above the RDA are proposed to support higher lean mass when stressors such as ER and/or physical activity occur (12–15). Several previous meta-analyses reported that consuming higher- compared with lower-protein diets favors changes in lean mass when consumed during periods of stress (16–18). However, these reviews also contained studies prescribing diets containing less than the RDA in the lower protein comparator group. Including groups with prescribed dietary protein intakes below the RDA may skew the effect size toward supporting that higher protein intakes are beneficial for lean mass, when in fact consuming less may be detrimental. A meta-analysis that specifically compares higher protein intakes with the RDA on body composition, especially lean mass, during periods with and without purposeful stressors is needed. Therefore, the purpose of this systematic review and meta-analysis of randomized controlled trials is to compare the effects of consuming greater than the protein RDA with those of consuming the RDA on lean mass changes in adults. We hypothesized that protein intakes greater than the RDA would result in beneficial lean mass changes among all studies combined, regardless of the presence or absence of anabolic and/or catabolic stressors.

Methods

This systematic review and meta-analysis followed the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses report (19). The procedures for identification, screening, data extraction, and analysis were agreed upon in advance among all authors. The research question was defined by using the PICOS (population, intervention, comparison, outcome, and setting) criteria (**Table 1**). Details of the methods were documented in a protocol that was registered at the International Prospective Register of Systematic Reviews (PROSPERO, CRD 42018106532) before data analysis.

Inclusion criteria

Randomized controlled trials with a parallel study design that were published in English were included. All articles must have had a comparison group with a prescribed diet meeting the protein RDA (0.8 ± 0.05 g · kg^{-1} · d^{-1}) and an intervention group consuming greater than the RDA (>0.85 g · kg^{-1} · d^{-1}). When information about the prescribed diet was not available, the actual relative protein intake, as calculated by each article's authors, based on baseline body weight determined eligibility. The dietary intervention periods needed to be ≥6 wk long with no upper limit, with or without ER or exercise training (resistance and/or aerobic exercises). Acceptable forms of body composition measurement included DXA, air-displacement plethysmography, hydrostatic weighing, and total body potassium. Articles were excluded if they were not original research (e.g., reviews of literature); included participants <19 y old; did not have or could not provide lean or fat-free mass change values; or prescribed very-low-energy diets (<800 kcal/d). There was no lower limit for publication date.

Search strategy

Two independent reviewers conducted a systematic search of the literature on 20 December, 2017 using the PubMed, Scopus, CINAHL, and Cochrane databases (JLH and YW). The same search was conducted again on 3 August, 2018 for updates (YW and REB). The search terms and search parameters specific to each database are in **Supplemental Table 1**. Additional articles were identified through manual searches and reference lists of previously published review articles (16–18, 20–31).

Article identification and data extraction

A multiple-pass method was used to identify 1520 articles from the database searches (**Figure 1**). After duplicates were removed, the first pass involved screening titles and abstracts to identify and exclude clearly irrelevant articles. If there was insufficient information to categorically exclude an article, the full text of the article was reviewed in the second pass. The 2 independent reviewers crosschecked their results after each pass and differences were discussed and reconciled with a third reviewer.

Only 6 articles contained the necessary data for inclusion in the meta-analysis that originated from the database searches (32–37). Another 52 authors representing their respective articles were contacted via email to acquire unpublished data to determine their eligibility. Forty articles were excluded because either the authors did not respond, the

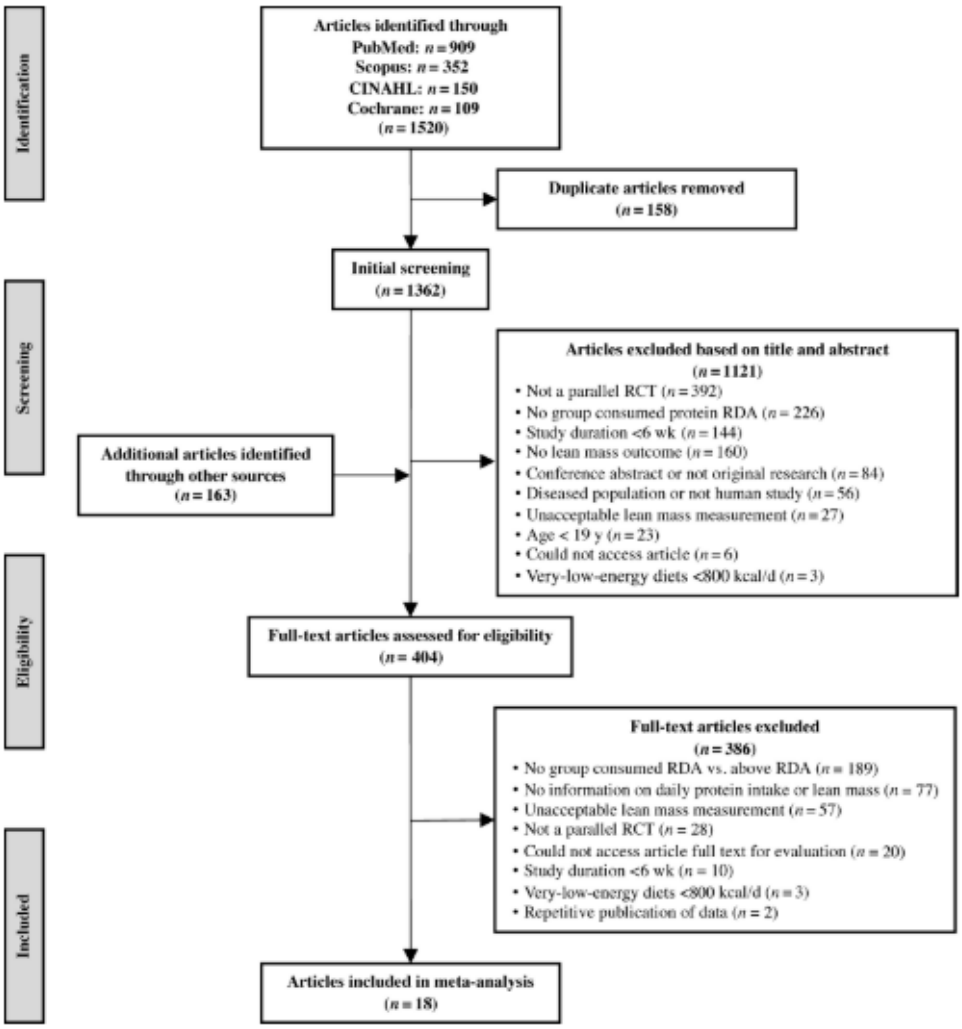

FIGURE 1 Flowchart of the literature search process. RCT, randomized controlled trial.

outcome data were not available, or the articles did not meet the inclusion criteria after information was received. Authors of another 12 articles, 7 from the original database search (38–44) and 5 from external sources (45–49), provided data via email, met the inclusion criteria, and had their articles included in the final meta-analysis for 18 total articles.

The following information was extracted from selected articles independently by both reviewers: first author's last name; publication year; title; body composition assessment method; sample size of each intervention group; mean age, sex ratio (number of females compared with males), and BMI of participants; intervention duration; exercise characteristics and modality; energy and macronutrient intakes; energy status; techniques for dietary control and monitoring compliance; and pre- and postintervention and net changes in whole-body lean mass (post minus pre).

Data synthesis

When articles included multiple treatment arms, each treatment arm was treated as a distinct intervention. For articles including multiple groups that would classify as a comparator group (i.e., groups consuming the RDA), the group most closely matching the treatment was used. For articles including multiple groups that would classify as an intervention group (i.e., groups consuming greater than the RDA), each intervention group was compared with the control group in a separate comparison (32, 33, 35). If an article had multiple time points, only the mean change from pre- to postintervention was retrieved for the outcomes. Only 3 articles had study designs with multiple phases [i.e., controlled feeding and ad libitum feeding phases (32) or weight loss and weight maintenance phases (33, 39)] and each phase was treated as a distinct intervention. Only the controlled feeding (32) and weight maintenance (33) phases

TABLE 2 Summary of the characteristics for the comparator (RDA) and intervention (>RDA) groups[1]

							Prescribed diet				Achieved diet			
Authors (ref)	Group	Duration, wk	ER	RT	*n*	Age, y	Energy, kcal	Protein, g · kg^{-1} · d^{-1} (%)	CHO, %	Fat, %	Energy, kcal	Protein, g · kg^{-1} · d^{-1} (%)	CHO, %	Fat, %
Porter Starr et al. (37)	RDA	24	+	−	26	68.7 ± 1.2	500 deficit	0.8 (15)	55	30	~1458[2]	0.77 (~20)[2]	~50[2]	~3[2]
	>RDA	24	+	−	41	67.9 ± 0.8	500 deficit	1.2 (30)	40	30	~1489[2]	~1.1 (~30)[2]	~40[2]	~30[2]
Tang et al. (36)	RDA	12	+	−	21	44.8 ± 3.6	750 deficit	0.8 (15)	60	25	2273 ± 78	~0.79 (~14)[3]	~61[3]	~25[3]
	>RDA	12	+	−	22	51 ± 2.6	750 deficit	1.4 (25)	50	25	2254 ± 75	~1.41 (~25)[3]	~50[3]	~25[3]
Josse et al. (34)	RDA	16	+	+	13	26 ± 1	750 deficit	(15)	55	30	1430 ± 42	0.84 ± 0.02 (18 ± 1)	58 ± 1	24 ± 1
	>RDA	16	+	+	14	30 ± 1	750 deficit	(30)	40	30	1500 ± 36	1.33 ± 0.03 (28 ± 1)	41 ± 1	31 ± 1
Wycherley et al. (44)	RDA	12	+	−	21	45.9 ± 1.8	~1600	0.85 (17)	58	25	1729 ± 138	0.81 ± 0.09 (25.1 ± 0.6)	51.2 ± 0.8	25.1 ± 0.6
	>RDA	12	+	−	21	47.7 ± 1.7	~1600	1.3 (35)	40	25	1677 ± 156	1.2 ± 0.23 (27 ± 0.7)	38.3 ± 0.8	27 ± 0.7
Verreijen et al. (43)	RDA	13	+	+	30	63 ± 1.1	600 deficit	—	—	—	1662 ± 63	0.85 ± 0.04 (18.3 ± 0.7)	47.8 ± 0.9	29.3 ± 0.8
	>RDA	13	+	+	30	63.7 ± 1.1	600 deficit	—	—	—	1823 ± 103	1.11 ± 0.05 (22.9 ± 0.6)	42.0 ± 1.1	29.2 ± 0.7
Mitchell et al. (48)	RDA	10	−	−	15	74.7 ± 1.0	—	0.8	—	28–31	2695 ± 154	0.9 ± 0.03 (11.7 ± 0.4)	56.6 ± 0.7	31.7 ± 0.3
	>RDA	10	−	−	14	73.7 ± 0.9	—	1.6	—	28–31	2779 ± 46	1.7 ± 0.03 (20.6 ± 0.4)	51.1 ± 0.6	28.3 ± 0.4
Bhasin et al. (45)	RDA	24	−	−	21	71.3 ± 0.9	—	0.8	—	—	2423 ± 79	0.81 ± 0.02	—	—
	>RDA	24	−	−	21	73.5 ± 1.2	—	1.3	—	—	2369 ± 104	1.17 ± 0.03	—	—
Leidy et al. (47)	RDA	12	+	−	25	53 ± 3	750 deficit	0.8 (18)	57	25	1560 ± 60[4]	0.92 ± 0.02 (18.2 ± 0.1)[4]	~57[3,4]	~25[3,4]
	>RDA	12	+	−	21	46 ± 2	750 deficit	1.4 (30)	45	25	1540 ± 60[4]	1.52 ± 0.02 (29.5 ± 0.1)[4]	~44[3,4]	~26[3,4]
Layman et al. (46)	RDA	12	+	−	12	50.1 ± 1.1	500 deficit	0.8	—	<30	1659 ± 167	~0.8 (16)	58	26
	>RDA	12	+	−	12	50.1 ± 1.1	500 deficit	1.6	—	<30	1670 ± 197	~1.5 (30)	41	29
Wycherley et al. (49)	RDA	52	+	−	33	50.2 ± 1.6	~1600	0.85 (17)	58	25	1731 ± 129	~0.82[3] (20.4 ± 0.3)	35.9 ± 0.6	27.7 ± 0.6
	>RDA	52	+	−	30	51.3 ± 1.6	~1600	1.3 (35)	40	25	1737 ± 198	~1.25[3] (30.7 ± 0.6)	47.3 ± 0.7	29.8 ± 0.7
Aldrich et al. (32)	RDA	8	+	−	6	51.3 ± 0.9	~800 deficit	0.8 (15)	55	30	1601 ± 8	0.8 (~15[5])	~53.8[5]	~29.5[5]
	>RDA (mixed protein)	8	+	−	6	49.6 ± 1.4	~800 deficit	1.52 (30)	40	30	1607 ± 2	1.52 (~30[5])	~41[5]	~29[5]
	>RDA (whey protein)	8	+	−	6	49.2 ± 0.7	~800 deficit	1.52 (30)	40	30	1601 ± 12	1.52 (~30[5])	~40[5]	~30[5]
Pal et al. (35)	RDA	12	−	−	25	48.4 ± 0.9	—	—	—	—	1732 ± 338	~0.82[3] (15.8 ± 0.7)	51.5 ± 1.1	30.1 ± 0.9
	>RDA (casein)	12	−	−	20	48.4 ± 0.9	—	—	—	—	1607 ± 236	~1.6[3] (32.9 ± 0.8)	35.3 ± 1.3	29.3 ± 1.0
	>RDA (whey)	12	−	−	25	48.4 ± 0.9	—	—	—	—	1794 ± 385	~1.59[3] (31.9 ± 0.8)	34.9 ± 1.3	29.7 ± 0.9
Layman et al. (39)	RDA	16	+	−	51	46 ± 1	500 deficit	0.8 (15)	55	30	1482 ± 0	~0.73[3] (~15[5])	~59[5]	~26[5]
	>RDA	16	+	−	52	45.2 ± 1.2	500 deficit	1.6 (30)	40	30	1609 ± 0	~1.26[3] (~27[5])	~40[5]	~33[5]
Campbell et al. (38)	RDA	12	−	+	6	66.0 ± 1.4	—	0.8	—	—	—	—	—	—
	>RDA	12	−	+	6	64.0 ± 1.6	—	1.62	—	—	—	—	—	—
Melanson et al. (41)	RDA	12	+	−	41	37.9 ± 1.1	—	—	—	—	—	~0.78[6]	—	—
	>RDA	12	+	−	36	38.8 ± 1.2	—	—	—	—	—	~0.95[6]	—	—
Claessens et al. (33)	RDA	12	−	−	16	46.0 ± 2.2	—	—	≥55	30	1868 ± 142	~0.83[3] (15.8 ± 0.6)	62.7 ± 2.4	21.2 ± 1.5
	>RDA (casein)	12	−	−	14	45.4 ± 2.2	—	(≥25)	—	30	1848 ± 108	~1.85[3] (34.5 ± 1.3)	42.3 ± 1.2	23.5 ± 1.5
	>RDA (whey)	12	−	−	18	44.9 ± 2.0	—	(≥25)	—	30	1812 ± 103	~1.84[3] (35.2 ± 1.6)	42.1 ± 1.1	24.3 ± 1.7
McMillan-Price et al. (40)	RDA (high GI)	12	+	−	27	31.8 ± 1.7	1400 (F), 1900 (M)	(15)	55	30	—	~0.73[6] (18 ± 1)	60 ± 1	19 ± 1
	>RDA (high GI)	12	+	−	31	30.2 ± 1.5	1400 (F), 1900 (M)	(25)	45	30	—	~1.08[6] (28 ± 1)	42 ± 1	27 ± 1
	RDA (low GI)	12	+	−	30	30.5 ± 1.4	1400 (F), 1900 (M)	(15)	55	30	—	~0.79[6] (19 ± 0)	56 ± 1	22 ± 1
	>RDA (low GI)	12	+	−	27	34.6 ± 1.5	1400 (F), 1900 (M)	(25)	45	30	—	~1.05[6] (26 ± 1)	40 ± 2	29 ± 1
Reimer et al. (42)	RDA	12	−	−	26	40.4 ± 2.7	—	—	—	—	1772 ± 102	0.84 ± 0.06 (~12)	45[5]	38[5]
	>RDA	12	−	−	22	38.7 ± 2.6	—	—	—	—	1940 ± 140	1.08 ± 0.07 (~30)	34[5]	36

[1]Values are mean ± SE. CHO, carbohydrate; ER, energy restriction; GI, glycemic index; ref, reference; RT, resistance training.
[2]Estimated using baseline and change values provided in the article.
[3]Estimated protein intake (g · kg^{-1} · d^{-1}) using week 12 protein intakes (g/d) and baseline body weights.
[4]Dietary intake measured at the end of the 12 wk.
[5]Calculated percentage of intake.
[6]Received individual data.

TABLE 3 Summary of the lean mass results from the overall and subgroup meta-analyses between the comparator (RDA) and intervention (>RDA) groups[1]

	n	WMD (95% CI), kg	*P* value[2]	τ^2	I^2, %	χ^2	*P* value[3]
All	22	0.324 (0.011, 0.637)	0.043	0.258	51.9	43.66	0.003
With ER	14	0.361 (0.056, 0.666)	0.02	0.066	20.6	16.38	0.229
With RT	2	0.65 (0.16, 1.14)	0.009	0.000	0.0	0.92	0.338
Without RT	12	0.25 (−0.094, 0.60)	0.153	0.056	15.3	12.99	0.294
Without ER	8	0.23 (−0.44, 0.89)	0.503	0.643	73.3	26.26	0.000
With RT	1	—	—	—	—	—	—
Without RT	7	0.08 (−0.59, 0.75)	0.810	0.572	72.8	22.1	0.001
With RT	3	0.77 (0.23, 1.31)	0.005	0.044	18.4	2.45	0.294
Without RT	19	0.22 (−0.12, 0.56)	0.198	0.250	49.4	35.6	0.008
Age							
Minimum inclusion age >50 y	5	0.91 (0.24, 1.60)	0.008	0.339	59.6	9.89	0.042

[1] ER, energy restriction; RT, resistance training; WMD, weighted mean difference.
[2] *P* value for WMD.
[3] *P* value for χ^2.

qualified based on our criteria and adequate data were only available for the weight loss phase (39).

Fourteen studies included in this review measured body composition using DXA (32, 34–36, 39, 40, 42–49). Two more utilized air displacement plethysmography (37, 41) and 2 used hydrostatic weighing (33, 38) to measure body composition. There were some discrepancies in how lean mass was reported, with 12 articles using the term "lean mass" or "lean body mass," 5 articles using "fat-free mass," and 1 article using "muscle mass." Although precisely these terms are not interchangeable—lean mass may or may not include bone mass, whereas fat-free mass does not include essential fat in the organs and bone—for this review, these terms were considered synonymous, and "lean mass" is used consistently for clarity. Articles that included bone mineral content within lean mass were included in the analyses because bone mineral content only accounts for ~5% of total lean mass (50); moreover, bone turnover (remodeling) is very slow, requiring a minimum of 4–6 mo (51).

Change value means and SDs for lean mass were extracted when available. Otherwise, change means and change SDs were calculated from pre- and post-intervention values when raw data were provided by an article's authors.

Meta-analyses

Random-effect meta-analyses were conducted in Stata/SE 15.1 software (StataCorp LP) using the metan function, and results are reported as the weighted mean differences (WMDs) and 95% CIs. A positive WMD value was considered a beneficial effect of consuming greater than the RDA on lean mass changes. The SE for mean difference within each comparison was calculated as the squared SEM of the difference using the following formula:

$$\text{SEM} = \sqrt{\left[\left(S_1{}^2/n_1\right) + \left(S_2{}^2/n_2\right)\right]} \quad (1)$$

where S_1 and S_2 are the SDs for the change means of the intervention and comparator groups, respectively.

Heterogeneity and risk of bias

Heterogeneity was assessed by χ^2 and I^2 statistics and significance was set at $P < 0.05$. Risk of bias for each article was assessed using a domain-based evaluation and was independently assessed by the reviewers (**Supplemental Table 2**). Funnel plots were visually inspected to determine publication bias (**Supplemental Figure 1**). Sensitivity analyses were performed by removing each comparison one by one. Sensitivity analyses of the influence from removal of each individual study on the WMD and heterogeneity are included in **Supplemental Table 3**.

Subgroup analyses

Subgroup analyses were determined a priori to elucidate possible modifiers of any observed effects in the overall analysis. Subgroup analyses were performed on changes in lean mass with ER, without ER, with physical activity, and with no physical activity, and the 4 permutations of ER and physical activity statuses (8 subgroup analyses total). All articles with a physical activity component prescribed resistance training (RT). As such, the physical activity subgroups are labeled as being with RT or with no RT. Subgroup analyses were also planned for studies with inclusion criteria that demarcated participants as either >50 or <50 y of age.

Results

Study characteristics

The study characteristics of the 18 parallel randomized controlled trials that met all inclusion criteria, representing 934 participants, are described in **Table 2**. Four articles each contributed 2 comparisons (32, 33, 35, 40) for 22 total comparisons on changes in lean mass in the overall analysis. Regarding the subanalyses, 14 comparisons were classified as with ER, 8 were with no ER, 3 were with RT, 19 were with no RT, 2 were with ER + RT, 12 were with ER + no RT, 1 was with no ER + RT, and 7 were with no ER + no RT (**Table 3**).

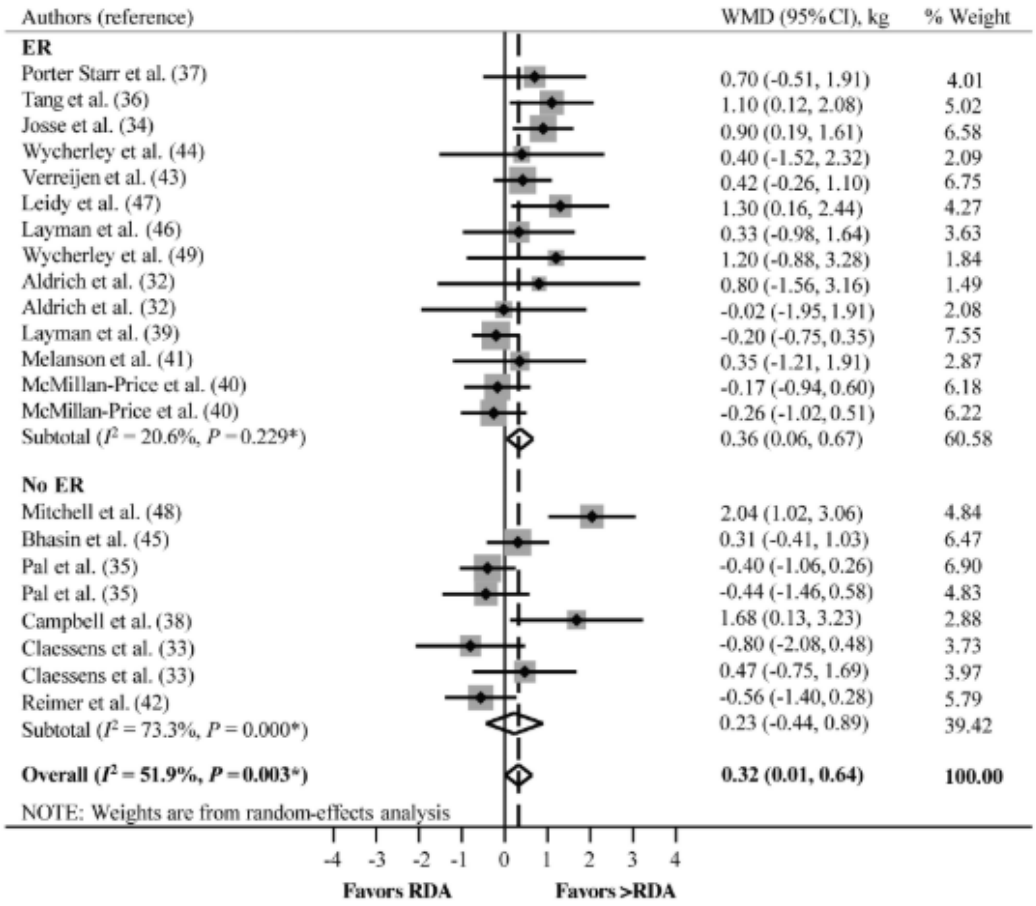

FIGURE 2 The overall and subgroup analyses with ER and with no ER on the effect of consuming greater than the protein RDA compared with the RDA on lean mass changes. *P value for χ^2 test for heterogeneity. ER, energy restriction; WMD, weighted mean difference.

Of the 22 comparisons available in the overall analysis, only 5 comparisons were eligible to be included in the subgroup analysis of older adults whose minimum inclusion age was >50 y. Only 2 comparisons in younger adults qualified for meta-analysis because their maximum inclusion age was <50 y (results not shown).

Publication dates ranged from 1994 to 2018, study durations ranged from 8 to 52 wk, cohort mean age ± SD ranged from 26 ± 1.0 y to 75 ± 1.0 y, and 3 and 5 comparisons included female and male only participants, respectively. All 18 articles reported some amount of dietary control, with 5 articles indicating that all foods and beverages were provided to the participants, 11 providing a portion, and 2 providing menus and counseling. Dietary intakes were measured via food records (n = 9), menu check-off sheets (n = 2), compliance questionnaires (n = 1), dietary recalls and food records (n = 1), food records and menu checkoff sheets (n = 4), and 1 supervised meal consumption (Supplemental Table 2). In the overall analysis, total protein intakes averaged ~0.80 g · kg^{-1} · d^{-1} in the RDA group and ~1.30 g · kg^{-1} · d^{-1} in the >RDA group and were comparable in the subgroup analyses.

Heterogeneity and risk of bias

There was significant heterogeneity in the overall analysis on lean mass (χ^2 = 43.7, I^2 = 51.9%, P = 0.003). The heterogeneity became nonsignificant when only comparisons with ER (χ^2 = 16.4, I^2 = 20.6%, P = 0.229) and with RT (χ^2 = 2.45, I^2 = 18.4%, P = 0.294) were analyzed, but remained significant among comparisons with no ER (χ^2 = 26.3, I^2 = 73.3%, P = <0.001) and no RT (χ^2 = 35.6, I^2 = 49.4%, P = 0.008).

Four and 14 articles had low and unclear risk of selection bias, respectively, based on the information provided in the articles regarding randomization and allocation concealment (Supplemental Table 2). Eight and 10 articles had low and unclear risk of performance bias, whereas 6 and 12 articles had low and unclear risk of detection bias, respectively. Thirteen, 4, and 1 articles had low, unclear, and high risk of

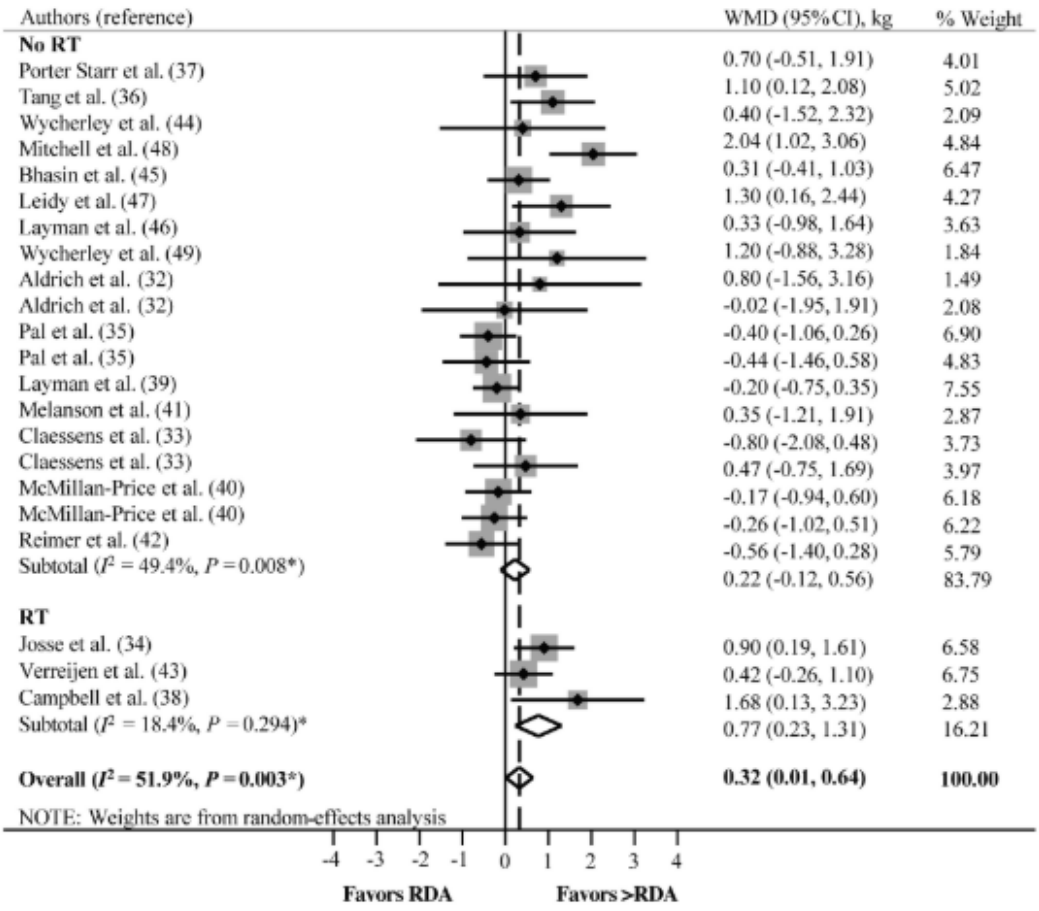

FIGURE 3 The overall and subgroup analyses with RT and with no RT on the effect of consuming greater than the protein RDA compared with the RDA on lean mass changes. *P value for χ^2 test for heterogeneity. RT, resistance training; WMD, weighted mean difference.

attrition bias, respectively. Nine and 9 articles had low and unclear risk of reporting bias, respectively.

Overall effect of consuming greater than the protein RDA

A random-effect analysis of all comparisons showed that consuming greater than the protein RDA benefitted changes in lean mass relative to consuming the RDA (WMD: 0.32 kg; 95% CI: 0.01, 0.64 kg; $P = 0.043$, $n = 22$) (**Figure 2**; Table 3).

Effect of consuming greater than the protein RDA relative to specific stressors

Random-effects analyses of specific subgroups showed that compared with the RDA, consuming greater protein than the RDA attenuated lean mass loss after ER (WMD: 0.36 kg; 95% CI: 0.06, 0.67 kg; $P = 0.020$, $n = 14$), but did not influence lean mass change with no ER (WMD: 0.23 kg; 95% CI: −0.44, 0.89 kg; $P = 0.503$, $n = 8$) (Figure 2; Table 3). Protein intakes greater than the RDA increased lean mass with RT, relative to no change when the RDA was consumed (WMD: 0.77 kg; 95% CI: 0.23, 1.31 kg; $P = 0.005$, $n = 3$), but did not influence changes in lean mass with no RT (WMD: 0.22 kg; 95% CI: −0.12, 0.56 kg; $P = 0.198$, $n = 19$) (**Figure 3**, Table 3).

When ≥2 comparisons were available, we performed further subgroup random-effects analyses to delineate any potential effect of consuming greater than the protein RDA on the changes in lean mass induced by ER and/or RT independently or combined. Consuming greater than the protein RDA did not affect lean mass loss with ER + no RT (WMD: 0.25 kg; 95% CI: −0.09, 0.60 kg; $P = 0.153$, $n = 12$), but promoted lean mass gain with ER + RT (WMD: 0.65 kg; 95% CI: 0.16, 1.14 kg; $P = 0.009$, $n = 2$) (**Figure 4**; Table 3). With no ER + no RT, chronically consuming greater than the protein RDA, compared with the RDA, did not differentially affect lean mass (WMD: 0.08 kg; 95% CI: −0.59, 0.75 kg; $P = 0.810$, $n = 7$) (**Figure 5**; Table 3).

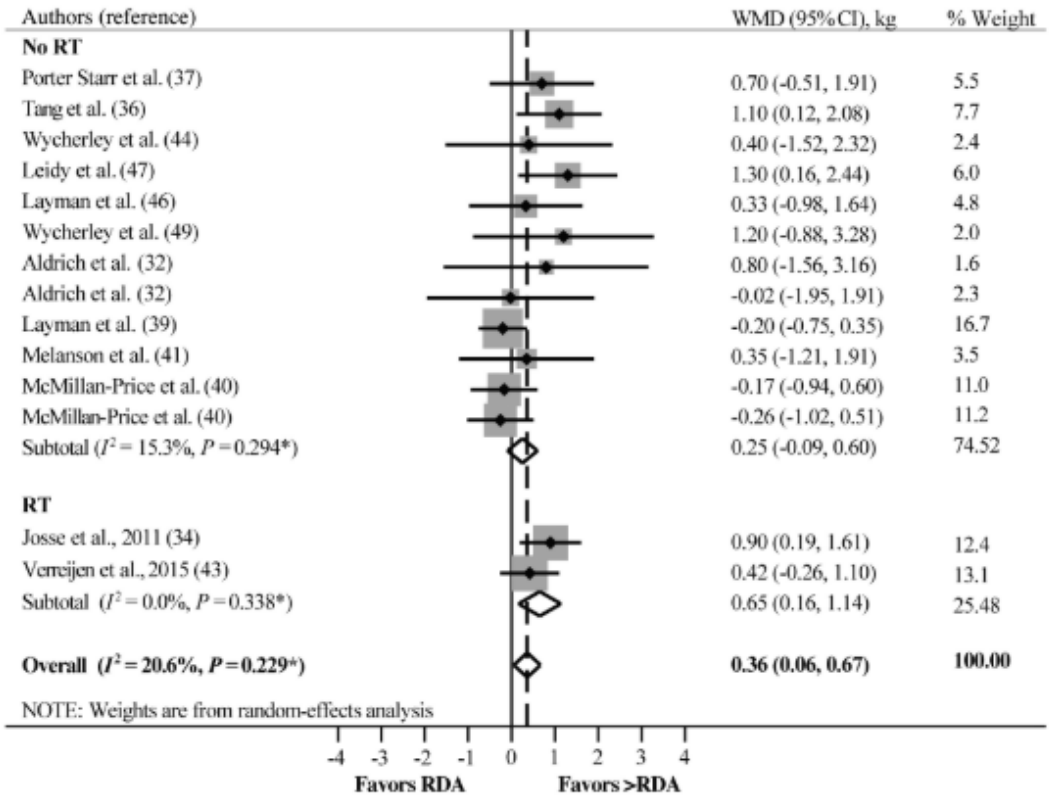

FIGURE 4 The effect of consuming greater than the protein RDA compared with the RDA with ER + RT and with ER + no RT on lean mass changes. *P value for χ^2 test for heterogeneity. ER, energy restriction; RT, resistance training; WMD, weighted mean difference.

Effect of consuming greater than the protein RDA in older adults

In older adults, consuming greater than the protein RDA compared with the RDA resulted in higher lean mass after the interventions (WMD: 0.91 kg; 95% CI: −0.24, 1.60 kg; $P = 0.008$, $n = 5$) (Table 3).

Discussion

This systematic review and meta-analysis of randomized controlled trials was designed and conducted to quantify the overall effect of consuming greater than the protein RDA compared with the RDA on whole-body lean mass among the available literature. In the overall meta-analysis totaling 981 participants from 18 articles that did and did not include purposeful ER or RT, consuming protein in excess of the RDA (~1.3 g · kg^{-1} · d^{-1}) influenced beneficial changes in lean mass in adults. In analyses stratified by specific stressors, protein intakes greater than the RDA influenced beneficial changes in lean mass when adults were purposefully stressed by the catabolic stimulus of dietary ER. However, the beneficial effect of consuming protein in excess of the RDA on lean mass was lost in the absence of purposeful stressors—no ER + no RT. Our results suggest that the beneficial effect of protein intakes greater than the RDA on lean mass may only manifest during stressful periods.

Several narrative reviews and perspective articles suggest that consuming greater than the current protein RDA would influence beneficial changes in lean mass that occur during periods with catabolic and anabolic stressors, such as ER or RT, respectively (4, 52, 53). Results from several systematic reviews and meta-analyses scientifically support these hypotheses; however, these articles typically contain comparator groups that consume less than the RDA (16–18). This could feasibly skew the effect sizes to support that consuming greater than the RDA is beneficial for lean mass when in fact consuming less may be detrimental. This systematic review and meta-analysis is the first to use groups that consumed the RDA as the comparator to assess whether consuming protein in excess of the RDA indeed influences beneficial lean mass changes. Results from this study support the results from previous meta-analyses (16–18) that consuming a higher-protein diet favors changes in lean mass that occur in response to ER regardless of RT status (n = 14 comparisons), ER + RT (n = 2), and RT regardless of ER status (n = 3).

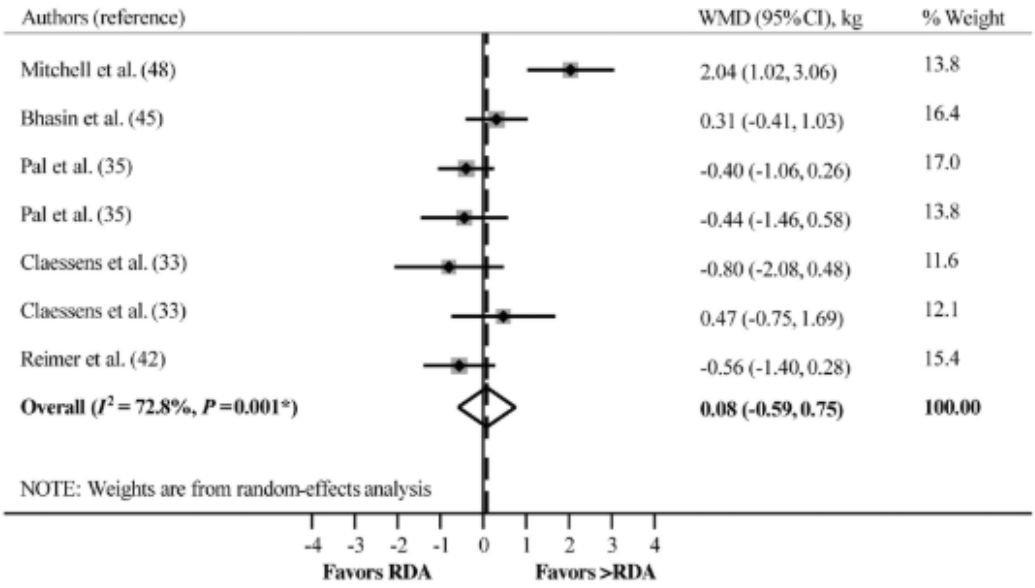

FIGURE 5 The effect of consuming greater than the protein RDA compared with the RDA in adults without purposeful stressors (with no energy restriction + no resistance training) on lean mass changes. WMD, weighted mean difference. *P value for χ^2 test for heterogeneity.

Protein requirements and allowances embedded within the DRIs are based on nitrogen balance values obtained in adults consuming adequate energy and high-quality protein sources (54). Under short-term experimental conditions, 0.8 g · kg^{-1} · d^{-1} protein would support nitrogen balance in 97.5% of healthy adults (1). Nitrogen balance studies and the subsequent protein reference values are frequently scrutinized (55), with researchers citing well-known limitations of the nitrogen balance methodology (5) resulting in an underestimation of the Estimated Average Requirement and RDA (3). Alternative estimates derived from indicator amino acid oxidation studies indicate that the protein RDA should be close to 1.2 g · kg^{-1} · d^{-1} (3). Although indicator amino acid oxidation is a valid technique for estimating individual amino acid requirements, its utility to accurately estimate whole-body protein requirements is unresolved. According to results from the NHANES, American adults consume a mean of ~1.1 g · kg^{-1} · d^{-1} protein (56). If estimates from indicator amino acid oxidation studies were accurate, there would be a high prevalence of protein undernutrition. There are no data to support that such a public health issue exists. Coincidentally, the mean protein intake for the higher-protein group was ~1.3 g · kg^{-1} · d^{-1}. On a morphologic scale, our results showed that, in adults not purposefully stressed (no ER + no RT), chronically consuming protein in excess of the RDA during an intervention did not affect changes in lean mass compared with consuming 0.8 g · kg^{-1} · d^{-1}. These results support that the current RDA is sufficient to retain lean mass, and that higher protein intakes do not influence maintaining lean mass when an individual is not purposefully stressed.

This review is subject to the standard limitations of systematic reviews and meta-analyses such as the selection biases and discrepancies in experimental design. In an effort to address these concerns, 3 separate reviewers searched multiple databases and conducted manual searches of relevant systematic reviews and meta-analyses. Some unpublished data were also retrieved from authors through email: 24 responses from 52 requests (46% response rate). A funnel plot analysis was used to visually inspect publication bias. In the overall analysis, there was significant heterogeneity among the comparisons, which could affect the findings. Anticipating this potential limitation, a priori subgroup analyses were identified before searching the literature, which reduced the I^2 statistic in 2 of the 4 subgroups to <50%. The lack of data from RT literature that utilized the protein RDA limited our ability to quantify the independent effect of RT with or without ER (n = 3) with confidence. This review also only included 5 articles whose participants were greater than 50 y (mean age >60 y). Protein intake as it relates to aging adults and skeletal muscle quantity and performance is particularly important (45, 53), but the varied experimental designs in the 5 articles resulted in 4 iterations of energy balance and RT, which precluded aggregating the articles separately. More research is needed to document the impact of protein intake on skeletal muscle size, metabolic quality, and function, along with functional measures of daily living. At present, insufficient information exists to assess dietary protein adequacy compared with "optimal" sufficiency based on tissue-specific and health-related outcomes for older adults. The duration of the studies may be another limitation of this meta-analysis, ranging from 8 wk to 52 wk (mode:

12 wk). It is possible that the body composition techniques used to assess lean body mass changes are not precise enough to document a potential effect of protein quantity over the study durations, especially at lesser lengths. This would favor a null effect and may be more relevant in the subgroup analysis without apparent stressors that influence changes in lean mass. Caution is warranted to not over-generalize the results beyond the scope of the durations presented in this study.

The results of this systematic review and meta-analysis indicate that the RDA for protein adequately meets the needs of adults to maintain lean mass when they are not purposely stressed. Protein intakes greater than the RDA are shown to augment beneficial changes in lean mass over time when adults purposefully experience catabolic stressors, specifically weight loss. These findings underscore the need to update the DRIs for protein of the general population with consideration given to the energy and physical activity status of adults.

Acknowledgments

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