DIETARY PROTEIN FOR PERFORMANCE, HEALTH AND DISEASE MANAGEMENT

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DIETARY PROTEIN FOR PERFORMANCE, HEALTH AND DISEASE MANAGEMENT

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The Effects of a High-Protein Dairy Milk Beverage With or Without Progressive Resistance Training on Fat-Free Mass, Skeletal Muscle Strength and Power, and Functional Performance in Healthy Active Older Adults: A 12-Week Randomized Controlled Trial

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The study aimed to investigate the independent and combined effects of consuming a high-protein dairy milk beverage, twice daily, with or without a progressive resistance training (PRT) program on outcomes of age-related sarcopenia, in healthy active older (≥50 years) adults. In this 12-week, 2 × 2 factorial study, participants were randomly allocated into one of four groups: dairy milk beverage (DM), exercise and dairy milk beverage (EX+DM), exercise alone (EX), and control (CON). The EX group underwent a 12-week whole-body PRT schedule (three sessions/week) and a high-protein dairy milk beverage (DM) was consumed twice daily (30 g protein/day). At weeks 0, 6, and 12, body composition (iDXA), strength [one-repetition maximum (1RM): leg press, chest press, lateral (*lat*) pull-down, and handgrip], power (countermovement jump), cardiorespiratory fitness (VO_2), and physical performance (gait speed) were measured. Before measurements, blood samples were collected to determine the immune (i.e., leukocyte trafficking and inflammatory cytokines) and hormonal (i.e., insulin, cortisol, IGF-1, testosterone, and estradiol) profiles. Participants ($n = 37$) completed the study within the controlled experimental conditions. Protein intake increased in the EX+DM [mean ± SD, 1.2 ± 0.2 to 1.8 ± 0.4 g/kg body mass (BM) per day^{-1}] and DM (1.3 ± 0.5 to 1.8 ± 0.6 g kg^{-1} BM day^{-1}) groups during the intervention. Absolute fat-free mass increased in the EX+DM [mean (95% confidence interval) = 0.65 (0.25–1.0) kg] and EX [0.49 (−0.44 to 1.40) kg] groups ($P < 0.001$) compared to DM [−0.54 (−1.6 to 0.05) kg]. Relative fat mass decreased (group*time, $P = 0.018$) in DM [−1.8% (−3.3 to −0.35%)] and EX+DM [−1.3% (−2.3 to −0.31%)], which was a greater reduction than that in the CON [0.10% (−0.80 to 1.0%)] group ($P < 0.01$). Relative maximal strength increased in both the EX and EX+DM (≥35%, $P < 0.05$) groups, but not in the DM and CON groups. The change in 1RM strength outcomes was higher in EX+DM compared to all other

groups (53–78%, $P < 0.01$). There was an increase in resting plasma IL-10 concentration in EX+DM (88%), compared to all the other groups ($P = 0.016$). No other differences in systemic inflammatory cytokines were observed. There were no significant changes in all hormone concentrations measured among all groups. In conclusion, a high-protein dairy milk beverage providing additional protein did not further enhance the effects of PRT on outcomes of fat-free mass, power, or physical performance. However, there was a significant augmentative effect for high-protein dairy milk consumption on changes to maximal strength outcomes during PRT in healthy active older adults.

Keywords: leucine, calcium, inflammatory cytokines, insulin, insulin-like growth factor, testosterone, estradiol, cortisol

INTRODUCTION

There has been considerable research exploring the age-related decline in skeletal muscle mass and function (e.g., strength, power, and performance measures), collectively known as sarcopenia (1, 2). The multifactorial (e.g., training status, biological sex, age, and nutrition status) and dynamic (e.g., hormonal and immunological) pathophysiological process of sarcopenia is complex, and currently, there is limited evidence to support the efficacy of pharmacological treatments (3, 4). Therefore, there has been an increased interest in modifiable lifestyle factors such as exercise (e.g., resistance training) and nutrition (e.g., dietary protein) for the treatment and management of age-related sarcopenia. To date, there have been numerous randomized controlled trials and meta-analyses that have consistently reported that progressive resistance training (PRT) can effectively improve gains in fat-free mass (FFM) (∼1.2 kg), maximal strength (≥25%), and physical functional performance (e.g., gait speed) in older adults (>50 years) (5–7). There is no consensus regarding the effect of protein supplementation [e.g., whey protein, casein, essential amino acids (EAAs), and/or leucine] on augmenting further adaptations of FFM, skeletal muscle strength, and power following PRT (8–10). Many studies are confounded by the inclusion of frail institutionalized or sedentary community-dwelling adults, often referred to as "older adults," who are predominantly aged ≥60 years, the varied use of supplementation (e.g., type, form, dose, and frequency) and outcome measures, and the large variations of baseline habitual protein intakes (2, 8, 9). Active older adults (≥50 years) who regularly engage in physical activity—from 150 min/week of light-intensity [e.g., 3–5 metabolic equivalents (METs)] to moderate-intensity (e.g., 6–9 METs) physical activity or 75 min/week of vigorous-intensity (e.g., >9 METS) physical activity (11), either recreationally or competitively—still show signs of age-related sarcopenia (12). Although active older adults do not have the confounding variables attributed to frailty, sedentary behavior, and/or disease (pathogenic hormonal and/or inflammatory status), they are currently underrepresented in sarcopenia research. While ≥50 years is not considered "older" in the spectrum of sarcopenia research, it is the age at which sarcopenia begins to be noticeable (5–7). Furthermore, given the potential efficacy of pairing PRT with protein supplementation, nutritional interventions in active older adults are limited and require further exploration in order to examine their effectiveness in this population.

Higher daily protein intakes [>1.2 g/kg body mass (BM) per day] have been suggested for active older adults, exceeding the current recommended dietary allowance (RDA) of protein (e.g., 0.8 g kg^{-1} BM day^{-1}), to overcome the increased requirements of amino acid utilization from the exercise stimulus and the blunted response to muscle protein synthesis (MPS) known as "anabolic resistance" (13, 14). Moreover, evenly distributed relative and absolute protein intakes per meal have been advocated, as there is an observed maximum capacity for the utilization of EAAs (15). Optimal doses of protein per meal to elicit a near-maximal response have been reported at ∼25–35 g/meal (∼10 g EAA) (16, 17) or relative amounts of 0.40 g/kg BM per meal (18). Numerous cross-sectional studies have observed skewed distributions of protein intake across the day, often not reaching the adequate threshold at breakfast and lunchtime for older adults (19, 20). The unevenness of the protein distribution across the day has been associated with higher levels of frailty (21) in older (≥75 years) community-dwelling individuals. However, in a cohort of "healthier" older adults (75–85 years), there were no observed associations between protein distribution and the outcomes of skeletal muscle mass and strength (22). Noting that these findings are mostly drawn from observational research, there is a lack of data from randomized controlled trials supporting the consumption of ≥1.2 g kg^{-1} BM day^{-1} in a dietary intake with a balanced protein distribution on outcomes of skeletal muscle mass and physical function in healthy active older adults.

The majority of recent research regarding protein requirements for older adults derives from single-type protein supplementation sources [e.g., whey protein isolate; (9, 10)]. However, there has been increased interest in the use of whole foods (e.g., dairy milk) as a protein source to facilitate gains in skeletal muscle mass and strength with PRT in older adults (23). Dairy milk (e.g., bovine), which comprises both whey (20%) and casein (80%), is considered a high-quality protein source as it contains all the EAAs and high levels of leucine (24). There

Abbreviations: BIA, bioelectrical impedance; BMI, body mass index; BM, body mass; CI, confidence interval; CON, control; DEXA, dual-energy X-ray absorptiometry; EAA, essential amino acid; EX, exercise; FM, fat mass; FFM, fat-free mass; DM, high-protein dairy milk beverage; MPS, muscle protein synthesis; MRI, magnetic resonance imaging; PRT, progressive resistance training; SD, standard deviation.

is limited research using dairy milk alone, without additional fortification, in older adults (25–28). Studies in younger athletic populations have shown promising outcomes using unfortified dairy milk beverages on outcomes of skeletal muscle mass, strength, and physical function (29, 30). Despite these observed benefits of dairy milk beverages in younger active adults, an investigation of the effects of dairy milk on these same outcomes in active older adults remains a research gap.

Aging is characterized by a decline in anabolic hormones [e.g., testosterone and insulin-like growth factor-1 (IGF-1)] and a state of chronic low-grade inflammation, a term known as "inflammaging" (31, 32). Raised levels of systemic inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6, and a reduction of anti-inflammatory cytokines such as IL-10 are a common feature (31, 32). Low-grade inflammation in older adults has been associated with the acceleration of the aging process, leading to a decline in skeletal muscle mass and function (32, 33). In multiple observational studies, older adults (>60 years) have an inverse dose–response relationship between physical activity and systemic inflammatory biomarkers even at modest activity levels (33), whereas in exercise intervention studies that have provided resistance training, raised plasma concentrations of IL-10 have been reported following 16–24 weeks of training in older adults (34, 35), suggesting that exercise has the ability to prompt anti-inflammatory processes. Considering that dairy milk contains components associated with anti-inflammatory (e.g., casein-derived bioactive peptides) and immunomodulatory effects, together with resistance training, this combination may act synergistically to reduce inflammaging (23). However, the majority of studies exploring this interaction are mainly based on observational findings with limited evidence on the effects of dietary and exercise interventions on anabolic and cytokine outcomes.

The current study aimed to determine the independent and combined effects of a high-protein dairy milk beverage provided at breakfast and lunch (or after resistance exercise), with or without PRT, on outcomes of FFM, skeletal muscle strength and power, and physical performance in a cohort of healthy active older adults. We sought to evaluate the study hypothesis that providing a high-protein milk on its own would maintain outcomes of FFM, skeletal muscle strength and power, and physical performance compared to those that receive no intervention (e.g., control). In comparison, we hypothesized that a high-protein dairy milk beverage in conjunction with PRT would further enhance the effects of PRT, leading to augmented gains in FFM and skeletal muscle strength and power and an improved physical performance compared to PRT alone.

MATERIALS AND METHODS

The study protocol obtained approval from the Monash University Human Research Ethics Committee (project number 12812), in accordance with the Helsinki Declaration for human research ethics. Informed written consent was obtained from all participants before they were enrolled in the trial. The study was registered with the Australian and New Zealand Clinical Trial Registry as ANZCT12618001088235.

Participants and Study Design

Older adult males and females (≥50 years old, with no age upper limit) performing exercise training for recreational fitness and/or sports competitions (e.g., endurance runners or aerobic gym goers) three or more structured exercise sessions per week, totaling ≥90 min/week of structured exercise duration, plus additional unstructured physical activity that accounted for meeting the Australian physical activity guidelines (36), were recruited from metropolitan Melbourne and surrounding areas in Victoria, Australia. Interested participants were initially screened over the telephone and excluded based on the following criteria: (1) dairy protein allergy or known lactose intolerances; (2) currently using dietary protein supplements; (3) any injuries preventing safe exercise; (4) had surgery in the past 12 months; (5) had an acute coronary (e.g., myocardial infarction) or vascular event in the last year, as well as uncontrolled coronary heart disease; (6) had a stroke in the past 2 years; (7) have orthopedic limitations that limit participation in the exercise program; (8) been diagnosed with or taking medication for thyroid condition; (9) had weight loss of more than 5% of body weight over the last 6 months; (10) take medications that could interfere with skeletal muscle mass structure and/or function (e.g., corticosteroids, testosterone replacement, or anabolic drugs); (11) currently undergoing immunosuppressive therapy or hormone replacement therapy; (12) have any chronic diseases, such as diabetes mellitus or gastrointestinal diseases/disorders; (13) consume more than two standard drinks of alcohol/day or 14 drinks of alcohol/week; (14) were a smoker; (15) had a BMI >30 kg/m^2; and (16) had participated in a structured resistance training program in the past 12 months. Once participants were deemed eligible, data were collected during the period from September 2018 to January 2020.

A total of 65 participants expressed interest in participating; of these, 51 were eligible to participate and were randomly assigned into one of four groups: high-protein dairy milk beverage alone (DM), exercise and high-protein dairy milk beverage (EX+DM), exercise alone (EX), and control (CON) (**Figure 1**). Participants in CON were free-living, with self-selected physical activity and food/fluid intakes that were assessed in the laboratory at baseline and at 6 and 12 weeks as per the other groups. Randomization was carried out by a researcher blinded to the allocation using a block randomization table scheme with stratification by age and sex. Of the 51 randomized participants, five ceased the trial due to restrictions imposed by the COVID-19 pandemic. Other reasons for withdrawal from the study are provided in **Figure 1**. Due to the timeline of the data and sample collection, participants did not liaise with each other within or outside the experimental procedures. In case of close contact with participants (e.g., crossover time during PRT), the participants were advised not to discuss study participation with others.

Preliminary Data

Prior to commencing any physical activity, participants filled out a physical activity readiness questionnaire (PAR-Q), in which

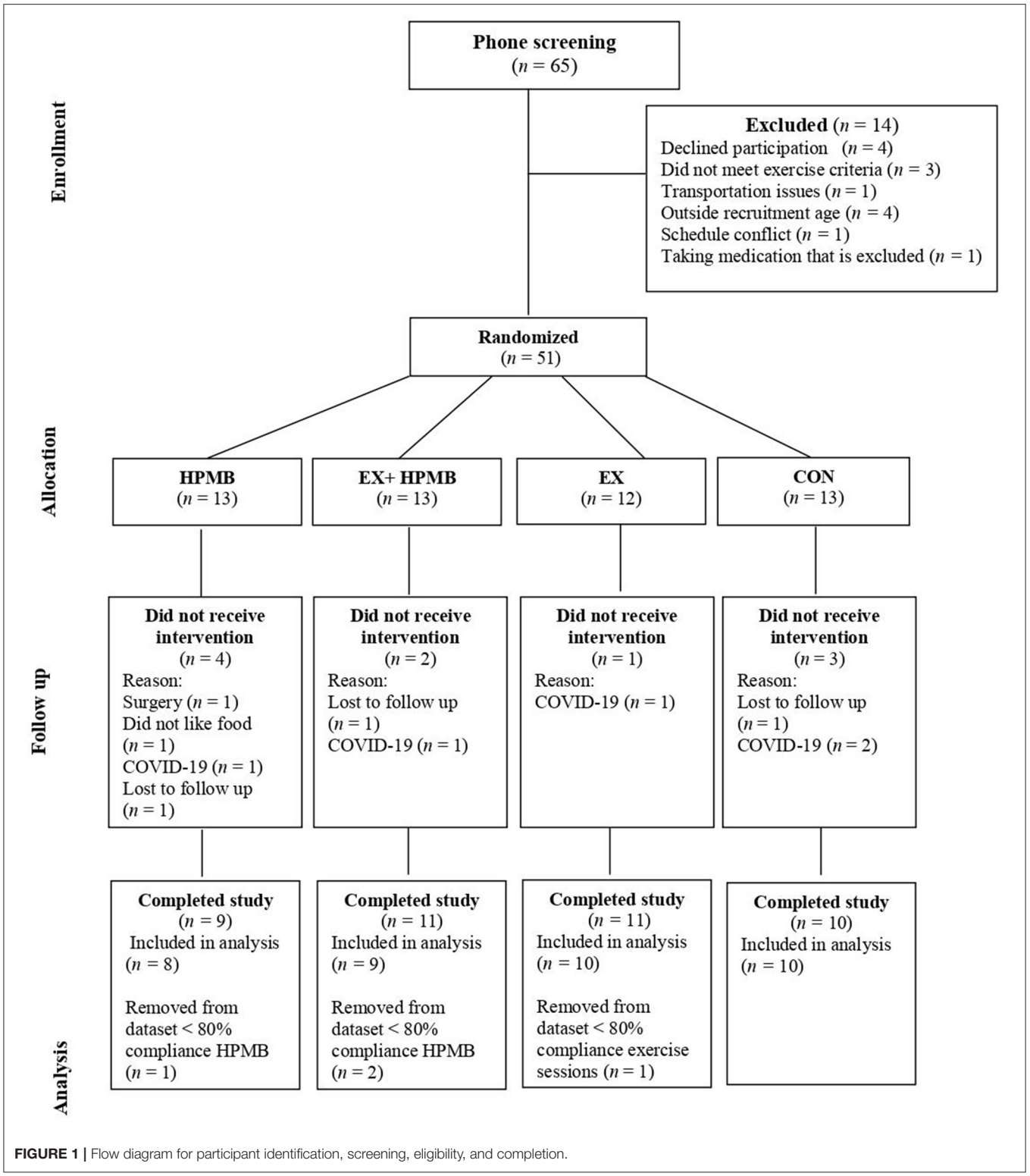

FIGURE 1 | Flow diagram for participant identification, screening, eligibility, and completion.

they self-reported their level of activity including exercise volume and type. The participants were asked to complete a 3-day food–fluid diary prior to their baseline visit, and again at 6 and 12 weeks during the intervention, as previously described (37). Food–fluid diaries were analyzed using FoodWorks v.10.0 nutritional analysis software (Xyris Software, 2019, Brisbane, Australia) based on the Australian Food Composition Database (AFCD) 2019. Total energy, macronutrients, and calcium intake

were obtained, and then dietary protein intakes distributed across breakfast, lunch, dinner, and snacks were extracted. Protein intakes per meal and per day were expressed as absolute (i.e., grams per day or grams per meal) and relative to body mass (i.e., grams per kilogram BM per day and grams per kilogram BM per meal).

High-Protein Dairy Milk Beverage

Participants assigned to DM and EX+DM were asked to consume 500 ml/day (2 × 250 ml) of reduced fat (1.5%) fresh dairy milk (Complete Dairy, Lion Dairy & Drinks, Melbourne, Australia). Participants were provided with a measuring cup and asked to consume 250 ml of dairy milk in the morning (with breakfast) and another at lunchtime (or supervised after resistance exercise in EX+DM, consumed within 10 min of completing their session). Each 250-ml cup of dairy milk contained: 535 kJ energy, 15.0 g protein (1.57 g leucine), 8.3 g carbohydrates (8.3 g lactose), 3.8 g fat, and 435 mg calcium. The participants received food provisions to deliver 100% of the total daily estimated energy requirements and 100% of the total daily estimated protein requirements (∼1.2 g kg^{-1} BM day^{-1}) over the entire duration of the experimental procedure, facilitated by an accredited practicing dietitian. Energy requirements were calculated using the participants' resting metabolic rate (RMR) scaled by an activity factor based on reported exercise (1.37 ± 0.09). RMR was determined by indirect calorimeter (Vmax Encore Metabolic Cart, Carefusion, San Diego, CA) in temperate ambient conditions (22.2 ± 1.4°C) and in accordance with best practice guidelines (38). Intake compliance of daily food provisions and dairy milk, and consumption of other foods/fluids, was recorded using a food–fluid diary. Milk bottles were returned weekly prior to collecting the participants' subsequent week's dairy milk and food provisions. An outline of the study protocol is depicted in **Figure 2**.

Exercise Protocol

Participants allocated to EX+DM and EX were required to attend supervised PRT sessions, on three non-consecutive days per week, for 12 weeks at the research laboratory. These sessions were conducted either in a morning session (7:00 a.m. to 10:00 a.m.) or afternoon session (1:00 p.m. to 3:00 p.m.), to account for participants' work–life schedule. During the course of the trial, all exercise sessions were instructed by a strength and conditioning qualified investigator to ensure correct lifting, to monitor the appropriate amount of exercise and rest intervals, and to check compliance. Each training session (30 ± 15 min)

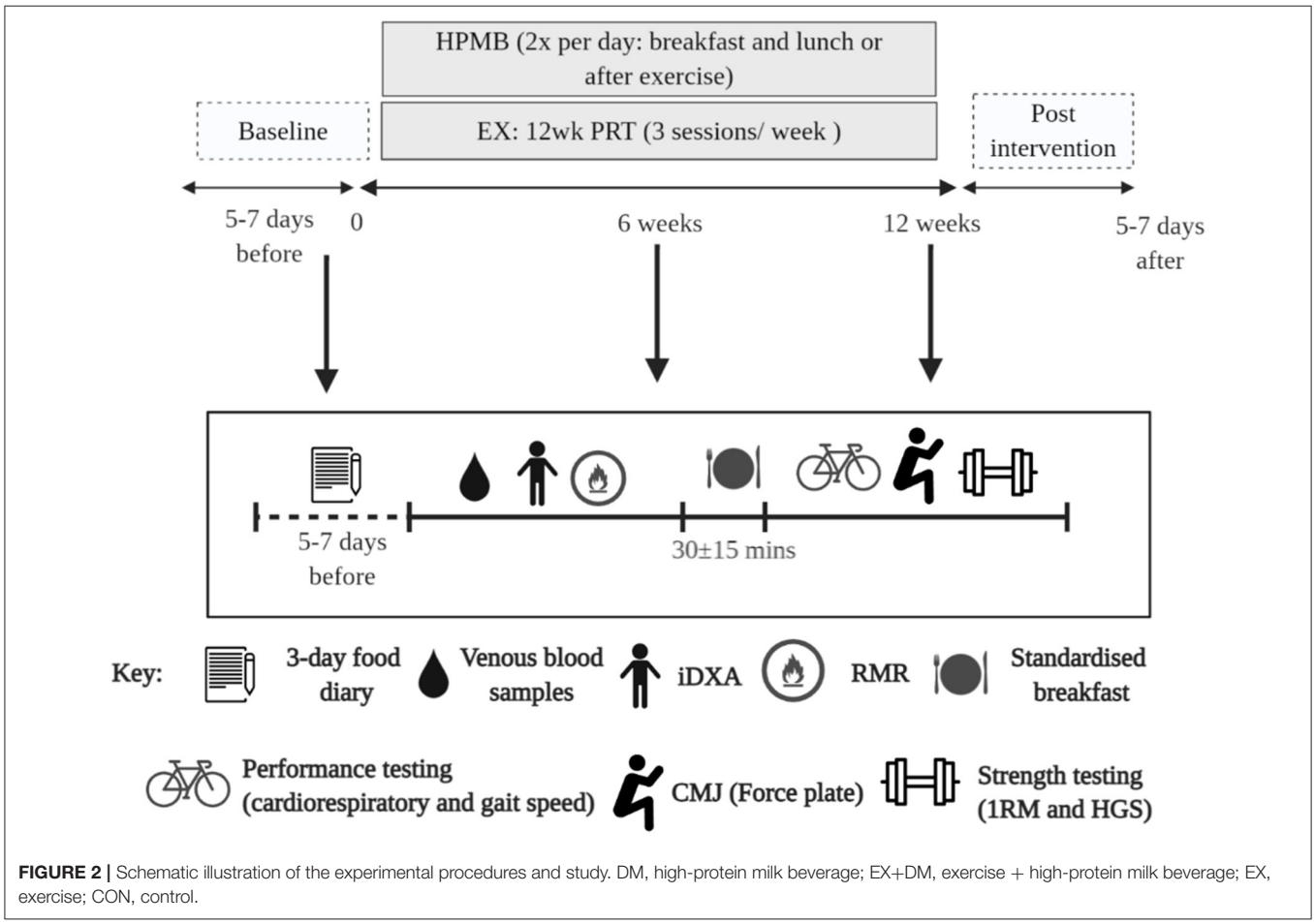

FIGURE 2 | Schematic illustration of the experimental procedures and study. DM, high-protein milk beverage; EX+DM, exercise + high-protein milk beverage; EX, exercise; CON, control.

consisted of full-body resistance training, which included leg press, *latissimus dorsi* (*lat*) pull-down, and chest press (Hammer Strength, LifeFitness, Sydney, Australia). Additional exercises including biceps curls, triceps extensions, shoulder raises, calf raises, cable deadlifts, leg curls, back rows, and abdominal exercises were used on a cable machine (Infinity Series Functional Trainer, Keiser, Fresno, CA) and rotated throughout the program to ensure the development of muscle balance. During the first 2 weeks of the PRT program, the participants completed three sets of 10–15 repetitions of 50–60% of their one-repetition maximum (1RM) with 2-min rest intervals. For the following 4 weeks, the training volume was set at three sets of 8–12 repetitions at an intensity of 69–75% of 1RM, which increased to 80–95% for six to eight repetitions for the remaining 6 weeks. Each week, weight progressively increased by 5–10%. For all exercises, the participants were instructed to perform each repetition in a slow, controlled manner, with a rest of 2 min between sets. Testing for participants' 1RM occurred at baseline and week 6; weights were adjusted according to their new 1RM to account for strength gains throughout the protocol. Participants in the exercise groups (i.e., EX+DM and EX) had 2 ± 5 days between their last exercise session and their mid- and post-assessment to minimize a carryover effect. Exercise compliance was determined by the number of sessions attended. All participants in the exercise groups (i.e., EX+DM and EX) were instructed to continue their normal physical activity outside the PRT program.

Activity Tracking

All participants were asked to resume their usual lifestyle activity levels and were required to wear an activity monitor (ActiGraph wGT3X-BT, ActiGraph, Pensacola, FL) on their non-dominant wrist. Participants were instructed to wear the activity monitor during the course of the intervention trial from waking to bedtime and to take the monitor off only when engaged in aquatic activities. A new activity monitor was provided every 2 ± 1 weeks due to the limited (25 days) battery life. Data were uploaded to analytical software (ActiLife 6 v.6.1.3, ActiGraph, Pensacola, FL). A valid day was defined as ≥80% wear time.

Anthropometry and Body Composition

The participants arrived at the laboratory between 7:00 a.m. and 9:00 a.m. in a fasted state [plasma osmolality = 296 ± 5.6 mOsmol/kg (Osmomat 030, Gonotec, Berlin, Germany) and total body water = 53.3 ± 6.4% (Seca 515 MBCA, Seca Group, Hamburg, Germany)]. All participants were required to avoid strenuous exercise for a 24-h period prior to all laboratory assessments. Height was assessed using a fixed stadiometer (Holtain, Crosswell, Crymych, UK). BM was measured (Seca 515 MBCA) to the nearest 0.1 kg using standardized anthropometrical procedures. Total (in kilograms) and relative (in percent) fat mass (FM) and FFM and bone mineral content (BMC) were assessed by a trained radiographer using a dual-energy X-ray absorptiometry (iDXA; Prodigy, GE Lunar, Madison, WI, with analysis software 14.10). Appendicular lean mass (ALM) was determined by adding the total arm and leg mass, and then it was adjusted for height (ALM/ht^2).

Submaximal Incremental Bike Test

To track changes in cardiorespiratory fitness along the experimental timeline, submaximal aerobic fitness was determined using an incremental bike test using a cycle ergometer (Corival, Lode, Groningen, Netherlands) and a metabolic cart (Vmax Encore Metabolic Cart, Carefusion, San Diego, CA). Procedures were adjusted from standard fitness testing protocols (39). The initial workload began at 1 W per kilogram of FFM (W/kg FFM) and increased by 0.5 W/kg FFM every 3 min until the participants could not maintain the speed at ≥60 rpm, they reached a rating of perceived exertion (RPE) of 15–17, and/or obtained a respiratory exchange ratio (RER) of 1.000 (40). Heart rate (HR) (Polar Electro, Kempele, Finland), RPE, VO_2, and RER were measured every 3 min in real time. Cardiorespiratory fitness was expressed as the VO_2 in milliliters per kilogram per minute at which the RER reached 1.000.

Countermovement Jump

A force plate (400s+ Performance Force Plate, Fitness Technology, Adelaide, Australia) was used to measure relative muscle power (in watts per kilogram), jump height (in centimeters), and velocity (in meters per second) during a countermovement jump (CMJ) test. The participants were asked to start in a full erect standing position in the middle of the force plate and then instructed to dip to a self-selected depth and to perform a static squat jump. Hands were kept on the hips to minimize any influence of arm swing (41). The participants were asked to perform three attempts of a CMJ with a 1-min rest in between jumps. The force plate was interfaced with computer software (Ballistic Measurement System; Fitness Technology, Adelaide, Australia); the best of the three jumps was selected for further analysis.

Gait Speed Measurement

To assess gait speed, a walking course of 4 m length was marked on the floor. The participant was instructed to walk from one end of the course to the other at their usual walking pace. The timer began as the participant started walking and the timer stopped with the first footfall after the 4-m line. The test was repeated twice and the fastest time of the two scores was recorded (1).

Skeletal Muscle Strength Outcomes

Strength was assessed by performing a 1RM according to previously described protocols (42). During a familiarization trial, proper lifting technique was demonstrated, and then participants were familiarized with each resistance machine (Hammer Strength, LifeFitness, Sydney, Australia) by performing 8–10 repetitions of a light load (∼50% of predicted 1RM). After the successful completion of a further five to six repetitions at a heavier weight selected by the instructor, the workload was increased incrementally until only one repetition with the correct technique could be completed. Participants were given 3–5 min rest in between attempts (43). The value of 1RM was the highest load that could be raised in one single repetition using the correct technique. Leg press, *lat* pull-down, and bench press exercises were measured. The 1RMs were normalized by body weight (1RM/BM). Hand grip strength (HGS) was measured

using a digital hand dynamometer (Jamar® Plus+ Digital Hand Dynamometer, Sammons Preston, Bolingbrook, IL). HGS was measured in a standing position with the participants elbow by their side and flexed to 90° and a neutral wrist position. The participants were asked to apply the maximum grip strength by squeezing the dynamometer with as much force as possible using their dominant hand. This was repeated three times with a 1-min rest in between attempts. HGS was defined as the highest value for their dominant hand (44).

Blood Collection and Analysis

Blood glucose concentration, hemoglobin, and the total and differential leukocyte counts (i.e., neutrophils, lymphocytes, and monocytes) were determined by the HemoCue system (Glucose 201+, Hb201, and WBC DIFF, respectively; HemoCue AB, Ängelholm, Sweden) in duplicate from heparin whole blood samples. The coefficients of variation (CVs) for blood glucose concentration, hemoglobin, and total leukocyte counts were 3.0, 1.5, and 4.6%, respectively. Hematocrit was determined using the capillary method in triplicate (CV = 1.1%) from heparin whole blood samples and using a microhematocrit reader (ThermoFisher Scientific). Hemoglobin and hematocrit values were used to estimate changes in plasma volume relative to baseline and used to correct plasma variables. The remaining heparin whole blood samples were centrifuged at 4,000 rpm (1,500 × g) for 10 min within 15 min of sample collection. Aliquots of heparin plasma were placed in 1.5-ml microstorage tubes and frozen at −80°C until analysis, except 2 × µl plasma was used to determine plasma osmolality in duplicate (CV = 1.1%).

Circulating concentrations of cortisol (DiaMetra, Perugia, Italy), insulin (Crux Biolab, Scoresby, Australia), IGF-1 (Crux Biolab, Scoresby, Australia), testosterone (17b-OH-4-androstene-3-one; DiaMetra, Perugia, Italy), and estradiol (17β-estradiol; DiaMetra, Perugia, Italy) were measured by enzyme-linked immunosorbent assay (ELISA). The plasma concentrations of TNF-α, IL-6, IL-8, IL-2, and IL-10 were determined by high-sensitivity multiplex ELISA (HCYTOMAG-28SK, EMD Millipore, Darmstadt, Germany). All assays were performed as per the manufacturer's specifications, with standards and controls on each plate. The CV for the analyzed circulating biomarkers was ≤7.2% and for the systemic inflammatory cytokine profile was ≤13.5%. Systemic cytokine profile was established, as previously described (45).

Statistical Analysis

Only participants that attended ≥80% of the PRT sessions and consumed ≥80% of the DM beverage over the 12-week intervention were included in the data analysis. Based on the statistical test, mean, standard deviation, and effect size (i.e., small = 0.20, medium = 0.50, and large = 0.80) for outcomes of FFM, skeletal muscle strength, and physical performance and applying standard alpha (0.05) and beta (0.80) values, a sample size of $n = 36$ ($n = 8$ per group), using a randomized controlled design as reported in Hanach et al. (9), is estimated to provide adequate statistical power (0.80–0.99) to detect variable differences (G*Power 3.1, Kiel, Germany). Data in the text and tables are presented as either mean ± SD or mean and 95% confidence interval (CI), as indicated. For clarity, data in figures are presented as mean ± standard error of the mean (SEM). Only participants who completed the experimental design, adhered to the controlled intervention conditions, and with full datasets within each specific variable were included in the data analysis, as indicated in the table and figure legends. All data were checked for distribution using the Shapiro–Wilk test of normality. Variables with singular data points were examined using a one-way ANOVA or non-parametric Kruskal–Wallis test, when appropriate. Variables with multiple data points were examined using a two-way repeated-measures ANOVA with a matrix including group (DM, EX+DM, EX, and CON) and time [baseline (week 0), week 6, and week 12]. Assumptions of homogeneity and sphericity were checked, and when appropriate, adjustments to the degrees of freedom were made using the Greenhouse–Geisser correction method. Significant main effects were analyzed using a *post-hoc* Tukey's HSD test. Statistics were analyzed using SPSS statistical software (v.25.0, Chicago, IL) with significance accepted at $P \leq 0.05$.

Furthermore, correlations between changes in the primary variables (e.g., FFM, skeletal muscle strength, power, and physical performance) and the inflammatory and hormone markers were conducted at 6 and 12 weeks. This was carried out with a Pearson's or Spearman's correlation test, based on the data distribution. Significance was accepted at $P \leq 0.05$. Additionally, Cohen's d was applied to determine the magnitude of effect size for significant differences, with $d \geq 0.20$ for small, $d \geq 0.50$ for medium and $d \geq 0.80$ for large effect size.

RESULTS

Baseline Characteristics

The participants from this study came from a variety of sporting backgrounds, including endurance runners/race walkers (61%), cyclists (9%), aerobic gym goers (16%), or a combination of multiple activities (14%). The dropout rate for the current study was 20% between all four groups, with the dropout reasoning depicted in **Figure 1**. At the end of the study, the groups were composed as follows: DM, $n = 8$; EX+DM, $n = 9$; EX, $n = 10$; and CON, $n = 10$. Participants' baseline variables, based on group allocation, are summarized in **Table 1**. There were no significant differences in the baseline characteristic variables between groups.

Progressive Resistance Training and Food Provisions Compliance

In EX+DM and EX, the PRT was well-tolerated, with the average compliance for participants in the exercise program being 89% (95% CI = 85–93%), and did not differ between groups ($P = 0.538$). In DM and EX+DM, the average compliance for the high-protein dairy milk beverage provisions, according to the food diaries and milk bottle returns, was 93% (88–97%) and did not differ between groups ($P = 0.969$). Average adherence to the standardized meal plan and food provisions, based on food diaries, was 81% (76–85%) and did not differ between groups ($P = 0.821$).

TABLE 1 | Baseline characteristics of the participants according to randomized group selection.

	DM ($n = 8$)	EX+DM ($n = 9$)	EX ($n = 10$)	CON ($n = 10$)	*P* value
Males, *n*	7	6	8	7	
Females, *n*	1	3	2	3	
Age (years)	59.7 (52.9–67.0)	63.6 (57.4–70.0)	58.0 (53.0–67.0)	56.1 (51.5–60.6)	0.147
Height (m)	1.7 (1.6–1.8)	1.7 (1.6–1.7)	1.7 (1.7–1.8)	1.6 (1.6–1.7)	0.105
BM (kg)	78.3 (65.6–91.0)	71.6 (63.0–80.1)	70.0 (64.4–89.5)	67.8 (63.2–72.4)	0.333
BMI (kg/m^2)	24.6 (22.2–27.0)	24.9 (22.0–27.8)	25.3 (22.5–28.1)	24.1 (23.0–25.3)	0.876
Self-reported structured exercise (min/week)	233 (125–340)	189 (144–264)	273 (210–336)	215 (137–293)	0.378

Values shown are the mean (95% CI).
BM, body mass; BMI, body mass index; EX+DM, exercise and high-protein milk beverage; DM, high-protein milk beverage; EX, exercise; CON, control.

Dietary Intake

A group*time interaction ($P = 0.048$) was observed for energy intake, indicating a significant increase in the DM (19%) and EX+DM (22%) groups at 12 weeks compared to baseline (**Table 2**). Consumption of the high-protein dairy milk led to a significant increase in absolute (in grams per day; $P = 0.001$) and relative protein intake (in grams per kilogram BM per day; $P < 0.001$) in the DM and EX+DM groups at 6 weeks (38 and 35%, respectively) and 12 weeks (44 and 45%, respectively). Similarly, there was a group*time interaction for calcium intake ($P = 0.007$). Further analysis indicated that DM and EX+DM had a significant increase in calcium intake at 6 weeks (88 and 99%, respectively) and 12 weeks (120 and 112%, respectively) compared to baseline (**Table 2**). Based on the protein intake relative to BM, a group*time interaction effect was observed for protein (in grams per kilogram BM) at breakfast ($P = 0.005$) and dinner ($P = 0.012$) and toward significance at lunch ($P = 0.055$). Further analysis indicated that, compared to baseline, protein at breakfast significantly increased at 6 and 12 weeks in DM ($\geq$44%) and EX+DM ($\geq$55%) compared to EX and CON. Whereas, there was a significant decrease of relative protein intake (in grams per kilogram) at dinner in the EX+DM (50%) and EX (10%) groups at 6 and 12 weeks compared to baseline.

Physical Activity

At baseline, the reported physical activity did not differ between groups (**Table 1**). The average compliance based on the wear time for the ActiGraph was 93% (90–95%) and did not differ between groups ($P = 0.754$). Based on the analysis of the accelerometer over the intervention period, there were no differences between groups for the amount of hourly kilocalories, time in sedentary, or time in light physical activity (**Table 3**).

Body Composition

A significant group*time interaction was observed for BM ($P = 0.029$), absolute FFM ($P = 0.051$), and absolute and relative FM ($P = 0.013$ and $P = 0.043$, respectively; **Table 4** and **Figure 3**). BM decreased in DM at week 6 (−2.2 kg) and week 12 (−2.7 kg), which significantly reduced more than all the other groups at both time points. Absolute FFM significantly increased in EX+DM at both time points (weeks 6 and 12) and in EX at week 12. This increase was significantly greater than that in DM, which showed a significant decline in FFM at week 6 (−0.26%) and week 12 (−0.96%). Absolute FM significantly decreased over time at week 6 in DM and at 12 weeks in the DM, EX+DM, and EX groups. DM had the greatest loss in absolute FM at 6 weeks (−1.4 ± 1.2 kg) and 12 weeks (−2.1 ± 2.6 kg). At 12 weeks, there was a significant decline in relative FM of $\geq$1.0% in the DM, EX+DM, and EX groups compared to baseline. No significant main effects or interactions were observed for regional body composition, ALM/ht^2, bone mineral density (BMD), or resting metabolic rate (**Table 4**).

Skeletal Muscle Strength

There was a significant group*time interaction for absolute and relative maximal 1RM leg press ($P < 0.001$ and $P = 0.006$), chest press ($P < 0.001$ and $P < 0.001$), and *lat* pull-down ($P = 0.007$ and $P < 0.001$, respectively; **Figure 4**). A significant change in absolute maximal 1RM strength was observed in the EX+DM and EX groups at 6 and 12 weeks from baseline (**Figure 4**). The significant change in relative strength was observed in the EX+DM (range = 53–78%) and EX (35–36%) groups from baseline to 12 weeks (**Figure 5**). The change in relative 1RM strength was greater in EX+DM compared to all other groups at 12 weeks. There were no significant main effects or interactions for HGS ($P = 0.561$).

Skeletal Muscle Power and Physical Performance

For outcomes of muscle power (i.e., CMJ), cardiorespiratory fitness (i.e., submaximal VO_2), and physical performance (i.e., gait speed), there were no significant main effects or interactions from baseline to week 12 (**Table 5**).

Systemic Hormonal and Inflammatory Cytokine Profiles

There were no main effects or interactions observed for any of the hormonal biomarkers measured (**Table 6**). There was a group*time interaction for IL-10 ($P = 0.016$; **Table 7**), associated with the increase observed for the EX+DM group at 6 weeks (88%) and 12 weeks (46%). This increase was significantly higher than in all the other groups ($P < 0.01$). There were no main effects or interactions observed for any other immune biomarkers measured. There was no significant correlation

TABLE 2 | Baseline values and the mean within-group changes at weeks 6 and 12 for total dietary energy and macronutrient intake and relative protein intake based on each meal according to randomized allocation.

	DM ($n = 8$)	EX+DM ($n = 9$)	EX ($n = 10$)	CON ($n = 10$)
TOTAL ENERGY AND MACRONUTRIENT INTAKE				
Energy intake (MJ/day)				
Baseline	8.6 (6.7–10.6)	8.1 (6.0–9.3)	9.1 (7.0–10.0)	9.6 (9.0–10.5)
6 weeks	10.0 (7.6–14.0)	9.4 (6.0–13.7)**	8.6 (5.7–11.4)^{aa}	9.6 (4.3–13.0)
12 weeks	11.0 (8.0–14.0)**	9.5 (7.5–12.4)**^{aa}	8.9 (4.3–11.8)^{aa}	9.0 (6.1–12.0)^{aa}
Total protein intake (g/day)				
Baseline	101 (81.7–120)	86.4 (67.6–105)	95.4 (77.1–113)	108 (91.0–127)
6 weeks	125 (105–148)**	115 (72.0–161)**	94.0 (54.3–119)^{ab}	112 (52.2–159)^{ab}
12 weeks	127 (118–152)**	123 (94.0–153)**	106 (57.0–145)^{ab}	98 (62.0–145)^{ab}
Relative protein (g kg^{-1} BM day^{-1})				
Baseline	1.3 (1.0–1.7)	1.2 (1.0–1.4)	1.4 (1.0–1.7)	1.6 (1.2–2.0)
6 weeks	1.7 (1.2–0.2.9)**	1.6 (1.0–2.2)**^{aa}	1.2 (0.80–1.9)^{aa}	1.6 (0.9–2.5)^{aacc}
12 weeks	1.8 (1.4–3.2)**	1.8 (1.2–1.8)**^{aa}	1.4 (0.70–1.8)^{aa}	1.4 (0.9–1.4)^{aacc}
Protein (%)				
Baseline	27.0 (19.4–34.5)	29.1 (20.6–37.5)	23.0 (17.0–27.0)	32.2 (26.8–37.6)
6 weeks	35.5 (26.0–57.0)**	34.0 (23.0–46.0)**	21.5 (10.3–31.0)*^{ab}	32.3 (17.0–48.3)^{ac}
12 weeks	37.0 (28.4–37.0)**	38.6 (31.0–39.0)**	24.4 (14.0–38.0)*^{ab}	29.3 (17.5–29.3)^{abc}
Carbohydrates (g/day)				
Baseline	206 (128–308)	192 (80–297)	229 (108–331)	202 (155–249)
6 weeks	304 (187–305)**	106 (68–144)**	237 (187–363)^{ab}	208 (68–305)^{ab}
12 weeks	218 (93–330)**	100 (48–151)**	267 (171–384)^{ab}	210 (68–348)^{ab}
Fat (g/day)				
Baseline	81.6 (66.0–97.0)	70.5 (53.5–87.7)	78.6 (67.0–90.5)	92.0 (81.5–102)
6 weeks	64.0 (33.0–83.0)	16.3 (−100 to 132)	70.0 (24.0–98.0)	66.0 (44.0–122)
12 weeks	64.0 (35.0–100)	33.6 (−90.0 to 159)	75.0 (29.3–74.5)	87.4 (51.2–118)
Calcium (mg/day)				
Baseline	1,011 (675–1,347)	749 (394–1,104)	1,117 (807–1,426)	1,022 (789–1,256)
6 weeks	1,911 (1,467–2,458)**	1,629 (941–2726)**	1,006 (547–1613)^{aabb}	1,262 (582–2,074)^{aabb}
12 weeks	2,037 (1,449–2,849)**	1,695 (1,245–2,704)**	1,135 (605–1,592)^{aabb}	1,134 (462–2,151)^{aabb}
RELATIVE PROTEIN INTAKE BASED ON EACH MEAL				
Protein at breakfast (g/kg BM)				
Baseline	0.25 (0.10–0.40)	0.21 (0.12–0.30)	0.30 (0.15–0.40)	0.30 (0.21–0.36)
6 weeks	0.36 (0.20–0.60)**	0.33 (0.30–0.40)*	0.22 (0.0–0.50)	0.28 (0.10–0.50)
12 weeks	0.34 (0.10–0.60)**	0.36 (0.20–0.50)*	0.25 (0.10–0.40)*	0.31 (0.10–0.50)
Protein at lunch (g/kg BM)				
Baseline	0.40 (0.21–0.60)	0.31 (0.18–0.43)	0.40 (0.25–0.60)	0.37 (0.22–0.51)
6 weeks	0.50 (0.30–0.70)*	0.50 (0.40–0.60)$^{\#}$	0.34 (0.10–0.50)	0.45 (20–0.70)
12 weeks	0.50 (0.30–0.70)*	0.44 (0.30–0.50)**	0.31 (0.10–0.0.70)^{b}	0.40 (0.10–0.60)^{abc}
Protein at dinner (g/kg BM)				
Baseline	0.50 (0.31–0.65)	0.64 (0.50–0.80)	0.74 (0.40–1.1)	0.64 (0.50–0.80)
6 weeks	0.40 (0.20–0.50)	0.35 (0.20–0.50)*	0.70 (0.30–1.0)*^{b}	0.70 (0.20–1.2)^{ab}
12 weeks	0.40 (0.30–0.41)	0.35 (0.20–0.50)*	0.60 (0.40–1.0)^{b}	0.60 (0.30–1.2)^{b}

Values shown are the mean (95% CI).

BM, body mass; CON, control; EX, exercise; EX+DM, exercise and high-protein dairy milk beverage; DM, high-protein dairy milk beverage.

*Within-group changes: **$P < 0.01$ and *$P < 0.05$ vs. baseline; Between-group changes: $^{aa}P < 0.01$ and $^{a}P < 0.05$ vs. DM; $^{bb}P < 0.01$ and $^{b}P < 0.05$ vs. EX+DM; $^{cc}P < 0.01$ and $^{c}P < 0.05$ vs. EX.*

between the changes in any of the primary outcomes (e.g., FFM, skeletal muscle strength, power, and physical performance) and any of the systemic hormonal and inflammatory cytokine markers.

DISCUSSION

This study aimed to determine the independent and combined effects of a high-protein dairy milk beverage provided at

TABLE 3 | Average daily physical activity measured by an accelerometer over the 12-week experimental procedure.

	DM ($n = 8$)	EX+DM ($n = 9$)	EX ($n = 10$)	CON ($n = 10$)
Sedentary time (min/day)				
0–6 weeks	923 (844–978)	863 (672–1,035)	782 (351–933)	844 (697–941)
6–12 weeks	904 (848–978)	896 (706–1,064)	785 (152–1,017)	877 (785–985)
Time in light physical activity (min/day)				
0–6 weeks	281 (193–366)	340 (158–842)	368 (245–883)	280 (230–363)
6–12 weeks	282 (214–374)	352 (136–938)	359 (215–900)	275 (208–342)
Time in moderate physical activity (min/day)				
0–6 weeks	171 (130–218)	206 (130–315)[a]	219 (153–280)[a]	216 (174–323)[a]
6–12 weeks	170 (131–213)	210 (115–317)[a]	217 (128–310)[a]	214 (158–277)[a]
Time in vigorous physical activity (min/day)				
0–6 weeks	11.1 (0.0–65.2)	19.0 (0.0–58.0)	13.3 (0.0–43.8)	24.5 (0.50–55.4)
6–12 weeks	13.8 (0.0–60.0)	18.0 (0.0–47.6)	22.1 (0.0–45.3)	32.2 (0.00–57.3)
Total steps, (*n*/day)				
0–6 weeks	24,582 (9,793–11,112)	13,360 (9,723–18,505)	14,670 (11,734–17,000)	14,126 (11,787–19,928)
6–12 weeks	12,323 (10,000–15,145)	13,811 (8,742–20,175)	14,516 (10,700–17,510)	14,152 (11,220–15,686)

Values shown are the mean (95% CI).
DM, high-protein dairy milk beverage; EX, exercise; EX+DM, exercise and high-protein dairy milk beverage; CON, control.
Between-group changes: [a]$P < 0.05$ vs. DM.

TABLE 4 | Baseline values and within-group changes at weeks 6 and 12 for total body and regional composition, bone mineral density, and resting metabolic rate according to randomized allocation.

	DM ($n = 8$)	EX+DM ($n = 9$)	EX ($n = 10$)	CON ($n = 10$)
Total body FFM (%)				
Baseline	73.0 (67.4–79.4)	69.9 (60.1–79.1)	75.7 (40.2–80.5)	76.0 (69.1–82.2)
6 weeks	75.7 (62.0–84.2)	70.1 (54.0–87.3)	75.7 (61.2–89.3)	76.5 (62.7–89.5)
12 weeks	75.5 (60.4–87.0)	70.5 (53.6–86.4)	76.6 (62.4–89.4)	76.4 (63.6–91.7)
Total body FM (%)				
Baseline	26.6 (20.3–32.9)	31.5 (21.6–41.4)	25.5 (19.9–31.2)	24.6 (18.1–31.1)
6 weeks	25.2 (16.0–38.0)**	31.5 (17.3–48.0)	25.1 (11.7–39.4)	24.4 (10.3–37.7)
12 weeks	25.0 (12.9–40.1)**	31.0 (14.0–47.0)**[a]	24.5 (11.3–37.9)[a]	24.7 (9.8–37.2)
Arm LM (kg)				
Baseline	5.1 (3.0–7.0)	5.1 (3.0–6.9)	6.3 (4.0–8.6)	5.6 (3.3–8.4)
6 weeks	6.0 (3.2–7.5)	5.1 (3.0–7.0)	6.3 (4.0–9.0)	5.6 (3.3–8.4)
12 weeks	6.0 (3.1–8.0)*	5.2 (3.0–7.2)	6.5 (3.8–9.2)	5.7 (3.3–8.4)
Arm FM (kg)				
Baseline	2.0 (1.6–3.7)	2.3 (1.1–4.3)	2.0 (0.8–4.0)	1.8 (1.3–2.8)
6 weeks	2.0 (1.4–3.5)	2.3 (0.8–4.1)	2.1 (0.8–3.7)	1.8 (0.7–2.8)
12 weeks	2.0 (1.1–3.4)	2.4 (1.0–4.3)	2.1 (1.0–4.0)	1.9 (0.9–3.0)
Leg LM (kg)				
Baseline	18.8 (12.0–23.0)	16.1 (11.0–20.4)	18.5 (14.1–24.3)	17.0 (11.8–23.2)
6 weeks	18.6 (12.2–23.0)	16.1 (11.3–21.0)	18.6 (14.2–25.0)	17.1 (12.0–24.4)
12 weeks	18.4 (12.1–22.3)	16.1 (11.0–20.5)	18.5 (14.5–24.6)	17.0 (12.0–23.1)
Leg FM (kg)				
Baseline	5.6 (3.6–8.0)	5.6 (3.1–10.0)	5.5 (2.4–13.0)	6.3 (2.5–14.0)
6 weeks	5.5 (4.2–7.5)	5.5 (3.0–10.1)	5.6 (2.2–13.0)	6.3 (2.3–14.0)
12 weeks	5.1 (3.4–7.3)	5.5 (3.0–10.4)	5.3 (2.0–13.0)	6.3 (2.4–14.0)
Trunk LM (kg)				
Baseline	26.0 (17.6–29.6)	22.6 (14.7–28.1)	26.0 (20.5–35.0)	25.1 (16.5–35.0)
6 weeks	26.0 (17.6–30.0)	23.0 (15.1–28.0)	26.0 (21.0–34.5)	25.1 (18.4–34.2)
12 weeks	26.0 (17.6–30.0)	23.0 (15.0–28.0)	26.2 (20.1–35.0)	24.2 (12.0–34.0)
Trunk FM (kg)				
Baseline	11.6 (4.7–21.5)	13.0 (3.5–23.6)	11.0 (2.2–20.0)	7.8 (2.7–11.0)
6 weeks	10.5 (2.3–22.0)	12.2 (2.4–23.5)	10.54 (2.0–19.6)	7.7 (2.7–11.0)
12 weeks	10.2 (3.0–21.5)	12.0 (2.2–23.0)	10.6 (1.9–20.6)	7.8 (2.2–11.0)
ALM/ht^2				
Baseline	7.7 (6.5–9.0)	7.3 (5.1–9.1)	8.3 (5.8–11.2)	7.8 (5.6–9.4)
6 weeks	7.7 (6.6–8.6)	7.4 (5.2–9.0)	8.3 (5.8–11.2)	7.8 (5.7–9.8)
12 weeks	7.6 (6.5–8.4)	7.4 (5.2–9.0)	8.3 (5.8–11.0)	7.8 (5.7–9.3)
BMD (g/cm)				
Baseline	1.2 (1.0–1.4)	1.1 (0.9–1.4)	1.3 (1.0–1.6)	1.2 (0.9–1.4)
6 weeks	1.2 (1.0–1.4)	1.1 (0.9–1.4)	1.3 (1.0–1.6)	1.2 (0.9–1.4)
12 weeks	1.2 (1.0–1.4)	1.2 (0.9–1.5)	1.3 (1.0–1.4)	1.1 (0.1–1.4)
RMR (MJ/day)				
Baseline	6.1 (4.2–7.6)	5.5 (4.5–7.3)	6.3 (4.3–8.4)	5.7 (4.7–7.4)
6 weeks	6.1 (4.2–7.6)	5.5 (4.2–6.7)	6.3 (4.3–8.0)	5.8 (4.7–7.4)
12 weeks	6.0 (4.0–7.8)	5.4 (4.3–6.7)	6.2 (4.3–8.0)	5.6 (4.4–7.2)

Values shown are the mean (95% CI).
DM, high-protein dairy milk beverage; EX, exercise; EX+DM, exercise and high-protein dairy milk beverage; CON, control; ALM/HT, appendicular muscle mass/height; BM, body mass; BMD, bone mineral density; FFM, fat-free mass; FM, fat mass; LM, lean mass; RMR, resting metabolic rate.
*Within-group changes: **$P < 0.01$ and *$P < 0.05$ vs. baseline. Between-group changes: [a]$P < 0.05$ vs. DM.*

breakfast and lunch (or after resistance exercise), with or without PRT, on outcomes of FFM, skeletal muscle strength and power, and physical performance in active older adults. In conflict with the hypotheses, a high-protein dairy milk beverage did not influence gains in FFM, skeletal muscle strength, power, or performance compared to the control. Whereas, in accordance with the hypotheses, a high-protein dairy milk beverage provided and consumed twice daily, in conjunction with PRT, resulted in significant increases in strength (i.e., 78% leg press, 56% chest press, and 53% *lat* pull-down) compared to PRT alone, but did not further augment changes in FFM, power, or physical performance. Moreover, the consumption of a high-protein dairy milk beverage during the PRT period resulted in significant increased levels of cytokine IL-10, suggesting an anti-inflammatory effect at this intervention, but it did not result in any anabolic hormone enhancements compared to other interventions or the control. Overall, these results suggest that the consumption of a high-protein dairy milk beverage, in combination with PRT, elicits greater effects on skeletal muscle strength outcomes than consuming the dairy milk beverage or PRT in isolation. This suggests that the DM does not seem to impact FFM, power, or physical performance any more than the CON in healthy active older adults. Therefore, high-protein dairy milk in combination with PRT may be an effective strategy in the

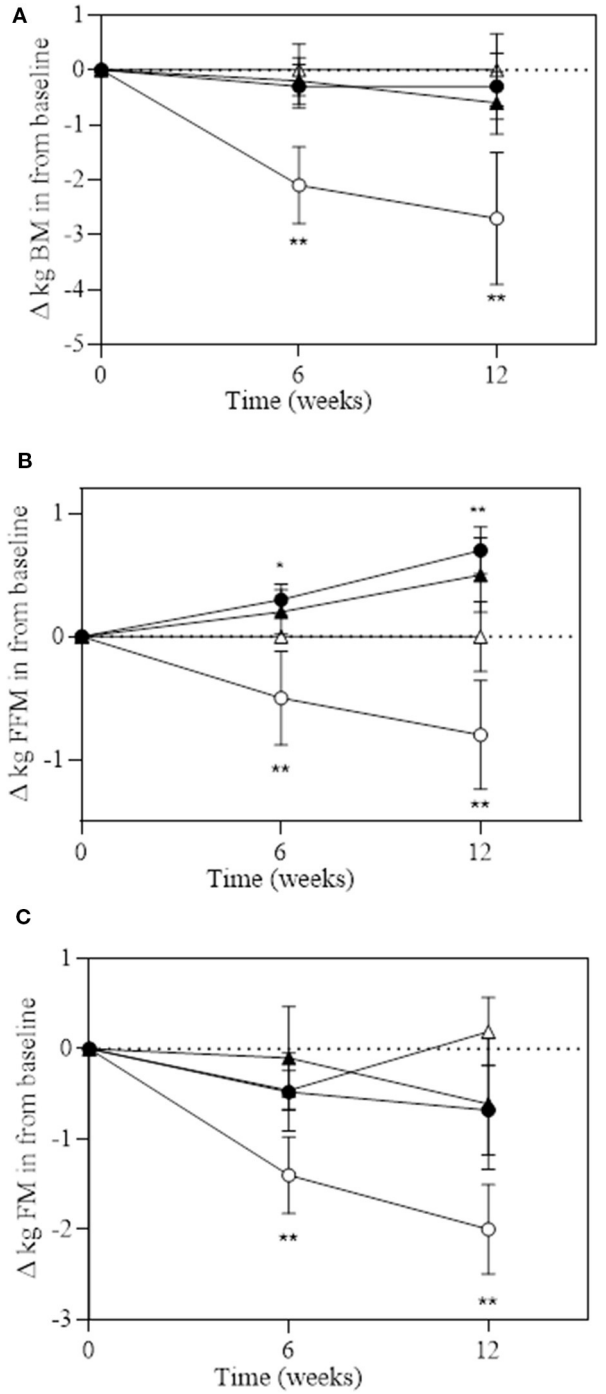

FIGURE 3 | Change over 6 and 12 weeks of intervention trial from baseline for **(A)** total body mass (in kilograms); **(B)** fat-free mass (in kilograms); and **(C)** fat mass (in kilograms) according to group: DM (high-protein milk beverage, *open circle*), EX+DM (exercise + high-protein milk beverage, *filled circle*), EX (exercise, *filled triangle*), and CON (control, *open triangle*). Mean ± SEM: $^{**}P < 0.01$ and $^{*}P < 0.05$ vs. baseline.

prevention and management of age-related sarcopenia in the active aging population.

The progressive decline in strength and FMM begins to be detectable from the age of ≥50 years (1). The rate of loss that occurs in skeletal muscle mass and strength is between 1–2% and 1.5–5.0% per year, respectively (46–48). Maintaining skeletal muscle strength is a key factor to maintaining functional capacity and independent living with increasing age (1). However, even in very physical active older adults (e.g., training four to five or more sessions per week), there have been observed declines in leg strength of 3–5% per year (48). In the current study, there was a significant increase in maximal 1RM lower and upper body strength observed in both groups that received PRT (EX+DM, ≥53%; EX, ≥35%). These findings align with previous studies that show maximal 1RM leg strength increases of >25% after 12 weeks of resistance training in older adults (49, 50). The improvement in maximal relative muscle strength as measured using 1RM (e.g., leg press, chest press, and *lat* pull-down) was significantly higher in EX+DM (53–78%) compared to EX (35–36%), DM (4–7%), and CON (7–11%), indicating an interaction effect. These findings align with a recent meta-analysis which reported that protein supplementation (20 ± 18 g protein/day) further augments strength (33%), as measured by 1RM leg press, in community-dwelling older adults (≥45 years) (10). However, these findings contradict previous exercise intervention studies that have not observed protein supplementation to further increase gains in maximal 1RM leg strength during resistance exercise training in healthy community-dwelling and active older adults compared to placebo or exercise-only groups (49, 50). One possible explanation for the positive finding from the current study, compared to the aforementioned studies, is the difference in the mean age (58 ± 7 years) compared to those in the previous studies (≥70 years). These age-related discrepancies may be due to the presence of anabolic resistance and the decreased work/power capacity that occurs with increasing age (51). Secondly, the amount of daily protein consumed in the supplement groups may have been inadequate to illicit a significant strength adaptation between groups. For example, cohorts receiving additional milk servings were consuming 1.3–1.4 g kg^{-1} BM day^{-1} of protein at baseline (49, 50). Although this is higher than the recommendations for older adults (≥1.2 g kg^{-1} BM day^{-1}) to treat sarcopenia, it is below (1.6 g kg^{-1} BM day^{-1}) the threshold recommended to support significant changes in muscle size and strength during prolonged resistance training in healthy active adults that are novice to weight training (52–55). Furthermore, in this current study, the addition of the high-protein dairy milk beverage increased the protein intake in the EX+DM and DM groups to 1.7 and 1.9 g kg^{-1} BM day^{-1}, respectively. While this is much higher than the reported amount needed for those that are novice to weight training (e.g., 1.3–1.8 g kg^{-1} BM day^{-1}), the lack of a further significant effect may be influenced by the CON and EX groups that were consuming high habitual protein intakes throughout the study intervention (e.g., ≥1.4 g kg^{-1} BM day^{-1}) (56). Although the EX group did habitually consume a higher amount than expected (1.4 g kg^{-1} BM day^{-1}), the EX+DM group still showed a significantly greater increase in maximal strength than the EX group. This may suggest that increasing protein intake by this magnitude with PRT can lead to additional adaptations in skeletal muscle strength, as previously reported in younger adults (52). The findings of this study may indicate that higher protein (e.g., ≥1.6 g kg^{-1} BM day^{-1}) intakes than those currently

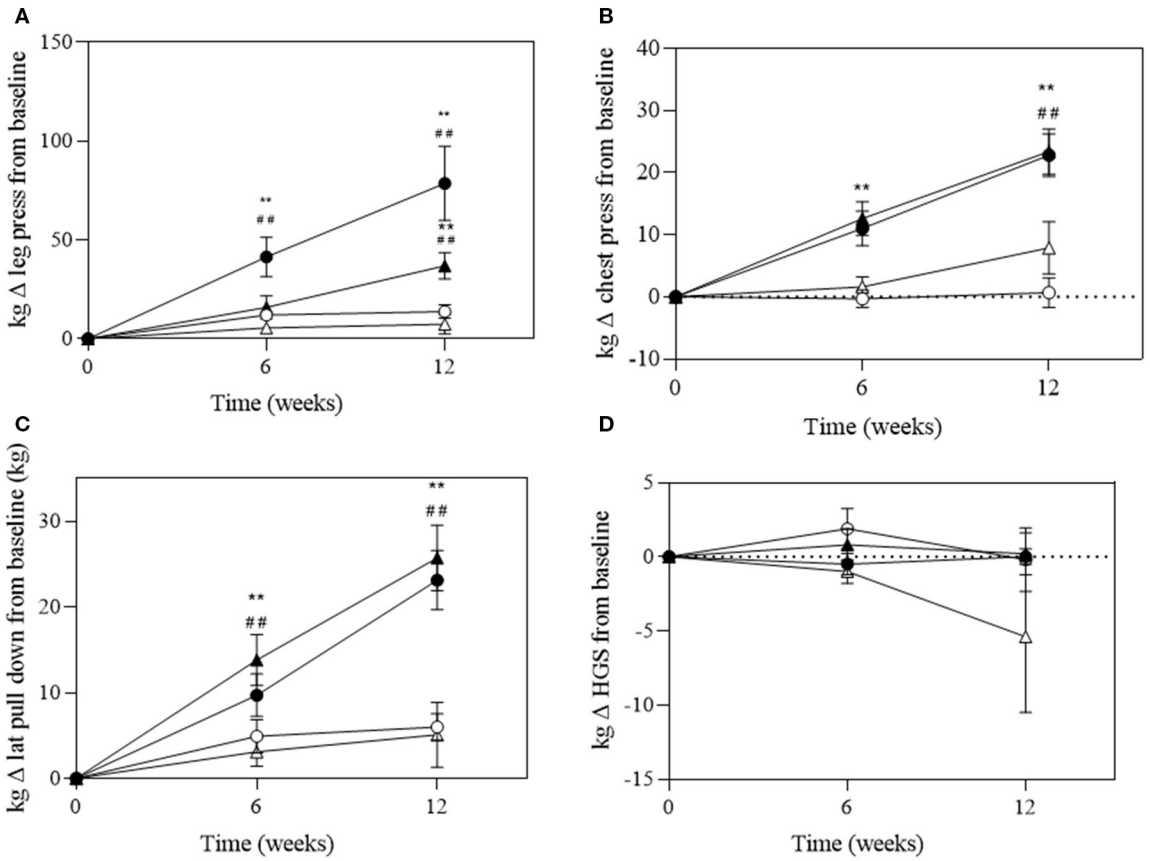

FIGURE 4 | Change of absolute strength (in kilograms) at 6 and 12 weeks of intervention trial from baseline for **(A)** lower body (leg press); **(B)** upper body (chest press); **(C)** back (*lat* pull-down) strength; and **(D)** handgrip strength (HGS) according to group: DM (high-protein milk beverage, *open circle*), EX+DM (exercise + high-protein milk beverage, *filled circle*), EX (exercise, *filled triangle*), and CON (control, *open triangle*). Mean ± SEM: $^{**}P < 0.01$ vs. baseline; $^{\#\#}P < 0.01$ vs. 6 weeks.

recommended for active older adults ($\geq$1.2 g kg^{-1} BM day^{-1}) may be required to see optimal strength adaptations. This requirement is in accordance with the nutrition guidelines for strength and power athletes for adaptations in skeletal muscle strength following resistance training (54, 55).

The current study provided participants with a 15-g protein (1.57 g leucine) dose at breakfast and lunch (or after resistance exercise). This significantly increased the relative protein intakes at those meal times ($\geq$25%) in DM and EX+DM compared to baseline. The provision of this protein dose at these time points was based on previous reports suggesting that the distribution of protein is often inadequate at those times in older adults (56). Additionally, cross-sectional reports have indicated that, in healthy active older adults that consume sufficient protein, if one meal reaches this proposed threshold, it may be sufficient to elicit favorable results in FFM, skeletal muscle strength and power, and physical performance (57). Considering the significant increases in FFM and strength observed in EX+DM compared to other studies that only provided protein supplementation post-training (49, 50), this may suggest that the distribution of protein may be more relevant in older adults already consuming adequate amounts of total daily protein (i.e., $\geq$1.2 g kg^{-1} BM day^{-1}). Resistance training acutely sensitizes skeletal muscle mass to anabolic effects of ingested protein (58). When considering a chronic response, PRT and nutritional supplementation have an additive effect on skeletal muscle strength (10, 57). Therefore, regular intakes of protein throughout the day increase the number of opportunities to maximally stimulate myofibrillar MPS. This accumulation of myofibrillar MPS stimulation throughout the day is likely to lead to long-term positive protein balance, which may facilitate adaptations in skeletal muscle mass and strength in active older adults. The findings of this current study may indicate that older adults who are already active and consuming adequate amounts of protein may need to consider the distribution of protein to gain further benefits from PRT.

There was a significant increase in absolute FFM in the EX+DM (1.2%) and EX (0.85%) groups at 12 weeks from baseline, whereas the DM group had a significant decrease in FFM (−1%) over the course of the intervention trial. The

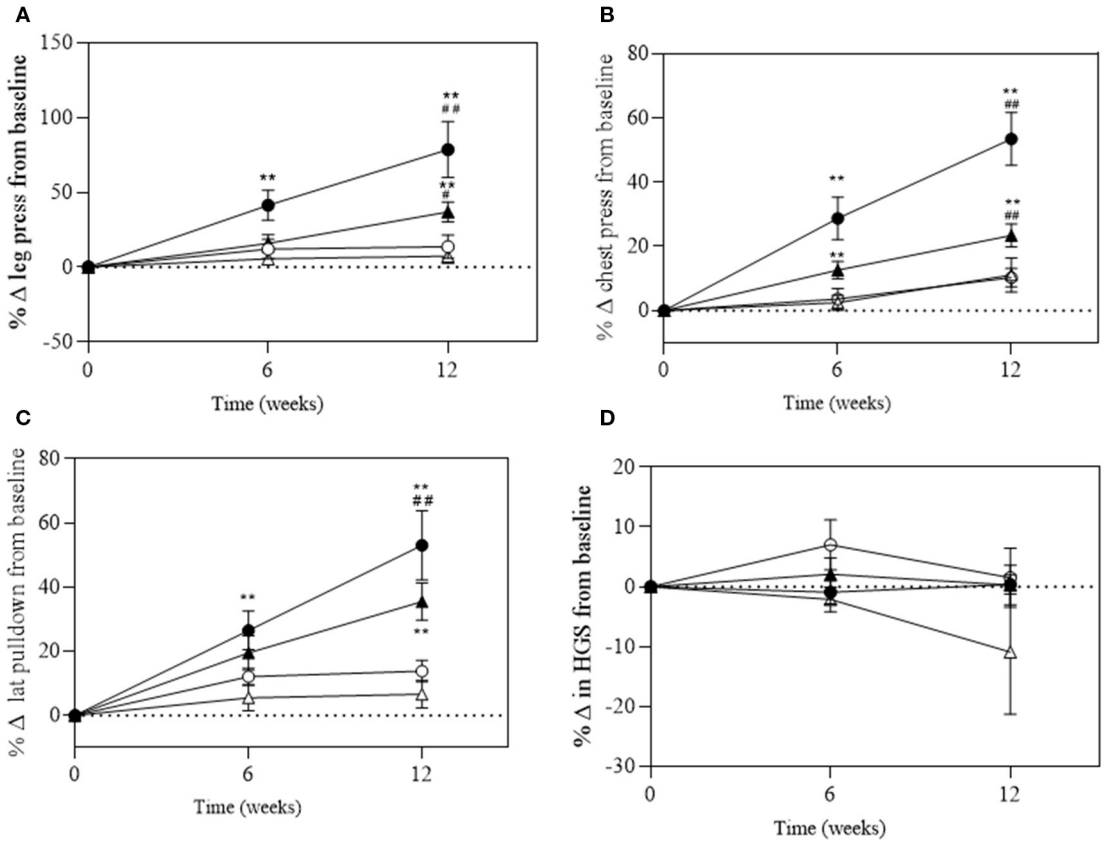

FIGURE 5 | Change of relative (in kilograms per body mass, BM) at 6 and 12 weeks of intervention trial from baseline for **(A)** lower body (leg press); **(B)** upper body (chest press); **(C)** back (*lat* pull-down) strength; and **(D)** handgrip strength (HGS) according to group: DM (high-protein milk beverage, *open circle*), EX+DM (exercise + high-protein milk beverage, *filled circle*), EX (exercise, *filled triangle*), and CON (control, *open triangle*). Mean ± SEM: $^{**}P < 0.01$ vs. baseline; $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ vs. 6 weeks.

absence of any greater increase in FFM from the consumption of additional protein in DM aligns and conflicts with previous findings in studies that investigated PRT and protein intake in healthy community dwellers (10, 25, 27, 51) and active older adults (8, 50). The discrepancies among these studies are likely due to the large heterogeneity within the study designs, such as the use of supplementation (e.g., plain dairy milk and protein-fortified dairy milk), methods of outcomes measured (e.g., iDXA, BIA, and MRI), and participant fitness status (e.g., community-dwelling and institutionalized). Furthermore, there is emerging evidence to suggest that whole foods such as dairy may exert a greater stimulatory effect on MPS than do isolated protein supplements. For example, a review by Burd et al. (59) compared between studies the MPS response to different protein sources and showed that skim milk was greater compared to whey protein or casein. Dairy milk also contains non-protein components that act directly as anabolic signaling molecules and can regulate nutrient activity due to the "food matrix effect" (60). However, more studies are needed to confirm this, especially in older active adults. Furthermore, while there were no significant interaction effects observed on outcomes of FFM, this present study confirms that a PRT can increase FFM by 0.8–1.2% in active older adults. Although not statistically significant, this has translational practice significance in the clinical setting, as the reported loss of skeletal muscle mass observed in older adults is 1–2% per year (45–47). This could indicate a saving of 1–2 years of skeletal muscle mass with a 12-week PRT intervention and therefore has great practical and clinical significance for potentially reducing age-related muscle loss in older adults.

Aging is associated with the redistribution of FM, characterized by an increase in the abdominal region (visceral fat) and a decrease in the appendicular (mostly subcutaneous fat) (61). This study observed the greatest loss in absolute FM in DM (−2.0 kg), followed by the EX+DM and EX groups (−0.68 and −0.61 kg, respectively). The greatest loss of FM was observed in the trunk region in both the DM and EX+DM groups (5–10%). While not statistically significant, these findings align with previous exercise and protein intervention trials (62, 63). One of the most cited plausible mechanisms proposed to contribute toward the decrease in FM is that dietary protein stimulates the release of satiety hormones, increases the thermic effect of food, and stimulates protein-induced alterations in gluconeogenesis (64). In the current study, the DM group increased their RMR

TABLE 5 | Baseline values and the mean within-group changes at weeks 6 and 12 in skeletal muscle power, cardiorespiratory fitness, and physical function outcomes according to randomized allocation.

	DM ($n = 8$)	EX+DM ($n = 9$)	EX ($n = 10$)	CON ($n = 10$)
CMJ (cm)				
Baseline	17.1 (8.4–28.0)	13.5 (3.0–21.0)	17.0 (10.3–24.2)	19.2 (10.3–26.0)
6 weeks	17.3 (7.5–22.3)	14.0 (3.3–22.1)	18.0 (12.0–24.0)	18.2 (10.3–26.0)
12 weeks	17.6 (6.7–24.0)	15.1 (2.3–29.0)	18.5 (13.3–24.0)	19.1 (11.0–25.2)
CMJ (W/kg BM)				
Baseline	28.9 (21.3–39.6)	25.4 (16.2–33.0)	31.7 (24.0–36.2)	29.7 (20.3–38.5)
6 weeks	28.5 (16.7–34.1)	25.4 (14.5–35.0)	31.0 (24.6–40.0)	30.2 (20.4–38.5)
12 weeks	29.0 (16.3–33.0)	28.2 (15.0–38.0)	31.4 (27.2–36.0)	30.5 (20.6–37.0)
Gait speed (m/s)				
Baseline	0.95 (0.80–1.20)	0.81 (0.60–1.10)	0.84 (0.70–1.00)	0.80 (0.70–0.90)
6 weeks	0.83 (0.70–1.00)	0.84 (0.70–1.10)	0.85 (0.60–1.00)	0.84 (0.70–1.00)
12 weeks	0.81 (0.60–0.90)	0.81 (0.60–0.90)	0.80 (0.60–1.00)	0.80 (0.70–0.90)
Submaximal VO_2 (ml/kg BM/min)				
Baseline	17.0 (11.6–22.3)	15.6 (9.7–21.5)	21.5 (17.0–26.0)	25.0 (17.0–33.0)
6 weeks	17.6 (7.0–28.3)	17.0 (5.0–28.6)	20.0 (13.0–27.0)	27.2 (15.6–39.0)
12 weeks	18.2 (8.5–27.6)	18.0 (5.7–30.0)	20.2 (12.0–28.6)	24.0 (11.0–37.1)

Values shown are the mean (95% CI).
DM, high-protein dairy milk beverage; EX, exercise; EX+DM, exercise and high-protein dairy milk beverage; CON, control; BM, body mass; CMJ, countermovement jump; FFM, fat-free mass; VO, maximal oxygen consumption.

TABLE 6 | Baseline values and the mean within-group changes at weeks 6 and 12 in biochemistry and hormonal markers according to randomized allocation.

	DM ($n = 8$)	EX+DM ($n = 9$)	EX ($n = 10$)	CON ($n = 10$)
Blood glucose (mmol/l)				
Baseline	5.0 (4.3–5.4)	5.0 (4.5–5.5)	5.1 (5.0–5.3)	4.7 (4.1–5.4)
6 weeks	4.5 (4.0–6.0)	4.1 (3.7–6.1)	4.6 (3.2–6.2)	4.7 (3.7–5.8)
12 weeks	4.5 (3.0–6.0)	4.8 (3.2–6.5)	5.1 (4.3–6.0)	4.7 (3.4–6.1)
Insulin (μIU/ml)				
Baseline	8.9 (4.0–14.0)	6.4 (4.4–8.3)	5.3 (3.6–7.0)	7.4 (5.0–10.0)
6 weeks	9.2 (1.0–18.0)	6.2 (2.7–9.6)	5.2 (2.0–8.5)	6.5 (2.0–11.2)
12 weeks	5.8 (0.6–24.0)	6.6 (3.2–10.0)	5.5 (2.6–8.4)	7.6 (3.0–12.3)
IGF-1 (pg/ml)				
Baseline	148 (28–268)	118 (28–264)	56 (17–94)	34 (22–47)
6 weeks	171 (15–328)	220 (0–654)	115 (0–310)	57 (16–100)
12 weeks	150 (0–344)	219 (0–622)	54 (0–26)	117 (52–183)
Estradiol (pg/ml)				
Baseline	60.0 (18.5–138.0)	35.2 (42.0–113.0)	42.3 (7.0–91.0)	14.7 (2.0–31.4)
6 weeks	52.0 (0.0–151.0)	8.0 (0.0–145.0)	29.5 (0.0–122.0)	6.3 (0.0–34.4)
12 weeks	38.0 (0.0–147.0)	17.0 (0.0–159.0)	2.0 (0.0–128.0)	8.7 (0.0–38.0)
Testosterone (ng/ml)				
Baseline	2.0 (0.7–3.1)	1.3 (0.4–2.3)	2.6 (1.5–3.7)	1.4 (0.4–2.3)
6 weeks	1.9 (0.2–3.4)	1.3 (0.2–2.4)	2.7 (0.4–5.1)	1.5 (0.3–2.4)
12 weeks	2.0 (0.2–3.6)	1.2 (0.0–2.9)	2.8 (0.9–4.8)	1.5 (0.1–2.8)
Cortisol (nmol/L)				
Baseline	396 (241.0–551.0)	393 (227.0–559.0)	301 (229.0–372.0)	397 (179.0–615.0)
6 weeks	254 (0.0–472.0)	344 (46.2–642.0)	365 (197.0–543.0)	374 (62.0–745.0)
12 weeks	383 (68.0–698.0)	404 (135.0–674.0)	333 (214.0–451.0)	275 (0.0–67.06)

Values are the mean (95% CI).
DM, high-protein dairy milk beverage; EX, exercise; EX+DM, exercise and high-protein dairy milk beverage; CON, control; IGF-1, insulin-like growth factor-1.

by 1% at 6 weeks; although this finding did not attain statistical significance, it could explain the significant FM and BM losses observed at that time point. Additionally, the greater FM loss observed in both groups that received the DM may be due, in part, to the greater calcium intake (∼983 mg/day) in EX+DM (941 ± 316 mg) and DM (1,011 ± 331 mg). Increasing dietary calcium, either through whole foods or supplementation, has been shown to increase fat oxidation (65) and fat excretion in the digestive tract (66). However, the findings of this current study contrast with the findings of Kukuljan et al. (25), who observed a significant increase in FM (1.3 kg) in healthy older community dwellers (50–70 years) who received a fortified milk beverage to consume twice daily for 18 months. The FM gain observed in the study by Kukuljan et al. (25) was likely due to the addition of extra energy intake (836 kJ, 200 kcal), which may have led to the excess energy intake leading to the gain in FM. This highlights one of the strengths of the current study, where there was a provision of food for 12 weeks, controlled for energy intake and provided 100% of the estimated total daily energy requirements, and estimated protein intakes for the participants in both the EX+DM and DM groups. Dietary control and food provisions also accounted for the extra energy provided by the high-protein dairy milk beverage. It is important to acknowledge that other studies have failed to provide adequate dietary controls. Therefore, the increase in calcium and protein intakes from DM may potentially explain the increased loss in FM observed in this current study despite the participants' balanced energy intake. Lastly, in relation to physical activity, DM had the lowest moderate physical activity and the highest sedentary physical activity compared to the other groups (**Table 3**). Therefore, the losses of FM and BM observed in DM cannot be due to the differences in habitual physical activity leading to increases in energy expenditure. Overall, there were no significant differences for markers (e.g., RMR, calcium, physical activity, and protein intake) individually, but combined they could have a substantial effect, which may explain the significant decreases in BM and FM observed in DM compared to the other groups.

The finding that the high-protein dairy milk beverage did not enhance the effects of PRT measures of HGS and performance outcomes (e.g., gait speed, countermovement jump, and cardiorespiratory fitness) is consistent with two meta-analyses that have reported mixed findings with regard to the benefit of additional dairy milk protein or protein supplementation on outcomes of physical performance (9, 67). In the current study, the lack of a significant change is likely due to the examination of active older adults who have physical performance measures higher than community dwellers or frail older adults, who are the typical participants in sarcopenia research (68). This highlights the lack of research in active older adults who are still prone to age-related sarcopenia (68). For example, Dulac et al. (69) recruited sedentary (<120 min/week of

TABLE 7 | Baseline values and the mean within-group changes at weeks 6 and 12 in cytokine response according to randomized allocation.

	DM ($n = 7$)	EX+DM ($n = 8$)	EX ($n = 8$)	CON ($n = 9$)
Leukocyte $\times 10^9$				
Baseline	5.8 (5.4–6.4)	5.3 (4.0–7.0)	5.3 (4.6–6.0)	4.7 (4.0–5.4)
6 weeks	5.0 (3.2–7.0)	5.0 (3.0–7.5)	4.6 (3.2–6.0)	3.8 (1.5–5.0)
12 weeks	4.8 (2.3–7.4)	5.0 (3.0–7.5)	5.9 (4.0–7.8)	4.0 (1.5–6.4)
Neutrophils $\times 10^9$				
Baseline	3.1 (2.3–4.0)	2.5 (1.7–3.4)	3.1 (3.0–3.5)	2.4 (2.8–3.0)
6 weeks	3.1 (1.8–4.5)	2.2 (1.0–3.5)	3.7 (2.0–3.4)	2.1 (1.5–3.6)
12 weeks	3.2 (1.4–5.2)	2.2 (0.7–3.0)	2.8 (1.5–4.5)	2.0 (1.5–3.3)
Lymphocytes $\times 10^9$				
Baseline	2.5 (2.0–3.0)	2.3 (1.4–3.1)	1.9 (1.5–2.4)	1.8 (1.5–2.1)
6 weeks	2.1 (1.0–3.2)	2.1 (0.7–3.3)	1.7 (1.0–2.5)	1.3 (0.1–2.4)
12 weeks	1.7 (0.3–3.2)	2.0 (0.5–3.2)	1.7 (0.4–3.2)	1.4 (0.5–2.4)
Monocyte $\times 10$				
Baseline	0.4 (0.3–0.4)	0.3 (0.2–0.0)	0.3 (0.2–0.4)	0.4 (0.2–0.6)
6 weeks	0.4 (0.2–0.6)	0.3 (0.1–0.5)	0.3 (0.1–0.5)	0.3 (0.0–0.8)
12 weeks	0.4 (0.3–0.6)	0.3 (0.1–0.5)	0.5 (0.1–0.8)	0.3 (0.0–0.7)
Neutrophil/lymphocyte ratio				
Baseline	1.4 (0.7–2.0)	1.1 (0.7–1.5)	1.7 (1.4–2.0)	1.2 (0.9–1.6)
6 weeks	1.4 (0.5–2.3)	1.1 (0.5–1.8)	1.4 (0.0–2.1)	1.3 (0.6–2.1)
12 weeks	1.1 (0.5–3.0)	1.0 (0.5–1.6)	1.9 (0.9–2.9)	1.1 (0.6–1.7)
IL-2 (pg/ml)				
Baseline	4.2 (1.9–6.5)	3.5 (1.9–5.1)	4.2 (3.0–5.4)	5.0 (2.5–7.6)
6 weeks	3.5 (0.0–7.3)	4.0 (1.2–6.7)	4.5 (2.2–7.0)	10.7 (0.0–21.4)
12 weeks	3.4 (0.2–6.7)	3.1 (1.0–6.7)	3.6 (1.1–6.2)	6.0 (0.5–12.0)
IL-6 (pg/ml)				
Baseline	13.0 (0.5–25.6)	2.0 (1.1–3.0)	8.0 (2.2–13.2)	4.0 (0.3–7.2)
6 weeks	11.6 (0.0–30.6)	2.4 (0.0–4.0)	7.1 (2.2–15.2)	3.8 (0.2–8.4)
12 weeks	11.5 (0.0–21.5)	2.0 (0.6–3.7)	7.2 (0.0–16.0)	3.7 (0.0–8.1)
IL-8 (pg/ml)				
Baseline	10.0 (2.0–18.0)	1.9 (1.3–2.6)	5.0 (2.6–7.0)	4.8 (0.74–10.3)
6 weeks	10.5 (0.0–22.5)	1.4 (1.2–3.7)	4.6 (1.5–7.2)	4.8 (0.0–11.8)
12 weeks	9.0 (0.0–22.1)	1.0 (0.8–3.2)	4.0 (0.0–9.0)	4.4 (0.0–11.3)
IL-10 (pg/ml)				
Baseline	16.0 (8.2–23.5)	14.1 (2.4–25.7)	20.4 (10.0–31.1)	18.6 (6.0–31.5)
6 weeks	16.0 (8.2–23.6)[b]	23.4 (3.3–43.4)*	20.4 (9.8–31.0)[b]	18.7 (5.4–42.0)[b]
12 weeks	16.0 (8.0–23.5)[b]	19.0 (1.0–136.0)*	20.3 (9.4–31.3)[b]	18.7 (5.6–31.0)[b]
TNF-α (pg/ml)				
Baseline	1.8 (1.5–2.3)	1.8 (1.4–2.3)	2.3 (1.4–3.2)	2.4 (1.1–3.6)
6 weeks	2.0 (1.4–2.8)	2.4 (1.6–3.3)	2.1 (0.6–3.7)	2.1 (0.0–4.7)
12 weeks	2.0 (1.3–3.0)	1.8 (1.2–2.0)	3.4 (1.3–4.1)	2.0 (0.0–4.3)
Systematic inflammatory response profile				
Baseline	48.0 (21.0–75.0)	16.5 (3.4–29.5)	41.0 (23.5–58.1)	37.7 (14.4–61.0)
6 weeks	48.2 (6.0–90.0)	29.0 (4.4–53.5)	42.0 (12.0–72.1)	32.1 (0.7–81.4)
12 weeks	40.6 (4.2–77.0)	24.5 (0.1–49.0)	32.3 (0.0–71.0)	33.0 (0.2–72.2)

Values are the mean (95% CI).

DM, high-protein dairy milk beverage; EX, exercise; EX+DM, exercise and high-protein dairy milk beverage; CON, control; IL, interleukin; IGF-1, insulin-like growth factor-1.

*Within-group changes: *$P < 0.05$ vs. baseline. Between-group changes: [a]$P < 0.05$ vs. DM.*

physical activity) older (69 ± 7 years) males and found significant gains in all groups for HGS (4–10%) and gait speed (−4 to −5%). Similarly, Daly et al. (63) found a significant increase in HGS (10–14%) and gait speed (−3%) in inactive (<7,500 steps/day) females. Considering that the self-reported baseline physical activity level for this current study was 227 ± 31 min/week, which is higher than the level of physical activity that is considered "active" for older adults [e.g., 150 min/week of light- to moderate-intensity or 75 min/week of vigorous-intensity physical activity; (11)] and higher than the previously mentioned studies, could indicate that the active older adults in this current study reached a "ceiling effect" in the outcomes of performance, similarly observed in high-functioning and highly trained older adults (68). For example, the average HGS values at baseline were 37 and 42 kg, for females and males, respectively. These are higher than the previous findings in community-dwelling females (≥27 kg) (69) and males (≥38 kg) (70). Moreover, gait speed has been found to have a non-linear relationship between leg strength, as indicated by a wide population variance [e.g., 22%; (68)]. Therefore, any changes in skeletal muscle mass and strength in active older adults are unlikely to show an improvement in gait speed or HGS. A review by Beaudart et al. (70) proposed that a gait speed test over a course of 400 m would be more clinically relevant and sensitive to detect changes in active older adults than would a 4-m distance. Previous works have suggested that measures of muscle power (e.g., CMJ) should be considered more clinically relevant in the active older population due to power declining at a faster rate than strength (51). Within this current study, there was an average increase by 10% in jump height in the groups that received PRT. Although this finding was not statistically significant, it aligns with Daly et al. (63), where a significant change in the CMJ height with a change of 3–6% was reported. The difference of results is likely due to the much larger sample size per group ($n = 108$) within the study by Daly et al. (63). Overall, the measurements of HGS and gait speed have been used as valid measurements in the clinical setting to detect age-related declines related to sarcopenia and may be more useful for use as an initial screening of participants, but may not be sensitive enough to detect meaningful changes in an intervention trial in an older population that is physically active.

Considering the role of systemic inflammatory responses in the pathophysiology of age-related sarcopenia (31, 32), the current study employed a human cyto/chemokine panel to determine intervention-induced changes in these immune response markers. Using a high-sensitivity multiplex assay, the results of the current study found a significant increase in the plasma IL-10 concentration in the EX+DM group (81%) at 12 weeks, which was greater than those in the EX (−6%), DM (−5%), and CON (−8%) groups and without any significant changes to any of the other cytokine markers measured (e.g., TNF-a, IL-2, IL-6, and IL-8). Previous studies have indicated that the anti-inflammatory cytokine IL-10 is the most sensitive cytokine marker in response to exercise stress, unlike the pro-inflammatory (TNF-a) and response (IL-6 and IL-8) cytokines which showed no to minimal responses to acute exercise (71–77). For example, studies that evaluate the cytokine response to resistance training in older adults

have reported a significant increase in the resting plasma IL-10 concentration (23–50%) following 16–24 weeks of resistance training compared to the controls (no training) (35, 36). The significant difference in the increase of plasma IL-10 concentration, suggesting a greater systemic anti-inflammatory effect in EX+DM compared to the EX group, is a novel finding, and the mechanism/s for such an outcome are yet unknown. Some plausible mechanisms have largely been explored in *in vitro* and *in vivo* models and could be possibly explained by the addition of the high-protein milk beverage. In particular, the addition of branched-chained amino acids (BCAAs) found in dairy milk acts as a substrate for the synthesis of short-chained fatty acids (SCFAs) such as butyrate (78). Butyrate and other derived SCFAs from BCAAs (e.g., isobutyric acid, 2-methylbutyric acid, and isovaleric acid) increase the expressions of IL-10 lymphocyte cells in the gut (79). Additionally, other constituents of dairy milk have immunomodulatory and anti-inflammatory properties (e.g., immunoglobulins, lactoferrin, and α-lactalbumin), which may act upon cytokine upregulation or downregulation (23). Galactooligosaccharides (GOS), which are prebiotic substrates derived from lactose found in dairy milk (80–82), have been found to promote the increase in the bacterial counts of bifidobacteria and lactobacilli, which have anti-inflammatory effects (81). GOS derived from dairy milk were found to play a direct role in the regulation of $CD4^+$ T cells, which are involved in the proliferation of IL-10 cytokines; however, this study was limited in animal models (82). Lastly, research has previously found that individuals who consume diets that are higher in GOS following a bout of strenuous physical activity showed lower levels of intestinal fatty acid binding protein (I-FABP; an indirect marker of intestinal epithelial injury and the regulatory point for luminal bacterial endotoxin translocation and subsequent systemic inflammatory responses) compared to those that followed a low-GOS diet [e.g., low FODMAPs; (71)]. These studies, along with our current findings, indicate a potential link between protein intake and skeletal muscle health in older adults. However, further research is needed to understand the mechanistic potential for dairy milk.

Anabolic resistance in aging individuals may be due to the changes in systemic anabolic hormones with increasing age (e.g., testosterone and IGF-1), which has a direct correlation with the onset of sarcopenia (83, 84). In addition, the decrease in estrogen levels associated with menopause may also play a role in the decline in skeletal muscle mass and skeletal muscle strength in aging females (85). In the current study, there were no significant changes in the outcomes related to systematic resting hormonal markers in any of the groups. Previous studies have found that resistance training alone can increase the circulating levels of IGF-1 (86) and testosterone (87) in older adults. In contrast, other studies have not found such effect (88, 89). In the current study, there was an increase in testosterone in both groups that received PRT (>7%). However, there was only an increase in IGF-1 in the EX+DM group (80%) at 12 weeks from baseline. West and Phillips (90) found in young males (18–30 years) that the associated effect (e.g., increase in skeletal muscle strength) of resistance training on circulating anabolic hormones was modest and explained 8–12% of the variance for changes in lean skeletal muscle. Considering that older adults have lower resting anabolic circulating hormones than their younger counterparts, the effect of PRT may be even less. Nonetheless, in older adults, even a modest effect on skeletal muscle strength or skeletal muscle may have practical implications as even small improvements could result in increased functional capacity for those at risk of sarcopenia.

Overall, the strengths of this study lie in its randomized controlled design, high study retention (≥80%) and compliance rate (≥80%) to the intervention, and in the comprehensive outcomes measured, including FFM, strength, power, and physical performance, accounting for outcomes that may be more relevant in an active aging cohort (e.g., CMJ). This study also controlled for variables such as dietary intake through using food provisions and monitoring food intake through diaries. Physical activity was monitored over the course of the clinical trial to ensure that changes in outcomes were due to the exercise intervention and not due to the participants increasing habitual exercise outside the trial, which other studies have failed to implement. The measurement of blood markers (e.g., cytokines and hormones) provided an extensive insight into the pathophysiology and potential mechanisms of the interventions. Previous nutrition and exercise intervention studies have shown significant interaction effects on sarcopenia outcomes (i.e., FFM, strength, and performance) with a study size of 6–196 participants per group, in two to four group studies (8, 9). In the present study, a larger sample size may have accounted for identifying subtle significant differences between the outcomes measured. From a clinical and practical perspective, these smaller changes that may have been observed in a larger sample size would possibly be of no clinical relevance beyond the magnitude of change that has already been observed in this current study that was sufficiently statistically powered. One limitation that should be acknowledged is that physical activity at baseline was self-reported. Therefore, it is unknown whether non-exercise activity or exercise activity, which can be significant components of energy expenditure, increased during the intervention period. This could have resulted in an increased energy deficit leading to more significant weight loss, as observed in DM (91). However, the most important limitation of this study is the large habitual protein intakes in the EX and CON groups (e.g., 1.4–1.6 g kg^{-1} BM day^{-1}). These participants were not provided a control diet based on previous studies that have suggested that up to 50% of active older adults do not meet the protein requirement of 1.2 g kg^{-1} BM day^{-1} (92). Therefore, an assumption was made that protein intake would be lower and unevenly distributed than our dietary intervention. However, the protein intake between groups was comprehensively managed, assessed, and analyzed, and a strength in comparison to previously published investigations of a similar nature (8, 9). Furthermore, unlike previous studies that used isolated protein supplementation, this study used whole foods (i.e., dairy milk), which are commercially available and accessible. The addition of 500 ml of dairy milk translates to two additional servings of dairy, consistent with the Australian Guidelines to Healthy Eating (93), which may provide a cost-effective solution to providing a high-quality

protein source and subsequent amino acids and were well-tolerated (compliance, 93 ± 8%).

CONCLUSION

This study showed that a daily consumption of two high-protein milk beverages at breakfast and lunch (or after PRT) significantly enhanced the effects of PRT on skeletal muscle strength outcomes in active older adults who already have high levels of protein intake ($\geq$1.2 g kg^{-1} BM day^{-1}). There was a significant increase in FFM following the PRT, but no augmented effect with the high-protein dairy milk beverage. There was a significant decrease in FM in those consuming the high-protein milk beverage. Potentially, the influence from the protein or other constituents of the high-protein milk beverage (e.g., calcium) may have contributed to the significant reduction in FM observed. Additionally, EX+DM led to a significant increase in resting anti-inflammatory cytokine (i.e., IL-10), which all may play a significant role in improving skeletal muscle mass and strength outcomes in active older adults (e.g., inflammaging). Finally, these results suggest that consuming a high-protein milk beverage at times that are usually inadequate in protein with PRT can facilitate gains in muscle strength, FFM, and reductions in FM and is well-tolerated by active older adults. Overall, the findings of this study are novel and define opportunities for future interventional studies examining age-related sarcopenia in the healthy active aging population.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Monash University Human Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZH and RC contributed toward the original research idea. ZH, RC, and JP contributed toward development of the experimental design. ZH, RC, and AP contributed toward various aspects of data collection, sample collection, and analysis. ZH and RC contributed toward the analysis of the raw data. ZH was responsible for the initial manuscript draft. All authors contributed to the critical review of the manuscript and approved the final manuscript for submission.

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Increased Leg Strength After Concurrent Aerobic and Resistance Exercise Training in Older Adults Is Augmented by a Whole Food-Based High Protein Diet Intervention

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Most studies in older adults have utilized powdered protein supplements or oral nutrition solutions as a source of additional dietary protein, but whole foods may provide a greater anabolic stimulus than protein isolated from food matrices. Therefore, the present study investigated a concurrent aerobic and resistance exercise training program in older adults, in the absence or presence of a high protein whole food-based dietary intervention, for effects on strength, physical function, and body composition. Community-dwelling older adults ($n = 56$; M/F, 28/28; age, 69.3 ± 4.0 years; BMI, 26.6 ± 3.7 kg m^{-2}) participated in a 12-week intervention after randomization to either nutrition only (NUTR; $n = 16$), exercise only (EX, $n = 19$), or nutrition plus exercise (NUTR + EX, $n = 21$) groups. NUTR and NUTR + EX followed a dietary intervention targeting an increase in protein-rich meals at breakfast, lunch, and dinner. Exercise training in EX and NUTR + EX consisted of 24 min sessions of concurrent aerobic and resistance exercise performed three times per week. Daily protein intake increased in NUTR and NUTR + EX, but not EX. The increase in 1RM leg press strength was greater (Interaction effect, $P = 0.012$) in NUTR + EX [29.6 (18.1, 41.0) kg] than increases observed in NUTR [11.1 (−1.3, 23.6) kg] and EX [12.3 (0.9, 23.8) kg]. The increase in 1RM chest press strength was greater (interaction effect, $P = 0.031$) in NUTR + EX [6.3 (4.0, 8.6) kg] than the increase observed in NUTR [2.9 (0.3, 5.5) kg], but not EX [6.3 (3.9, 8.7) kg]. Hand-grip strength and sit-to-stand performance were each improved in all three groups, with no differences observed between groups (interaction effect, $P = 0.382$ and $P = 0.671$, respectively). An increase in percentage body fat was observed in NUTR, but not in EX or NUTR + EX (interaction effect, $P = 0.018$). No between-group differences were observed for change in lean body mass (interaction effect, $P = 0.402$). Concurrent aerobic and resistance exercise training improves strength and physical function in older adults, but combining this training with an increase in daily protein intake through whole foods may be advantageous to increase lower limb strength.

Keywords: body composition, combined training, nutrition, physical function, lean body mass (LBM)

INTRODUCTION

Age-related declines in skeletal muscle strength and physical function are a major threat to healthy aging by increasing the risks of adverse outcomes such as falls and fractures, frailty, loss of independence, and reduced quality of life (Wolfe, 2006; Cruz-Jentoft et al., 2019). These declines are exacerbated by the loss of skeletal muscle mass, and when declines in physical function and muscle mass are advanced, this results in the diagnosis of sarcopenia (Cruz-Jentoft et al., 2019). Beneficial effects of exercise training and/or high protein intake ($>1.2\,g\,kg^{-1}\,d^{-1}$) for older adults are evident in both epidemiological (McLean et al., 2016; Stamatakis et al., 2018) and intervention studies (Norton et al., 2016; Timmons et al., 2018) and form the basis of recommendations for maintaining skeletal muscle health in older adults (Chodzko-Zajko et al., 2009; Deutz et al., 2014; Bauer et al., 2019).

Interest in the "optimal" approach to lifestyle intervention in older adults at risk for functional decline have typically centered on combined resistance exercise and nutrition co-interventions (Liao et al., 2017; Ten Haaf et al., 2018; Labata-Lezaun et al., 2020). Concurrent aerobic and resistance exercise training, however, provides benefits to both aerobic fitness and strength-based outcomes (Karavirta et al., 2011), and even when time-matched tends to provide the same or better benefits compared to either mode alone (Wood et al., 2001; Timmons et al., 2018). However, little research has investigated strength, physical function, or body composition outcomes in older adults after undertaking concurrent aerobic and resistance exercise training when this training has been undertaken combined with a dietary intervention to increase daily protein intake.

Moreover, many studies to date have utilized powdered protein supplements or oral nutrition solutions as the source of additional dietary protein (Liao et al., 2017; Ten Haaf et al., 2018; Labata-Lezaun et al., 2020), but accumulating evidence suggests that whole foods may provide a greater anabolic stimulus than protein sources isolated from traditional food matrices (Elliot et al., 2006; Burd et al., 2015; van Vliet et al., 2017; Abou Sawan et al., 2018). Consequently, recent reviews have proposed the need for protein-based dietary interventions focusing on whole food sources for the provision of additional dietary protein, at least in community-dwelling older adults, given this potentially additive anabolic effect (Burd et al., 2019; Marshall et al., 2020). To maximize the anabolic effect of feeding throughout the day in older adults, it is also suggested on a per meal basis to include $\geq$2.5 g of the amino acid leucine within a protein dose $\geq$0.4 g kg^{-1}, and for protein intake to follow an "even" distribution throughout the day (Traylor et al., 2019). Effects of such a pattern of intake from exclusively from whole food sources, either alone or in combination with exercise training, remain to be investigated.

Therefore, the present study investigated a concurrent aerobic and resistance exercise training program in older adults, in the absence or presence of a high protein whole food-based dietary intervention, for effects on strength, physical function, and body composition. The primary outcome under investigation was change in 1RM leg strength in response to intervention compared between groups. Leg strength was chosen as the primary outcome because of the observation of the age-related declines in muscle mass, muscle strength, and power being greater for the lower compared to upper limbs (Frontera et al., 1985; Lynch et al., 1985), and role of declining lower limb strength in the etiology of sarcopenia (Cruz-Jentoft et al., 2019). Secondary outcomes included changes in other measures of strength, physical function, body mass, and composition assessed both within and between groups. We hypothesized that this dietary intervention would augment exercise training-induced outcomes for leg strength and LBM compared to either exercise training or a high protein diet alone.

MATERIALS AND METHODS

Experimental Design and Participants

A randomized trial using a parallel group, pre-post design, and comprising a 12-week intervention investigated the separate and combined effects of high protein diet and concurrent aerobic and resistance exercise training performed in men and women aged $\geq$ 65 years. All experimental procedures were approved by the University College Dublin Research Ethics Committee (permit: LS-17-22-Timmons-Egan) in accordance with the *Declaration of Helsinki*. Participants provided written informed consent prior to participation. Recruitment was primarily through the University College Dublin Alumni newsletter seeking men and women aged $\geq$ 65 years who were medically stable (Greig et al., 1994), community-dwelling, independent, fully mobile, and capable of completing the proposed intervention. Participants were excluded if they reported a history of myocardial infarction, cardiac illness, vascular disease, uncontrolled metabolic disease, stroke, or major systemic disease; or if already engaging in two or more structured exercise sessions per week.

An *a priori* sample size calculation (G*Power v3.1) required a sample size of 63 participants based on a three-group design ($n = 21$ per group) assuming to detect an effect size f of 0.2 [partial eta squared (η_p^2) = 0.04; "small"] for a given parameter at a Type I error rate (α) of 0.05 and a power (1–β) of 0.8. Upon entry to the study, participants ($n = 63$) were randomly assigned to one of three groups: nutrition only group (NUTR), concurrent aerobic and resistance exercise training only (EX), nutrition and concurrent aerobic and resistance exercise training (NUTR+EX) (CONSORT flow chart as **Supplementary Figure 1**). Assignment to the groups was performed by an independent researcher using random number generation and included stratified randomization by sex. Five participants from NUTR were lost to follow-up or discontinued the intervention, and two participants dropped out of EX due to inability to maintain the training frequency, leaving a final n size of 56 (NUTR, $n = 16$; EX, $n = 19$; NUTR+EX, $n = 21$; **Table 1** and **Supplementary Figure 1**). Strength, physical function, and body composition were assessed before (PRE) and after (POST) 12 weeks of intervention. The POST assessment took place 48–96 h after the last training session for the EX and NUTR+EX groups.

TABLE 1 | Participant characteristics at baseline (PRE).

	NUTR ($n = 16$) mean ± SD	EX ($n = 19$) mean ± SD	NUTR+EX ($n = 21$) mean ± SD	ALL ($n = 56$) mean ± SD	*P* value ANOVA
M/F (*n/n*)	8/8	9/10	11/10	28/28	
Age (years)	69.3 ± 3.4	68.8 ± 3.8	69.7 ± 4.6	69.3 ± 4.0	0.769
Anthropometry					
Height (m)	1.69 ± 0.10	1.68 ± 0.10	1.69 ± 0.09	1.68 ± 0.09	0.933
Body mass (kg)	79.0 ± 8.8	72.5 ± 11.6	75.1 ± 13.0	75.3 ± 11.5	0.255
BMI (kg m^{-2})	28.0 ± 4.4	25.8 ± 3.6	26.3 ± 3.0	26.6 ± 3.7	0.197
Body composition					
Body fat (%)	33.8 ± 11.7	33.4 ± 7.5	34.0 ± 5.8	33.8 ± 8.2	0.978
Fat mass (kg)	26.36 ± 11.13	23.28 ± 6.50	24.47 ± 5.83	24.61 ± 7.84	0.519
LBM (kg)	49.92 ± 7.29	46.22 ± 9.05	47.66 ± 9.34	47.82 ± 8.67	0.459
ALM (kg)	22.28 ± 3.51	20.51 ± 4.76	22.11 ± 4.77	21.24 ± 4.42	0.501
Strength/physical function					
1RM leg press (kg)	129.9 ± 32.5	129.6 ± 56.1	129.4 ± 39.7	129.6 ± 43.6	0.999
1RM chest press (kg)	40.8 ± 16.8	39.4 ± 15.4	41.9 ± 16.0	40.7 ± 15.8	0.887
Hand-grip strength (kg)	31.9 ± 11.9	32.3 ± 11.7	31.7 ± 9.1	32.0 ± 10.6	0.984
Gait speed (m s^{-1})	1.97 ± 0.44	1.72 ± 0.35	1.96 ± 0.32	1.88 ± 0.38	0.077
Sit-to-stand (s)	10.64 ± 3.71	11.76 ± 2.32	10.85 ± 1.94	11.09 ± 2.67	0.422

1RM, one-repetition maximum; ALM, appendicular lean mass; BMI, body mass index; LBM, lean body mass; M/F, male/female. P values are reported from one-way ANOVA between groups.

Assessments

The assessment procedure was identical in content and sequence at PRE and POST and performed over two consecutive days by the same personnel. These personnel were unblinded to the intervention groups due to these personnel also being involved in the execution of the exercise and/or dietary interventions. On day one, participants arrived to the laboratory after an overnight fast (> 8 h), having consumed 500 mL of water 2 h prior to their visit and engaged in minimal morning ambulation. After voiding of the bladder, body mass (to the nearest 0.1 kg) using a calibrated digital scales (SECA, Germany), height (to the nearest 0.01 m) using a wall-mounted stadiometer (Holtain, UK), and body composition by dual-energy X-ray absorptiometry (DXA; Lunar iDXA, GE Healthcare, USA) were measured. Regional measures of LBM of the upper and lower limbs (arms and legs, respectively) were obtained from the DXA scan analysis in order to calculate appendicular lean mass (ALM). Participants then consumed a small snack (cereal bar plus banana) and were allowed water ad libitum. Next, hand-grip strength of the dominant hand was measured to the nearest 0.5 kg using a hydraulic hand dynamometer (JAMAR, USA) (Roberts et al., 2011) followed by habitual gait speed (3 m), and five repetition sit-to-stand (Guralnik et al., 1994). On day two, participants reported to the exercise training facility (Medfit Proactive Healthcare, Dublin) for the assessment of lower and upper limb strength by one repetition maximum (1RM) on leg press and chest press machines, respectively (Milon, Germany). One week prior to the assessment at PRE, a familiarization session was performed. In this session, each of the tests described above were performed, and the correct lifting technique was demonstrated and practiced for each strength exercise, after which maximum strength was estimated using the multiple repetitions testing procedure. This estimate, in turn, informed the subsequent assessment of 1RM performed at PRE.

Exercise Training Intervention

The exercise training intervention was fully supervised, small group ($n = 4$–6) training, and consisted of three exercise sessions per week (Monday, Wednesday, and Friday) of concurrent aerobic and resistance exercise training lasting ∼40 min per session, which included a standardized warm-up and cool-down. The warm-up employed RAMP principles (R, raise heart rate and core/muscle temperature; A, activate musculature; M, mobilization of joints to create full range of motion; and P, potentiate/increase intensity in preparation for exercise protocol) over the course of 5 min including 3 min of low-to-moderate intensity aerobic exercise and 2 min of low intensity bodyweight movements/calisthenics. The cool-down was 5 min in duration consisting of low intensity bodyweight movements/calisthenics and walking to gradually lower heart rate, and incorporated static stretching of the major muscle groups of the upper and lower limbs. All training sessions were supervised and performed on the Milon Circle (Milon, Germany). Each session consisted 3 × 4 min intervals of aerobic exercise (Cross Trainer and Stationary Cycle Ergometer) and two rounds of the six resistance exercise circuit (Leg Press, Seated Row, Chest Press, Lat Pulldown, Leg Extension, and Tricep Dips). The aerobic and resistance exercises were interspersed by having participants complete three resistance exercises, followed by one 4 min interval of aerobic exercise, and repeating this pattern twice before concluding with three resistance exercises. A rest period

of 30 s was taken in between each set of resistance exercise or interval of aerobic exercise.

For the aerobic exercise modes, the power output was adjusted to elicit a target intensity of 80% of age-predicted maximum heart rate for each 4 min interval throughout the training intervention in order to ensure that a progressive overload was continuously provided. For the resistance exercises, participants commenced training for weeks 1–4 with the prescription of 15 tempo-controlled repetitions of a given exercise in a 60 s period. The tempo for each 4 s repetition comprised of a 2 s eccentric movement, a 1 s pause, and a 1 s concentric movement and no pause between repetitions. For weeks 5–8, the prescription was adjusted to 12 tempo-controlled repetitions of a given exercise in a 60 s period. The tempo for each 5 s repetition comprised of a 3 s eccentric movement, a 1 s pause, and a 1 s concentric movement and no pause between repetitions. For weeks 9–12, the prescription was adjusted to 10 tempo-controlled repetitions of a given exercise in a 60 s period. The tempo for each 6 s repetition comprised of a 4 s eccentric movement, a 1 s pause, and a 1 s concentric movement and no pause between repetitions. Participants began the training intervention at ∼60% of 1RM, but once an exercise could be completed comfortably for the 60 s period, an ∼5% increment in weight to be lifted was added for the next training session in order to provide a progressive overload. For the weeks 5–8, and weeks 9–12, the load lifted was not prescribed based on %1RM but was manually adjusted by the practitioner according to the ability of each participant at the new prescription for repetitions/tempo, after which progressive overload was applied as described. The compliance with set duration and tempo was facilitated by the presence of a metronome and timer visible to participants on a digital display on each resistance training machine.

With 12 min of aerobic exercise and 12 min of resistance exercise, each training session, therefore, consisted of 24 min of active exercise, for a total of 72 min of active exercise each week (36 min aerobic exercise and 36 min resistance exercise). This exercise training program without dietary intervention has been previously shown by our group to elicit improvements in a range of measures of strength, physical function, and body composition in older adults (Timmons et al., 2018).

Dietary Intervention

The dietary intervention targeted a high protein intake by providing meal and recipe suggestions using a whole food-based approach (i.e., powdered protein supplements and oral nutrition solutions) to achieve ∼25–35 g (∼0.4 g kg^{-1}) of protein per meal. Each of these protein-rich meal recommendations also aimed to provide ∼3 g of leucine. Participants from NUTR and NUTR+EX initially attended a briefing session in groups of 4–6 participants during which the dietary intervention was explained in detail. Participants were instructed to consume a protein-rich meal at breakfast, lunch, and dinner every day for the 12-week period, and in NUTR-EX, for one of these protein-rich meals to be within 60 min of each training session. Participants were asked to consume the specified portion in one sitting, and were asked not to split the portion over different eating occasions. Identical meal and recipe suggestions were provided to the participants in NUTR and NUTR+EX fortnightly by email for the duration of the study. These suggestions were informed by the USDA Food Composition Database, by taking food combinations and translating these into user-friendly portion sizes, meals, and recipes. Compliance with the dietary intervention was determined using a tick-box checklist completed per meal on a daily basis. Because of attendance at the supervised exercise sessions, contact with the NUTR+EX participants was weekly and informal, whereas contact with the NUTR participants was maintained formally with a fortnightly phone call to encourage participants to comply with the intervention. The EX group were asked to not to make any changes to their habitual dietary intake for the duration of the study. All participants completed a 3-day (two weekdays, one weekend day) portion-estimate food diary at PRE, week 6 (MID), and POST, which were analyzed using Nutritics Dietary Analysis Software (Nutritics, Ireland).

Statistical Analysis

Data were evaluated using GraphPad Prism v8.4 (GraphPad Software, Inc., USA) and are presented as mean ± standard deviation (SD) at PRE and POST, and as mean difference (lower, upper 95% confidence limit of the mean difference) (95% CL) for data expressed as change from PRE. The data were tested for normality using the Shapiro-Wilk test prior to proceeding with the parametric tests described.

One-way analysis of variance (ANOVA) was performed to evaluate differences between groups at PRE for all parameters. Two-way (group × time) mixed ANOVA was performed to determine changes, if any, in response to intervention and differences, if any, between groups in those responses. When an interaction effect was indicated, between-group differences were evaluated using a one-way ANOVA performed on gain scores at POST with *post-hoc* comparisons performed with Tukey's correction applied, and for which multiplicity-adjusted P values are reported. Independent of the interaction effect, when a main effect of time was indicated, planned comparisons for within-group differences from PRE to POST were evaluated using *post-hoc* comparisons with Tukey's correction applied, and for which multiplicity-adjusted P values are reported. For all null hypothesis statistical testing, statistical significance was accepted at $P < 0.05$. Standardized differences in the mean were used to assess magnitudes of effects for between-group differences at POST, and for within-group changes from PRE to POST. These effect sizes were calculated using Cohen's d and interpreted using thresholds of *trivial* for < 0.2, *small* for ≥ 0.2 to < 0.5, *moderate* for ≥ 0.5 to < 0.8, and *large* for ≥ 0.8.

RESULTS

Compliance With Dietary and Exercise Training Interventions

There were no differences between groups at baseline for any parameter measured (**Table 1**). Attendance at the exercise training sessions averaged 87.4 ± 7.9% throughout the 12-week intervention, and did not differ by training group at 86.3 ± 10.2% and 88.7 ± 4.1%, for NUTR+EX and EX, respectively.

There was no change in dietary intake in EX throughout the intervention period whereas the dietary intervention was successful in increasing daily protein intake, and consequently daily energy intake, in NUTR and NUTR+EX (**Table 2**). Daily carbohydrate and fat intake did not differ between groups and remained similar over time (**Table 2**).

Strength Outcomes

For the primary outcome, EX [12.3 (0.9, 23.8) kg; $P = 0.031$; $d = 0.22$] and NUTR+EX [29.6 (18.1, 41.0) kg; $P < 0.001$; $d = 0.80$] resulted in increases in 1RM leg press strength at POST (Time effect, $P < 0.001$), but a directional increase in NUTR [11.1 (−1.3, 23.6) kg] did not reach statistical significance ($P = 0.093$; $d = 0.31$) (**Figure 1A**). The increase in 1RM leg press strength in NUTR+EX was greater (interaction effect, $P = 0.012$) than the increases observed in NUTR by 18.5 (1.9, 35.0) kg ($P = 0.026$; $d = 0.95$), and in EX by 17.3 (1.4, 33.1) kg ($P = 0.030$; $d = 0.70$) (**Figure 1B**).

1RM chest press strength was increased (time effect, $P < 0.001$) in all groups, i.e., NUTR [2.9 (0.3, 5.5) kg; $P = 0.026$; $d = 0.16$], EX [6.3 (3.9, 8.7) kg; $P < 0.001$; $d = 0.42$], and NUTR+EX [6.3 (4.0, 8.6) kg; $P < 0.001$; $d = 0.39$] (**Figure 1C**). The increase in 1RM chest press strength in NUTR+EX was greater (interaction effect, $P = 0.031$) than the increases observed in NUTR by 3.4 (0.0, 6.8) kg ($P = 0.026$; $d = 0.82$), but the greater increase in EX by 3.3 (−0.1, 6.7) kg compared to NUTR did not reach statistical significance ($P = 0.056$; $d = 0.92$] (**Figure 1D**).

Hand-grip strength improved in all groups (time effect, $P < 0.001$), and no differences were observed between groups (interaction effect, $P = 0.382$) (**Table 3**).

Physical Function Outcomes

Gait speed improved in EX ($P < 0.001$; $d = 1.01$) and NUTR+EX ($P < 0.001$; $d = 0.66$) (time effect, $P < 0.001$), but a directional improvement in NUTR did not reach statistical significance ($P = 0.105$; $d = 0.35$) (**Table 3**). Sit-to-stand improved in all groups (time effect, $P < 0.001$), and no differences were observed between groups (interaction effect, $P = 0.671$) (**Table 3**).

Body Mass and Body Composition Outcomes

Body mass increased in NUTR [0.93 (0.12, 0.73) kg; $P = 0.020$; $d = 0.11$], but not in EX [0.00 (−0.74, 0.74) kg; $P > 0.99$; $d = 0.00$] or NUTR+EX [0.52 (−0.18, 1.23) kg; $P = 0.202$; $d = 0.04$] (interaction effect, $P = 0.120$; **Table 3**). Although the interaction effect ($P = 0.067$) for fat mass and the directional increase in fat mass in NUTR [0.56 (−0.08, 1.19) kg] did not reach statistical significance ($P = 0.105$; $d = 0.05$) (**Table 3**), an interaction effect was observed for percentage body fat ($P = 0.018$), with the increase in percentage body fat in NUTR being greater than changes observed in EX by 0.99 (0.10, 1.89)% ($P = 0.027$; $d = 0.93$) and in NUTR+EX by 0.92 (0.04, 1.79)% ($P = 0.039$; $d = 0.94$) (**Figures 2A,B**).

There was no interaction effect observed for LBM (interaction effect, $P = 0.402$) indicating the absence of between-group differences in LBM in response to the interventions (**Figures 2C,D**). Independent of the absence of between-group differences, within-group PRE-POST comparisons revealed LBM was increased in NUTR+EX [0.56 (0.01, 1.11) kg; $P = 0.048$; $d = 0.06$], but not in NUTR [0.09 (−0.54, 0.73) kg; $P = 0.977$; $d = 0.01$] or EX [0.34 (−0.24, 0.92) kg; $P = 0.404$; $d = 0.04$] (time effect, $P = 0.021$; **Figures 2C,D**). Similarly, ALM was increased in NUTR+EX [0.45 (0.09, 0.81) kg; $P = 0.009$; $d = 0.09$], but not in NUTR [0.18 (−0.23, 0.59) kg; $P = 0.641$; $d = 0.05$] or EX [0.20 (−0.17, 0.58) kg; $P = 0.461$; $d = 0.04$] (time effect, $P = 0.003$; **Table 3**).

DISCUSSION

This present study confirms the efficacy of concurrent aerobic and resistance exercise training to improve physical function in older adults (Wood et al., 2001; Karavirta et al., 2011; Timmons et al., 2018), with the addition of high protein whole food-based diet intervention augmenting some, but not all, of the training-induced outcomes. Most notably, the dietary intervention augmented training-induced increases in lower limb strength. In the absence of exercise training, this dietary pattern resulted in some improvements in physical function, but notably also resulted in an increase in percentage body fat.

The effect of protein supplementation in combination with resistance exercise on changes in strength, physical function, and LBM in older adults has been widely examined, with meta-analyses reporting conflicting conclusions in terms of positive (Cermak et al., 2012; Liao et al., 2017) or equivocal effects (Ten Haaf et al., 2018; Labata-Lezaun et al., 2020). Potential explanations for these discrepancies are divergent inclusion criteria for analyses, in particular, the inclusion of healthy and/or non-healthy, active, and/or ambulatory individuals and different age cut-offs. Generally, the potential to benefit from an exercise and/or dietary intervention is often greater for those who are least healthy, have low habitual physical activity, and/or inadequate protein intake. In the present cohort, baseline daily protein intake was similar to that previously reported in Irish older adults (Hone et al., 2020), and the dietary intervention successfully increased this intake from ∼1.0 to ∼1.5 g kg^{-1} d^{-1} in both NUTR and NUTR+EX. This increase was equivalent to ∼40 to 55 g of additional protein per day. Moreover, daily energy intake was increased by ∼21% in NUTR and ∼34% in NUTR+EX. Dietary intervention alone (NUTR) resulted in improvements in several measures of physical function, i.e., chest press strength, hand-grip strength, and sit-to-stand. These outcomes are not unexpected as several studies of protein supplementation in the absence of exercise have demonstrated similar improvements in physical function in older adults (Bonnefoy et al., 2003; Tieland et al., 2012; Kim and Lee, 2013; Bauer et al., 2015; Chanet et al., 2017). However, the increase in body mass and percentage body fat in NUTR, albeit "trivial" in magnitude, may be considered a deleterious effect in the long-term if this results in adverse metabolic outcomes such as insulin resistance and/or anabolic resistance (Chang et al., 2012; Meex et al., 2019).

Concurrent aerobic and resistance exercise training is established as an efficacious strategy to improve strength, physical function, and body composition in middle-aged and

TABLE 2 | Dietary macronutrient intakes during the 12 weeks of concurrent aerobic and resistance exercise training with or without dietary intervention for the respective groups.

		Energy (kcal)	Carbohydrate (g)	Protein (g)	Protein (g kg^{-1})	Fat (g)
NUTR	PRE	1,648 ± 441	173.6 ± 59.0	73.4 ± 25.7	0.99 ± 0.34	64.3 ± 22.3
	MID	1,949 ± 428*	154.6 ± 43.4	119.9 ± 30.5**	1.52 ± 0.45**	78.4 ± 23.9
	POST	1,989 ± 439*	168.5 ± 51.9	113.1 ± 29.3**	1.43 ± 0.39*	79.8 ± 27.1
EX	PRE	1,823 ± 344$^{\#}$	188.0 ± 38.1	80.0 ± 18.1	1.14 ± 0.35	69.6 ± 18.1
	MID	1,777 ± 437	175.9 ± 53.4	77.4 ± 18.1$^{\#\dagger}$	1.10 ± 0.30	67.6 ± 23.4
	POST	1,793 ± 421	185.2 ± 63.7	75.6 ± 23.6$^{\#\dagger}$	1.05 ± 0.28	66.6 ± 16.8
NUTR+EX	PRE	1466 ± 371	152.0 ± 50.7	65.8 ± 13.8	0.90 ± 0.20	56.2 ± 19.7
	MID	1,873 ± 449*	151.6 ± 43.6	117.8 ± 23.7**	1.59 ± 0.28**	73.4 ± 22.7
	POST	1,971 ± 837*	151.4 ± 51.2	117.1 ± 39.3**	1.57 ± 0.49**	70.6 ± 23.9

*Data are mean ± SD. Statistical analysis was performed using two-way mixed ANOVA. Post-hoc pairwise comparisons with Tukey's correction were used to determine where differences existed between and within groups. Within-group differences compared to PRE are indicated by *$P < 0.05$ and **$P < 0.01$ for the annotated time point, and between-group differences are indicated by $^{\#}P < 0.05$ for EX compared to NUTR+EX, and $^{\dagger}P < 0.05$ for EX compared to NUTR for the annotated time point. No between-group differences were observed between NUTR and NUTR-EX.*

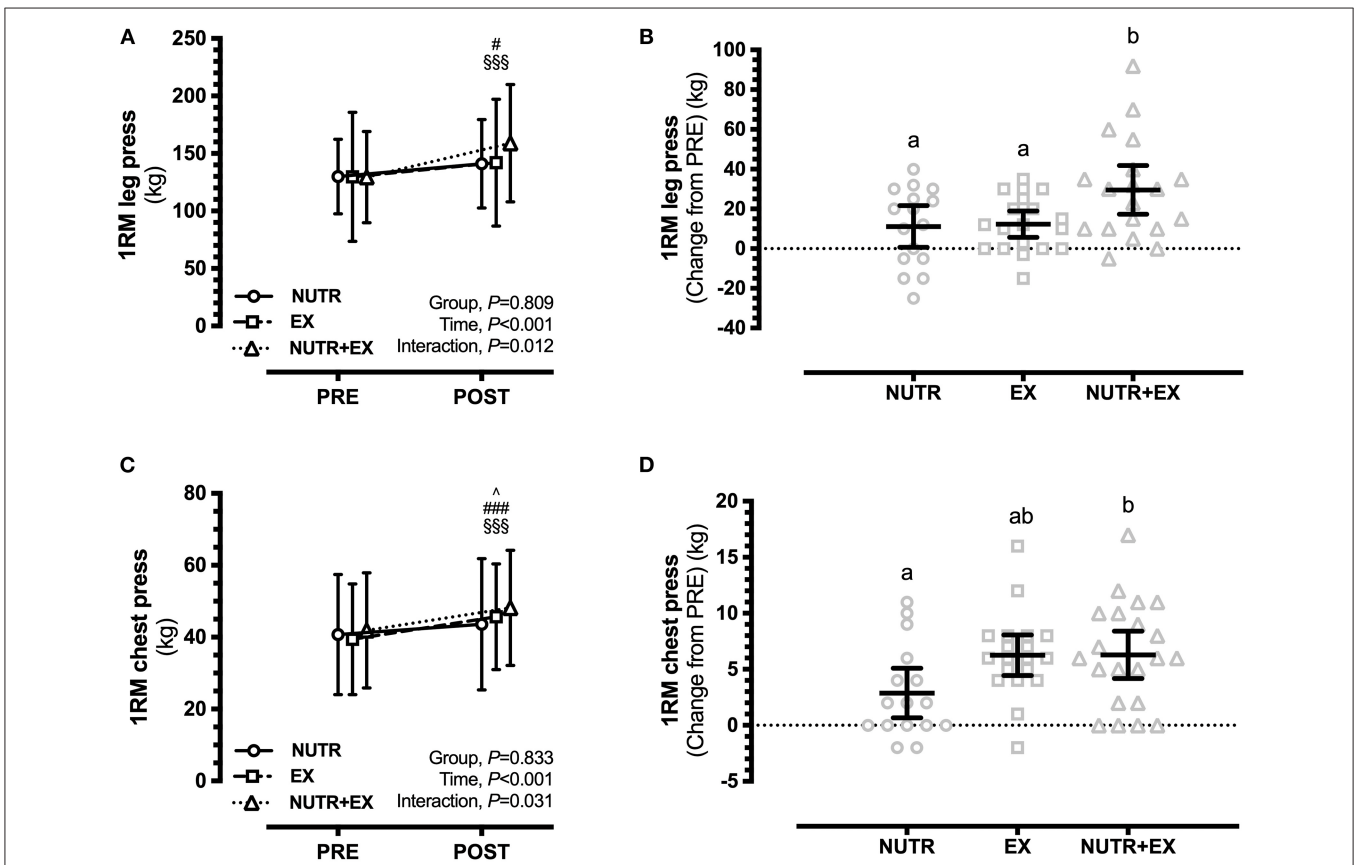

FIGURE 1 | Changes in lower and upper limb muscle strength assessed by 1RM in response to 12 weeks of concurrent aerobic and resistance exercise training with or without dietary intervention. **(A)** Group mean ± SD for leg press; **(B)** Individual data points and mean difference with 95% CI for gain scores from PRE for leg press; **(C)** Group mean ± SD for chest press; **(D)** Individual data points and mean difference with 95% CI for gain scores from PRE for chest press. ^ denotes significant difference from PRE to POST within NUTR; $^{\wedge}P < 0.05$; $^{\#}$denotes significant difference from PRE to POST within EX; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$; §denotes significant difference from PRE to POST within NUTR+EX; $^{\S\S\S}P < 0.001$. Dissimilar letters demonstrate between-group differences ($P < 0.05$) in panels (**B,D**).

older adults (Wood et al., 2001; Sigal et al., 2007; Davidson et al., 2009; Karavirta et al., 2011; Timmons et al., 2018). Similarly, in the present study, improvements in all strength (hand-grip strength, upper and lower limb strength) and functional (sit-to-stand and gait speed) outcomes were observed in both exercise training groups. However, the combination of both strategies

TABLE 3 | Changes from PRE to POST in body composition, strength, and physical function in response to the 12 weeks of concurrent aerobic and resistance exercise training with or without dietary intervention.

'	NUTR ($n = 16$)	EX ($n = 19$)	NUTR+EX ($n = 21$)	ANOVA *P* values
Body composition				
Body mass (kg)	0.93 (0.12, 0.73)*	0.00 (−0.74, 0.74)	0.52 (−0.18, 1.23)	Time, $P = 0.008$ Group, $P = 0.211$ Interaction, $P = 0.120$
Fat mass (kg)	0.56 (−0.08, 1.19)	−0.26 (−0.84, 0.33)	−0.05 (−0.61, 0.50)	Time, $P = 0.557$ Group, $P = 0.422$ Interaction, $P = 0.067$
ALM (kg)	0.18 (−0.23, 0.59)	0.20 (−0.17, 0.58)	0.45 (0.09, 0.81)**	Time, $P = 0.003$ Group, $P = 0.521$ Interaction, $P = 0.371$
Strength/physical function				
Hand-grip strength (kg)	4.1 (2.1, 6.0)***	3.0 (1.2, 4.8)***	2.6 (0.9, 4.3)**	Time, $P < 0.001$ Group, $P = 0.961$ Interaction, $P = 0.382$
Gait speed (m s^{-1})	0.14 (−0.02, 0.31)	0.34 (0.19, 0.49)***	0.24 (0.09, 0.38)***	Time, $P < 0.001$ Group, $P = 0.214$ Interaction, $P = 0.095$
Sit-to-stand (s)	−2.51 (−3.52, −1.51)***	−3.00 (−3.95, −2.06)***	−2.87 (−3.74, −1.99)***	Time, $P < 0.001$ Group, $P = 0.415$ Interaction, $P = 0.671$

*Data are reported as mean difference (95% CL). Statistical analysis was performed using two-way mixed ANOVA. For within-group differences, post-hoc comparisons with Tukey's correction were used to determine where differences existed compared to PRE as indicated by *P < 0.05, **P < 0.01, and ***P < 0.001 for the annotated time point.*

(i.e., NUTR+EX) resulted in a markedly larger increase in lower limb strength. While between-group comparisons of change in LBM in response to the interventions did not reveal any differences between groups, the within-group analysis revealed that NUTR+EX was the only intervention that resulted in increases in LBM and ALM. The lack of change in LBM with concurrent aerobic and resistance training in the absence of dietary change (i.e., EX) is consistent with our previous study using this training regimen (Timmons et al., 2018). Indeed, meta-analyses of the effects of resistance exercise training alone suggests that a >1 kg increase in LBM would take >20 weeks of training three times per week (Peterson et al., 2011; Borde et al., 2015). In this regard, the increase in LBM in NUTR+EX is notable for being ∼0.56 kg in 12 weeks, with ALM (∼0.45 kg) accounting for the majority of this increase, notwithstanding that the magnitude of the effect size is "trivial."

The markedly larger increase in lower limb strength in NUTR+EX (∼25%) compared to other groups (NUTR, ∼6%; EX, ∼13%) is notable as an augmentation of the response to exercise training when additional dietary protein is consumed. This positive effect is, however, discordant with the conclusions of recent meta-analyses that conclude that providing additional protein does not augment improvements in strength after resistance exercise training in community-dwelling, non-frail older adults (Ten Haaf et al., 2018; Labata-Lezaun et al., 2020). There are some key methodological differences between the present study and studies included in these meta-analyses, including that the present study was comprised of concurrent aerobic and resistance exercise training. There is a large degree of heterogeneity in the various study designs, but broadly speaking, similar studies often provide additional protein only on training days (∼3 days per week), or only achieve an increase of ∼15–30 g of additional protein per day (Ten Haaf et al., 2018; Labata-Lezaun et al., 2020). Our dietary intervention therefore differs to many previous studies in that the quantity of additional protein per day was ∼40 to 55 g, which was consumed on every day of week, and incorporated recent recommendations (Traylor et al., 2019) to provide an "even" distribution of protein throughout each day. These factors ultimately contributed to the average daily protein intakes reaching ∼1.5 g kg^{-1}, which is again greater than most previous studies in this domain (Ten Haaf et al., 2018; Labata-Lezaun et al., 2020). Notably, when a similar protein-enriched diet intervention using 2 × 80 g of cooked red meat 6 days per week was combined with thrice-weekly resistance exercise training for 16 weeks, leg extension strength increased by 28% in the meat plus exercise group as compared to 10% in the exercise only group (Daly et al., 2014). However, when the study was repeated with additional protein only on training days, no differences between groups was observed (Formica et al., 2020).

As for mechanisms by which additional dietary protein could augment the increase in strength in response to exercise training, the most likely explanation is a greater anabolic response both in the post-exercise period and generally on a per meal basis, together resulting in greater LBM accretion over time. While this was evident in the present study, it must again be acknowledged that the increase in LBM in NUTR-EX was "trivial" in magnitude, and was not significantly different between groups, so is unlikely to fully explain the differential effect observed on increased leg strength. That said, the present study assessed body composition *via* DXA, which is less sensitive for detecting small changes

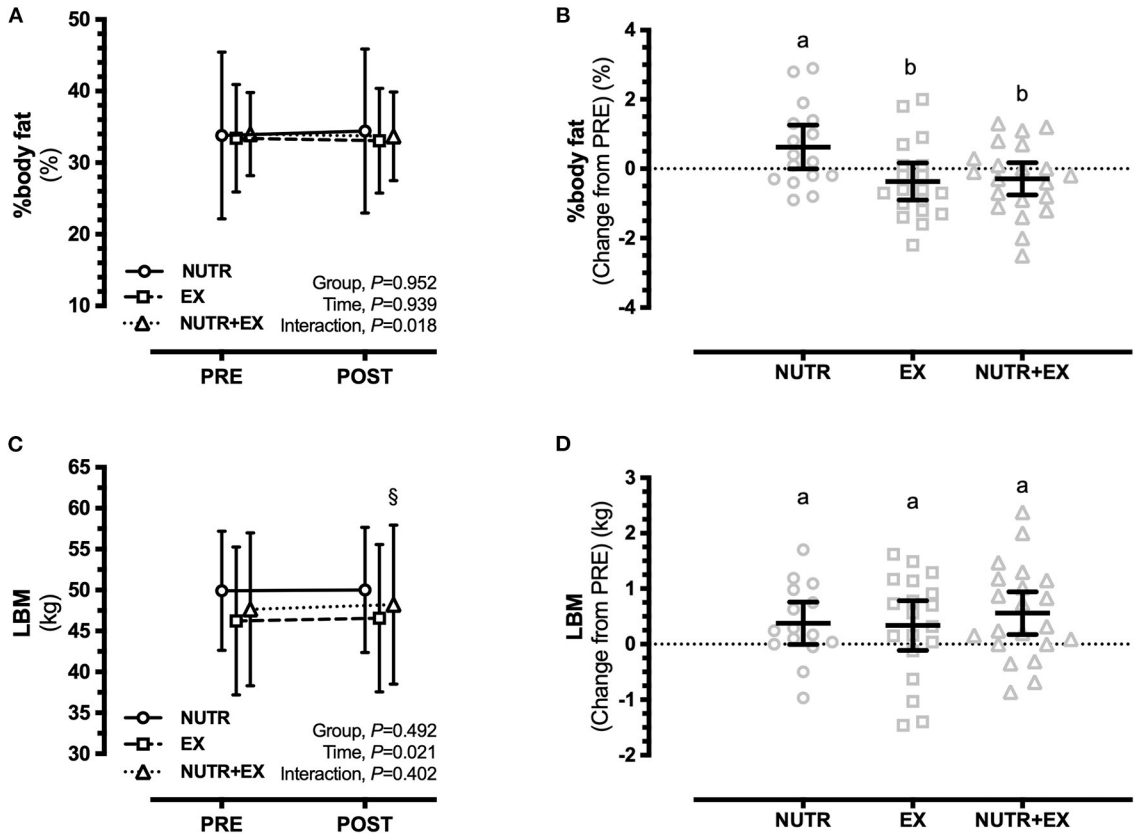

FIGURE 2 | Changes in body composition assessed by DXA in response to 12 weeks of concurrent aerobic and resistance exercise training with or without dietary intervention. **(A)** Group mean ± SD for percentage body fat; **(B)** Individual data points and mean difference with 95%CI for gain scores from PRE for percentage body fat; **(C)** Group mean ± SD for LBM; **(D)** Individual data points and mean difference with 95% CI for gain scores from PRE for LBM. §denotes significant difference from PRE to POST within NUTR+EX; §$P < 0.05$. Dissimilar letters demonstrate between-group differences ($P < 0.05$) in panels **(B,D)**.

in LBM over time compared to magnetic resonance imaging (Delmonico et al., 2008; Tavoian et al., 2019), which is a more sensitive method for detection of change in muscle size by cross-sectional area (Cooper et al., 2013). Alternatively, it is widely acknowledged that changes in muscle strength are not strongly correlated with changes in muscle size or LBM, especially in older adults (Visser et al., 2000; Hughes et al., 2001; Delmonico et al., 2009), and factors other than change in tissue mass are likely to contribute to increases in muscle strength. One example from dietary intervention in older adults is the observation of a larger increase in leg strength per kg LBM when resistance exercise training was supplemented with cysteine-rich whey protein compared to casein protein (43.3 vs. 30.0% increase, respectively), yet in the absence of differences in change in LBM between groups (Karelis et al., 2015). Other contributors to an increase in strength independent of a change in muscle size or LBM could include alterations to neuromuscular action and/or muscle quality. Declines in these two aspects of skeletal muscle physiology have been proposed as central to the etiology of age-related muscle weakness in older adults that has been termed dynapenia, and for which resistance exercise training is an important countermeasure (Clark and Manini, 2010). Overall the present results for lower limb strength and LBM suggests that there are synergies between dietary intervention and exercise training that can be realized in adaptive outcomes, but this effect very much depends on the parameter of interest.

Given that many studies investigating higher dietary protein intake rely on powdered protein supplements and oral nutrition solutions (Cermak et al., 2012; Liao et al., 2017; Ten Haaf et al., 2018; Labata-Lezaun et al., 2020), the present study is novel in the approach to employ an exclusively whole food-based dietary co-intervention with concurrent aerobic and resistance exercise training. This approach is timely given recent calls for protein-based dietary interventions to focus on whole food sources (Burd et al., 2019; Marshall et al., 2020), in recognition of a potentially additive anabolic effect of whole foods over isolated sources of protein (Elliot et al., 2006; Burd et al., 2015; van Vliet et al., 2017; Abou Sawan et al., 2018). The present data, however, cannot suggest that whole foods are more efficacious than isolated sources, as direct comparison of such approaches would be required. Many factors contribute to reduction in energy and protein intake in older adults including a decrease in appetite with advancing age, the higher

cost of more nutrient-dense foods, difficulty chewing fibrous foods, perceived food intolerances, and fear of eating excessive fat and cholesterol (Morley, 2005; Furman, 2006; Malafarina et al., 2013; Hung et al., 2019). In this context, 14% (3/21) of participants in the NUTR group failed to comply with the dietary intervention. Therefore, the translation of the present approach into other settings outside of a formal research trial would require cognizance of these issues, but clinicians should still consider emphasizing the significance of optimal daily protein intake when delivering advice around lifestyle change targeting skeletal muscle health.

The main limitation to the present study is the lack of a true, non-intervention control group, meaning that attributing within-group differences definitively to each intervention could be questioned. Factors such as the Hawthorne effect (McCambridge et al., 2014) resulting in generally better lifestyle habits during the intervention cannot be discounted, especially in NUTR, given that physical activity was not monitored in this study. In mitigation, our previous investigation in a similar cohort (age, sex, education, geographical location) employing many of the same assessments and the same duration of intervention observed no improvements in any of the measured outcomes in the non-intervention control group (Timmons et al., 2018). As the primary outcome was between-group differences in change in leg strength, the absence of a non-intervention control group does not impact the main conclusions from the present study. However, given that both energy intake and protein intake increased in NUTR+EX, it is not possible to attribute the benefits of the dietary intervention to protein specifically, or whether the general increase in energy availability supported the adaptive responses to exercise training. Lastly, one important caveat to this dietary approach is that it may only be appropriate for community-dwelling older adults given the challenges of nutrient provision in acute care settings and the myriad of factors influencing energy and protein intake in older adults (Morley, 2005; Furman, 2006; Malafarina et al., 2013; Hung et al., 2019). Caution may also be warranted in the case of excessive daily energy intake in the absence of exercise in this population.

CONCLUSIONS

The present study is novel in its methodology in view of participants achieving high protein intakes ($\sim$1.5 g kg^{-1} d^{-1}) exclusively through whole food sources, as opposed to supplementing with powdered protein supplements and oral nutrition solutions. While concurrent aerobic and resistance exercise training alone improved strength and physical function in older adults, combining an increase in dietary protein intake from whole foods with this type of exercise training was more advantageous for increasing lower limb strength and may support an increase in lean body mass, primarily in the form of appendicular lean mass. Such outcomes may be valuable in contexts such as during a period of rehabilitation after an adverse event that resulted in declines in muscle size and/or strength, or may inform clinicians and practitioners working in the field of exercise prescription, rehabilitation, and nutritional care of older adults.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University College Dublin Research Ethics Committee (permit: LS-17-22-Timmons-Egan) in accordance with the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JT and BE designed the study. JT, MH, and KC performed the assessments. JT, MH, and OD were responsible for performing the interventions. JT, MH, and BE analyzed the data. JT, MH, and BE wrote the first draft of the manuscript. KC and BE edited the manuscript. BE had the primary responsibility for final content. All authors contributed to editing and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fspor.2021.653962/full#supplementary-material

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REVIEW
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Protein Considerations for Athletes With a Spinal Cord Injury

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Athlete participation in the Paralympic games is steadily increasing; prompting research focused on the unique needs of this population. While the Paralympic Games includes a diversity of athletes, athletes with a spinal cord injury (PARA-SCI) represent a subgroup that requires specialized recommendations. Nutritional guidelines designed to optimize performance, in the context of the neurological impairments, are required. This narrative review summarizes the current literature regarding the importance of dietary protein for optimal health and performance. Factors with the potential to affect protein needs in PARA-SCI including loss of active muscle mass, reduced energy expenditure, and secondary complications are examined in detail. Furthermore, we analyze protein intakes in PARA-SCI from the available research to provide context around current practices and trends. In conclusion, we make the case that protein recommendations for able-bodied athletes may not be directly transferable to PARA-SCI. Consequently, PARA-SCI need their own guidelines to maximize performance and ensure long-term health.

Keywords: sports nutrition, spinal cord injury, paralympic athletes, amino acids, wheelchair athletes, dietary protein, performance nutrition

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INTRODUCTION

Participation in Paralympic games is steadily increasing, with the events in London in 2012 receiving mass-media coverage for the first time (1) and the 2018 Games in PyeongChang reporting 567 athletes from 49 delegations, the most ever (2). Moreover, the Paralympic Games may motivate children and young people with disabilities to participate in sport, increasing their quality of life and social participation. Qualitative research found that children with disabilities were inspired by Paralympic athletes, and that the Paralympic games provided them with more confidence should they wish to participant in sport (1). Furthermore, the worldwide incidence of spinal cord injuries (SCIs) is estimated to be up to 500,000 individuals each year (3). All considered, the number of athletes with a SCI (PARA-SCI) is predicted to increase and the level of competition will intensify. While wheelchair athletes include a diverse group (4), the focus of this article is specifically on PARA-SCI, defined as those with paraplegia or quadriplegia participating actively in wheelchair sports. The reason of the injury could be traumatic or non-traumatic with consequences in the motor, sensory, and autonomic systems. In parallel with the growth in wheelchair sports is a need for nutritional recommendations specific to PARA-SCI that will promote health and optimize performance. Presently, this population is understudied, and evidence-based recommendations are lacking; creating uncertainty among athletes, coaches, and other support personnel. Research conducted to date indicates that PARA-SCI are at risk for multiple nutritional deficiencies (5), which

can have a negative impact on their performance. Furthermore, recommendations specific to PARA-SCI are required, as those developed for able-bodied athletes may not be appropriate. Dietary protein is essential for athletes, as it has a role in energy production and synthesis of metabolic and contractile proteins (6, 7). Given the lack of recommendations for PARA-SCI, and the critical role of protein in exercise performance, the current article provides an overview of the physiology of PARA-SCI, considerations for protein recommendations, summary of reported intakes, and highlights future directions.

PHYSIOLOGICAL CONSIDERATIONS FOR PARA-SCI

A SCI will affect body function and composition, metabolism, and energy expenditure (8). Furthermore, PARA-SCI include a diverse group with different etiologies and physiological characteristics (9). For example, an individual with an acute SCI will present differently than an individual with a chronic SCI or an individual with tetraplegia compared with paraplegia.

An examination of the physiological adaptations following the injury describes the impact of a SCI on the body. Post-injury, there are dramatic changes with a partial or complete loss of neurological (motor and sensory) function and changes in the activation of the autonomic nervous system below the level of the injury. Muscle atrophy is severe, with an estimated 40% reduction in total skeletal muscle cross-section area at 6 weeks and a further 20% 2 years post-injury, resulting in a potential 60% reduction in total lean muscle mass (10, 11). Mechanistically, the reduced muscle mass and increased fat mass, often referred to as secondary sarcopenia (12), has been linked to a breakdown in the excitation–contraction coupling in the skeletal muscle, reductions in protein synthesis, decreases in anabolic hormones (e.g., testosterone, growth hormone, and insulin-like growth factor-1), and increased proteolysis in addition to the immobilization (12, 13). Body composition assessments in individuals with SCI show increased total fat mass and reduced total fat free mass (14) as compared with able-bodied controls. PARA-SCI have higher body fat percentages and lower lean body mass in the legs and entire body compared with able-bodied controls (15, 16). Alterations in body composition in PARA-SCI result in a reduction in resting metabolic rate (5). Finally, "sit-forms" of exercise result in reduced energy expenditure during physical activity (8).

Skeletal muscle atrophy and increased sedentary time increase the risk of glucose intolerance, potentially leading to insulin resistance (17). Osteoporosis, oxidative stress, chronic systemic inflammation, reduced cardiovascular efficiency, dyslipidemia, and cardiovascular disease (CVD) risk are also elevated with a SCI (17). While physical activity can positively impact the aforementioned health concerns, CVD risk remains elevated in Paralympic athletes with a SCI (18). A more recent assessment comparing Paralympic athletes with an amputation vs. a SCI found that PARA-SCI with a high lesion SCI had a higher platelet-derived cardiovascular risk as compared with those with an amputation, which the authors attributed to malnutrition (17).

A SCI significantly affects the gastrointestinal (GI) tract, with GI complications accounting for ∼11% of hospitalizations in individuals with a SCI (19). A SCI results in impaired colonic motility, vascular tone, and mucosal secretions (20). Individuals with a SCI often develop neurogenic bowel and an estimated 20–60% of the population experience changes in bowel function (19). Neurogenic bowel manifests as changes in the bowel physiology (e.g., colon motility and/or loss of anorectal sphincter function) and altered GI transit time (21). Often, this leads to constipation and impaction (19). In an effort to mitigate the constipating effects of neurogenic bowel, individuals with SCI may use suppositories, laxatives, and/or fiber supplements (20). A SCI can also increase the GI transit time (21), which may affect nutrient absorption and negatively impact mucosal function. In addition, changes in gut motility can lead to variations in the composition of the gut microbiota, creating a state of dysbiosis, whereby the balance between beneficial and pathogenic bacteria is negatively skewed (22). Furthermore, antibiotic use due to increased urinary tract infections, pneumonia, and pressure ulcers is elevated in this population (23). Consistent antibiotic use affects the beneficial gut bacteria in addition to the harmful strains, resulting in an unfavorable composition of the gut microbiota. Malnutrition, physical inactivity, and psychological stress can also exacerbate the effects of a SCI on the gut microbiome (24). Indeed, male patients with a SCI had increased gut dysbiosis as compared with an able-bodied control group (20). This creates a vicious circle, with a SCI causing gut dysfunction, leading to gut dysbiosis, subsequently impairing immune function, which, in turn, increases susceptibility to infections (22).

A SCI increases the risk of neurogenic bladder (25), whereby major urologic complications evolve including urinary tract infections, bladder diverticula, bladder stones, urethral trauma, bladder cancer, hydronephrosis, and renal failure. Athletes are at an elevated risk as for urinary tract infections and their presence impairs performance due to a loss of training days or withdrawal from competition (26). Another concern is upper GI dyspepsia, which is often treated with proton-pump inhibitors to reduce acid production (19). There is also evidence that sensitivity of vagal afferents to neuroactive peptides, neurotransmitters, and macronutrients may be diminished in a SCI (19), further complicating the matter. Finally, dysphagia, disordered swallowing function, is prevalent in this population, and consequently may affect food choices and eating habits (27). In conclusion, the physiological adaptations to the injury and its secondary complications may influence the protein needs of PARA-SCI.

ROLE OF DIETARY PROTEIN IN BODY COMPOSITION AND SPORT PERFORMANCE AS IT RELATES TO PARA-SCI

Research regarding the role of protein in PARA-SCI is limited; however, the effects of protein on sport and exercise performance in able-bodied athletes are well-researched. The role of dietary protein in sport is multifactorial, affecting muscle protein

synthesis (MPS), lean body mass, strength and power, energy production, and muscle damage/repair. With respect to body composition, skeletal protein turnover is a dynamic process that is highly responsive to exercise and dietary protein intakes. Muscle protein balance is negative in response to resistance exercise in the absence of feeding; however, it increases if amino acids are provided (28, 29). Protein/amino acid (AA) supplementation, in combination with resistance exercise, demonstrates a dose-dependent effect on MPS, with some suggesting 20 g of high-quality protein as optimal for able-bodied athletes (29). Mechanistically, compared with recovery from an acute bout of resistance exercise, in fasted or carbohydrate-fed state, protein supplementation results in higher activation of the mammalian target of rapamycin complex 1 (mTORC1), a crucial myocyte protein signaling MPS (30). In addition to promoting MPS, dietary protein may play a role in maintaining lean body mass, evidenced by studies looking at athletes aiming to lose weight while conserving muscle mass (31). Research regarding MPS and dietary protein in PARA-SCI is lacking; however, it is hypothesized that the aforementioned effects and mechanisms would apply to PARA-SCI in some capacity.

Performance wise, increased muscle strength was found when protein supplements were added to a resistance exercise program (32). The same systematic review concluded that protein supplementation could also improve aerobic and anaerobic power. The authors note, however, that the results were inconsistent in both cases (32). Protein also has a role in energy production. For example, leucine is an AA that can be oxidized during endurance exercise to a considerable extent (6). Studies have begun to explore the impact of protein on endurance exercise; however, results are inconclusive (28). Branched chain AA (BCAA)—leucine, isoleucine, and valine—intakes during exercise reduce perceptions of central fatigue and perceived exertion, and increase mental performance; however, other studies fail to support these improvements (33). Finally, protein combined with carbohydrate intake during ultra-endurance exercise may reduce subjective measures of muscle soreness and markers of muscle damage (28). Protein supplementation is also likely to be beneficial for PARA-SCI with respect to anaerobic power and possibly aerobic performance; however, insufficient evidence is available to make any strong conclusions.

To summarize, dietary protein plays an important role in muscle hypertrophy and remodeling, maintenance of lean body mass, and the optimal functioning of metabolic pathways. Emerging evidence suggests that protein has additional roles in exercise performance and training adaptations, which require further investigation.

PROTEIN RECOMMENDATIONS FOR ABLE-BODIED ATHLETES WITH RELEVANCE TO PARA-SCI

Determining protein needs for PARA-SCI is complex; consequently, protein recommendations for this population are not well-established nor is there universal agreement. Considering these limitations, we will outline the guidelines established for able-bodied athletes with relevance to PARA-SCI.

Protein recommendations for athletes should take into account the frequency, intensity, and type of exercise, with athletes focused primarily on strength sports and those desiring muscle hypertrophy typically requiring the highest amounts. The athlete's goals, as well as, their training phase are also critical considerations. Increasingly, there is a focus on periodized nutrition, whereby intakes are matched to the specific training sessions within a periodized plan rather than a general classification as strength or endurance athlete (34). All considered, however, recommendations for all athlete types are elevated as compared with non-athletes (34). Many of these same principles will likely apply to PARA-SCI, and there is evidence to suggest that individuals with a SCI experience muscle hypertrophy in response to exercise (35). However, there is little evidence in PARA-SCI athletes, and the role of dietary protein in MPS has not been determined in this population. While a similar response to able-bodied athletes is hypothesized, confirmation is required, and there is the possibility of differing responses including magnitude and amount of time required to see an effect.

Protein recommendations are made with respect to the quantity and timing of protein intakes. The recommended daily allowance (RDA) of the able-bodied population is 0.8 g/kg body mass per day (36). In able-bodied athletes, the amount of protein required each day ranges from 1.2 to 2.0 g/kg body mass (34). Typically, it is advised to space protein meals about 3 h apart, as well as before and after strenuous exercise (28). Importantly, even higher protein intakes, ranging from 2.3 to 3.1 g/kg body mass, may be advised for short time periods in resistance-trained athletes, when overall energy intakes are being reduced (e.g., during weight loss) (31). Endurance athletes are recommended to consume between 1.6 and 2.4 g/kg protein during caloric restriction (31). Conversely, protein intakes above 2.0 g/kg body mass in weight-stable, endurance athletes do not appear to improve performance (28). With respect to individual servings, for MPS, generally 0.25 g/kg of body weight or 20–40 g of protein is suggested (28). Further research is required to determine if these recommendations are relevant to PARA-SCI, while, as of yet unstudied, it is interesting to consider if protein recommendations based on total body mass are appropriate for PARA-SCI, as they are for able-bodied athletes, or if lean muscle mass would be more appropriate. Furthermore, the importance of acquired vs. congenital lesions and variance in functional muscle mass may affect protein recommendations.

The timing of protein intake is a key factor in optimizing performance, as MPS is upregulated for at least 24 h post-resistance training, and there is an increased sensitivity to dietary protein intakes during this time (34). Practically, protein intakes tend not to be evenly spaced throughout the day, and are often insufficient at breakfast and excessive in later meals. To take advantage of the increased sensitivity, multiple protein-containing meals and snacks post-exercise and during the day are advised. These recommendations hold true for all types of exercise, even if muscle hypertrophy is not the athlete's primary goal (34). Furthermore, the intake of protein or amino

acid combinations before and during resistance exercise will maximize muscle repair and hypertrophy (6, 7, 28). While these recommendations seem reasonable for PARA-SCI, to our knowledge, spacing and protein timing, as it relates to MPS, has not been studied in this population. Less is known regarding the potential benefit, or harm, of protein intake before and during endurance exercise. It is suggested that protein feeding during exercise might help maintain a favorable anabolic hormone profile, minimize increases in muscle damage, and increase time to exhaustion during prolonged running and cycling (28). Notably, time trials are considered to be a better indicator of performance than time to exhaustion tests. Here, a meta-analysis reported favoring a carbohydrate–protein combination; however, the individual studies were mixed (37). Given the potential for reduced muscle soreness, a recent position statement by the International Society of Sports Nutrition recommends 0.25 g/kg body mass of protein per hour of endurance exercise in combination with carbohydrate (28); however, caution is advised due to the potential for gastrointestinal discomfort. The applicability of dietary intakes during exercise for athletes with a SCI is questionable given the increased challenges regarding the gastrointestinal system. Finally, the potential benefits of protein intake pre-sleep have been investigated, and evidence suggests 30 g of protein, particularly casein, before going to bed may stimulate MPS and recovery (28). The effects of pre-sleep protein on PARA-SCI would be of interest, as this is an intervention that could be incorporated into daily routines.

Standard protein recommendations for athletes following a purely plant-based diet may not be optimal. Lynch et al. (38) and Rogerson (39) recommend increasing protein intake in vegan athletes up to 1.7–2.0 g/kg body mass, with an even higher amount during weight loss or energy restriction (e.g., up to 2.7 g/kg). A higher intake might be required to include a sufficient amount of essential AA through their diet. Furthermore, a wider variety of food sources seems to be needed to obtain this goal. The American Dietetic Association concluded, however, that if caloric requirements are met, protein or AA needs should be attained for vegan or vegetarian athletes (40). Studies assessing the effects of vegan or vegetarian PARA-SCI athletes are unavailable to our knowledge.

PROTEIN RECOMMENDATIONS FOR PARA-SCI

Protein intakes for PARA-SCI are undetermined with Goosey-Tolfrey et al. noting "there is a dearth of reported literature assessing the protein or amino acid requirements for athletes with SCI" (41). While there are recommendations for able-bodied athletes and limited guidelines for individuals with a SCI, PARA-SCI have received less attention. As PARA-SCI maintain active muscle mass in the upper body, we can imagine a similar response to strength training and MPS as shown in able-bodied athletes. We must acknowledge, however, that intervention trials, with a sufficient sample size, investigating the effect of strength training and protein supplementation on the stimulation of MPS in PARA-SCI are lacking. Considering the aforementioned physiological factors associated with a SCI, and the effect of sport on protein needs; we postulate that neither the able-bodied athlete recommendations nor non-athlete SCI recommendations be directly transposed onto the PARA-SCI population. In addition, athletes with SCI compete in different sports, which have different energy demands. Training volume, intensity, and exercise type as well as secondary conditions (e.g., gastrointestinal motility, pressure ulcer) will affect protein demands (**Figure 1**). Finally, the heterogeneity of PARA-SCI with respect to the level of injury, injury type (e.g., complete or incomplete), and the time since injury (5) adds additional challenges.

If one extrapolates from able-bodied athletes, the logical assumption would be to propose increased protein needs for optimal performance; however, rigorous scientific study is required to determine if the able-bodied recommendations are safe/appropriate or how they need to be adjusted.

Importantly, there are several factors unique to PARA-SCI that indicate caution before universally recommending higher intakes. The first consideration is the impact of total energy expenditure (TEE) on protein intakes. While a few studies have looked at estimating TEE in PARA-SCI (8, 42), additional work is required, especially in females. Ambiguity surrounding TEE is a barrier to the determination of protein needs, as it makes it difficult to determine the appropriate overall energy intake and subsequently protein needs. Regardless, the available evidence would suggest lower TEE in PARA-SCI than able-bodied athletes (5, 8), necessitating reduced food intakes. Reductions in TEE will make it difficult to obtain sufficient protein in the context of the overall macro- and micronutrient balance. For example, if energy intakes in PARA-SCI are lower than able-bodied athletes, due to reduced TEE, and protein recommendations remain unchanged, fat and/or carbohydrate intakes will need to be reduced. The importance of this is highlighted in context of elevated body fat found in PARA-SCI (11). Second, neurogenic bladder and associated complications could have implications on dietary protein intakes (e.g., decreased intake), as it seems possible that a high-protein diet might have some negative consequences in patients with kidney disease (43). In addition, bladder overactivity treated with anticholinergics can further diminish GI motility and affect diet due to food–drug interactions (44). Athletes should undergo a yearly medical screening including a check of bladder and kidney function, if they are considering a higher-protein diet. Finally, the impact of neurogenic bowel disease and gut dysbiosis should be considered, as this could affect fiber and hydration needs, ultimately affecting the overall diet and food choices.

The impact of high-protein diets, on the metabolic health of those with a SCI, is of interest; however, little is known. A recent study found that in five individuals with a long-standing SCI, an isocaloric high-protein diet (30% of total energy) resulted in reduced fat mass with no change in lean mass after an 8-week intervention (45). An additional study looking at a low-carbohydrate/high-protein diet is underway (46). To our knowledge, high-protein diets have not been tested in PARA-SCI. It should be noted, however, that studies suggest athletes with a SCI are at risk for overall low energy availability (47) and

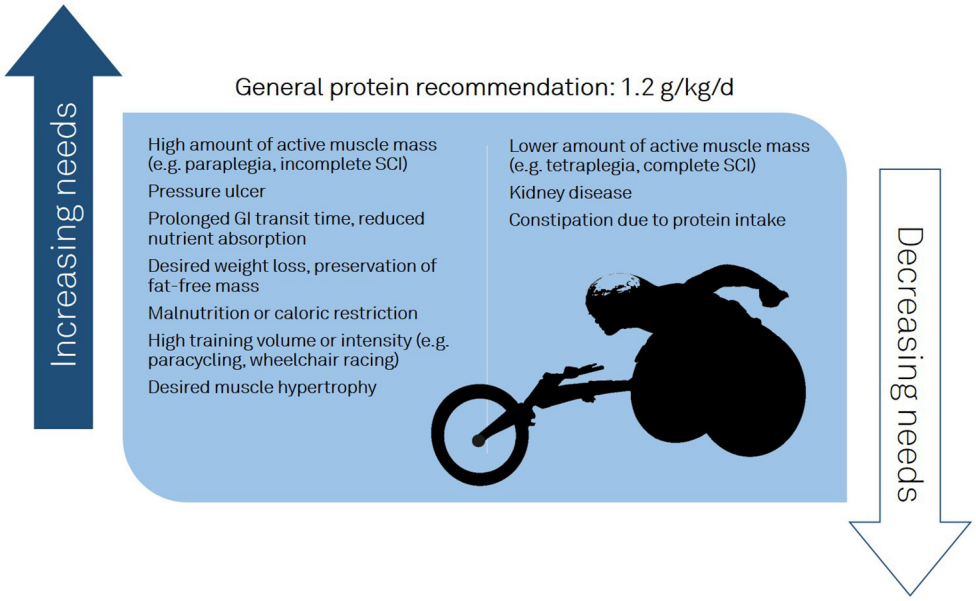

FIGURE 1 | Factors influencing protein recommendations in athletes with a spinal cord injury.

increased protein intakes could help when athletes are in caloric restriction or during phases of intended weight loss.

Another key factor in considering protein recommendations for PARA-SCI is pressure ulcers. Individuals with a SCI are at a high risk of developing pressure ulcers, which increases protein needs for wound healing (48). Conversely, nutritional factors, such as malnutrition and anemia, can further increase the risk of pressure ulcers (49). The treatment guidelines concerning nutritional therapy include a protein intake of 1.25–2.0 g/kg body mass for adults with a chronic SCI and a pressure ulcer or at risk to be malnourished, and 2.0 g/kg body mass for individuals with an acute SCI (50). Furthermore, the guidelines recommend the use of high-caloric, high-protein fortified foods or nutritional supplements in patients with a high risk for malnutrition or an existing pressure ulcer, especially if nutritional needs cannot be achieved through normal dietary intakes. In addition, the review states that arginine supplementation might be beneficial for the treatment of pressure ulcers (50). Brewer et al. (51) found supplementation with 9 g of arginine during the healing phase of a pressure ulcer significantly reduced the time required to heal as compared with controls from retrospective data (10.5 ± 1.3 weeks vs. 21.0 ± 3.7 weeks). In addition, a recent systematic review (52) revealed that arginine could potentially improve wound healing in malnourished and non-malnourished able-bodied individuals with a pressure ulcer. Considering the evidence available, arginine supplementation could be possibly applied in addition to the other nutritional guidelines (50).

At present, we are unable to present robust scientific evidence for protein intakes in PARA-SCI; however, we can extrapolate from recommendations for those with a SCI and able-bodied athletes. Evidence would indicate that in individuals with a chronic SCI, 0.8–1.0 g/kg/day of protein should be sufficient to achieve protein balance (53). Given that there is significant evidence that able-bodied athletes require elevated protein intakes, it is reasonable to assume an increased need in PARA-SCI as well. Furthermore, a minimum of 1.25 g kg BW is recommended for those with a SCI with pressure ulcers, suggesting this amount is safe for the SCI population (50). All considered, it would be reasonable to recommend 1.2 g kg BW as a minimum (**Figure 1**), particularly for those desiring muscle hypertrophy and those with a high TEE (e.g., high training volume/intensity, large amount of active muscle mass).

PROTEIN INTAKE REPORTED

Nutrient intake in individuals with a chronic SCI has been reported in previous studies (5). **Table 1** provides a summary of protein intakes in individuals with a SCI. Data are presented as total daily protein intake and protein intake per kilogram body mass. Total protein intakes ranged from 56 to 96 g/day in females and 63 to 95 g/day in males (**Table 1**). Generally, protein intakes were above the recommended intake of 0.8 g/kg body mass. Doubelt et al. (56) mention that 25% of the participants had intakes below 0.66 g/kg body mass, which corresponds to the estimated average requirement described by them. In addition, Gorgey et al. (57) showed an intake below 0.8 g/kg body mass in manual wheelchair users. When nutrient intakes in individuals with an acute or chronic SCI were compared, Perret and Stoffel (65) found no significant differences in protein intakes. Differences in protein intake between individuals with paraplegia or tetraplegia per kilogram body mass were not consistently observed; nor was gender a factor. However, the limited available literature does not allow any differentiated distinction between protein intake based on

TABLE 1 | Protein intake in individuals with chronic SCI (non-athletes).

References	Subjects	Number of subjects	Methods	Daily protein intake
Allison et al. (54)	Anti-inflammatory diet in chronic SCI:	20 (n = 12 intervention group, eight control group)	7-day food diary at baseline, 3-day food diary at 1, 2, and 3 months	Intervention group: Baseline 73 ± 24 g/day[a] 3 months: 95 ± 22 g/day[a] No data for control group shown
Barboriak et al. (55)	Individuals with a SCI (15 with paraplegia, 22 with tetraplegia)	37	24-h recall and checking and weighing leftover meals	Paraplegia: 95 ± 32 g/day = 1.3 g/kg/day Tetraplegia: 86 ± 28 g/day = 1.2 g/kg/day
Doubelt et al. (56)	Individuals with SCI (22 with tetraplegia, 12 with paraplegia, 94% male)	34	Food frequency questionnaire	82 g/day = ∼1.0 g/kg/day; 25% of the participants below 0.66 g/kg/day
Gorgey et al. (57)	Men with chronic motor complete SCI (10 with paraplegia, six with tetraplegia)	16	5-day food dietary log for 4 weeks	Manual wheelchair users: 65 g/day = ∼0.75 g/kg/day
Gorgey et al. (58)	Chronic SCI, five participants resistance training (RT), four participants in the control group (C)	9	Daily food diary for 12 weeks	RT: 1.1 ± 0.29 g/kg/day C: 1.09 ± 0.24 g/kg/day
Groah et al. (59)	Individuals with SCI (24 males with tetraplegia, 37 males with paraplegia, one female with tetraplegia, 11 females with paraplegia)	73	4-day food log	Male tetraplegia: 85.7 g/day[a] Male paraplegia: 87.6 g/day[a] Female tetraplegia: 95.5 g/day[a] Female paraplegia: 75.3 g/day[a]
Javidan et al. (60)	Patients with SCI (217 male, 48 female)	265	24-h dietary recall interviews	69.6 g/day = 1 g/kg/day 86.8% below 1.5 g/kg/day
Levine et al. (61)	Individuals with chronic SCI (24 male and 9 female)	33	7-day dietary record and a food frequency chart	Male: 69 g/day[a] Female: 56 g/day[a]
Lieberman et al. (62)	Individuals with chronic SCI	100	Food frequency questionnaire	100.3 g/day[a]
Nightingale et al. (63)	Individuals with paraplegia	33	Weighted food diary for 7 days	74.6 g/day = 0.98 g/kg/day
Pellicane et al. (64)	Inpatient rehabilitation (eight with paraplegia, eight with tetraplegia)	16	After meals, calculation of energy intake by examining food trays	All SCI: 71.5 ± 25.0 g/day Paraplegia: 0.86 ± 0.37 g/kg/day Tetraplegia: 0.92 ± 0.43 g/kg/day
Perret and Stoffel-Kurt (65)	Patients with an acute and a chronic SCI	24 (12 per group)	Daily food diary for 7 days	Acute: 74.6 ± 10.0 g/day = 1.07 g/kg/day Chronic: 71.4 ± 7.9 g/day = 1.07 g/kg/day
Sabour et al. (66)	Individuals with a chronic SCI	162	Semiquantitative food frequency questionnaire	Complete SCI: 64. 7 ± 23.2 g/day[a] Incomplete SCI: 64.3 ± 24.9 g/day[a] Tetraplegia: 63.3 ± 23.7 g/day[a] Paraplegia: 65.4 ± 25.4 g/day[a]
Tomey et al. (67)	Individuals with a chronic SCI	95	Semiquantitative food frequency questionnaire	82.3 ± 31.7 g/day[a]
Walters et al. (68)	Individuals with a chronic SCI (63 males, 14 females)	77	Multiple-pass 24-h recalls	Male: 81.8 g/day = ∼1.03 g/kg/day Female: 70.9 g/day = ∼1.0 g/kg/day

SCI, spinal cord injury.
[a]*Body mass not reported.*

lesion level (e.g., para- vs. tetraplegia) nor between men and women. In conclusion, the majority of this population exceeded the RDA of 0.8 g/kg body mass for healthy adults and individuals with a SCI, suggesting protein intakes are adequate in the general SCI population.

Protein intakes in PARA-SCI are presented in **Table 2**. Relative protein intakes ranged from 1.1 to 1.9 g/kg body mass. Gender differences are inconclusive, as Gerrish et al. (71) and Madden et al. (75) showed higher intakes in male athletes; whereas Krempien and Barr (74) found higher intakes in female athletes.

TABLE 2 | Protein intake in athletes with SCI.

References	Subjects	Number of subjects	Methods	Daily protein intake
Eskici and Ersoy (69)	Female wheelchair athletes	22	24-h retrospective diet recall	Women 1.6 ± 0.3 g/kg/day
Ferro et al. (70)	Male elite wheelchair basketball players	11	3-day food-weighing diary in 2 months during the pre-competitive period	May: 1.7 ± 0.6 g/kg/day June: 1.5 ± 0.5 g/kg/day
Gerrish et al. (71)	Canadian and US elite wheelchair athletes (tennis, track, basketball, and rugby)	19 women 20 men	Self-reported, single 24-h food journal in autumn and winter	**Autumn:** Women 1.1 ± 0.3 g/kg/day Men 1.7 ± 0.2 g/kg/day **Winter:** Women 1.3 ± 0.4 g/kg/day Men 1.3 ± 0.3 g/kg/day
Goosey-Tolfrey and Crosland (72)	Wheelchair Games player	14 women 9 men	7-day food-weighing diary over seven consecutive days	Women: 1.00 ± 0.29 g/kg/day Men: 1.37 ± 0.33 g/kg/day
Grams et al. (73)	Male wheelchair basketball players (5 amputees, 12 with SCI)	17	3-day weighed food journal over three consecutive days during three training camps over two consecutive years	**Training camp 1** 1.6 ± 0.7 g/kg/day **Training camp 2** 1.5 ± 0.5 g/kg/day **Training camp 3** 1.9 ± 0.7 g/kg/day
Krempien and Barr (74)	Elite athletes with a spinal cord injury	8 women 24 men	3-day self-reported food journal kept at home and training camp	**Training camp:** Women 1.7 ± 0.3 g/kg/day Men 1.4 ± 0.4 g/kg/day **Home:** Women 1.6 ± 0.6 g/kg/day Men 1.3 ± 0.4 g/kg/day
Madden et al. (75)	Various different wheelchair sports, mainly wheelchair basketball	22 women 18 men	3-day, consecutive self-reported food journal	Women 1.4 (1.1–1.6) g/kg/day Men 1.6 (1.4–2.2) g/kg/day

SCI, spinal cord injury.

Furthermore, the time point of the season at which protein intake was assessed seemed to influence the intake (70, 71, 73). Possible explanations for the effect of season include differences in training intensity, training focus (e.g., strength vs. endurance), or total training hours (e.g., difference in energy expenditure).

In comparison with the data presented in **Table 1**, protein intakes based on body mass appear to be higher in PARA-SCI compared with the general population with a SCI. A comparison with able-bodied athletes is difficult given variability in sport and the large range in reported intakes. However, a benchmark could be found in a Dutch study, including 553 data sets from different sports, where 80% of able-bodied athletes met the minimum recommendation of 1.2 g/kg body mass (76). To summarize, PARA-SCI typically meet or exceed the minimum able-bodied athlete recommendations for protein and intakes fall within a similar range.

PROTEIN SUPPLEMENTATION IN SCI

Research in able-bodied athletes indicates protein supplementation (e.g., whey) following a strength session or high-intensity interval training could increase post-exercise MPS, thereby enhancing training stimulus, recovery, and training adaptation (7, 28, 31). Only a few studies have investigated the effects of protein supplementation in individuals with a SCI (77–79). Kressler et al. (78) divided 11 individuals with a cervical lesion level (male and female) into two groups and supplemented them with whey protein immediately after a circuit training or on a rest day. Performance in a one-maximum repetition test significantly increased in both conditions, but anaerobic capacity and fatigue resistance might have been further enhanced in the circuit-training group. A very similar study (77) showed no effect of protein supplementation on fat oxidation. Both studies seemed to be underpowered, as evidenced by the small sample size; therefore, it is difficult to draw any definitive conclusions. Nash et al. (79) supplemented three subjects with either a whey + carbohydrate or a soy supplementation + sweetener in individuals with a lesion between C5 and T4 during ambulation. Some positive effects on time or distance of ambulation can be shown with the whey + carbohydrate compared with soy supplementation, but again, the results are limited by the small sample size. A similar conclusion was made by Navarrete-Opazo et al. (80) in their review article on protein supplementation in individuals with a SCI, as those studies show a risk of bias, small sample sizes, and missing data. Consequently, it would be premature to comment on the effectiveness of protein supplementation in PARA-SCI.

FUTURE DIRECTIONS

The field of performance nutrition in PARA-SCI is emerging and there is a need for future studies regarding optimization of dietary protein intakes. Going forward, nitrogen balance studies may be beneficial in determining overall protein needs in PARA-SCI, as they have been used in able-bodied athletes (81). Protein timing, dosage, and quality require exploration in

the context of MPS and muscle adaptation in PARA-SCI. The safety of elevated doses as well as appropriate screenings and protocols also need to be established in this population. Due to the effects of a SCI on the GI system (e.g., transit time) and nutrient absorption, the dosage and timing might play a role. Furthermore, studies measuring muscle hypertrophy and strength in response to protein intakes in PARA-SCI are also required, as nitrogen balance alone will likely be insufficient to determine optimal levels for performance (6). Critically, although challenging, the aforementioned studies will need to be undertaken with sufficiently large samples sizes and scientific rigor to ensure confidence in the conclusions. Finally, the available research does not adequately consider the impact of acquired vs. congenital lesions, lesion level or completeness, and variance in functional muscle mass. A breakdown of the role of protein and recommendations by these factors would be valuable.

CONCLUSION

To conclude, determining protein intakes for PARA-SCI will be challenging as optimal physical performance and physiological changes associated with a SCI need to be considered. The heterogeneity of the population will further complicate recommendations. The lower energy demands of a PARA-SCI and risk of experiencing secondary complications such as pressure ulcers (increased protein demands) or a kidney disease (reduced protein intake) may also alter protein recommendations. Future studies are needed to determine the effectiveness and safety of protein supplementation in PARA-SCI. While barriers exist to determining recommendations, they are essential and these challenges do not excuse the scientific community from working with PARA-SCI. Presently, in the absence of clinical trials, it seems prudent to recommend that PARA-SCI consume a minimum of 1.2 g/kg body mass, as this has been established as a minimum recommendation for able-bodied athletes. It can be assumed that intakes at this level will be well-tolerated in this population, as amounts in this range are commonly recommended for individuals with a SCI experiencing pressure ulcers.

AUTHOR CONTRIBUTIONS

JP and JF: conceptualization, writing—original draft, and writing—review and editing. Both authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Low Protein Diets and Energy Balance: Mechanisms of Action on Energy Intake and Expenditure

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Low protein diets are associated with increased lifespan and improved cardiometabolic health primarily in rodents, and likely improve human health. There is strong evidence that moderate to severe reduction in dietary protein content markedly influences caloric intake and energy expenditure, which is often followed by a decrease in body weight and adiposity in animal models. While the neuroendocrine signals that trigger hyperphagic responses to protein restriction are better understood, there is accumulating evidence that increased sympathetic flux to brown adipose tissue, fibroblast growth factor-21 and serotonergic signaling are important for the thermogenic effects of low protein diets. This mini-review specifically focuses on the effect of low protein diets with variable carbohydrate and lipid content on energy intake and expenditure, and the underlying mechanisms of actions by these diets. Understanding the mechanisms by which protein restriction influences energy balance may unveil novel approaches for treating metabolic disorders in humans and improve production efficiency in domestic animals.

Keywords: low protein, food intake, energy expenditure, neuroendocrine, energy balance

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INTRODUCTION

Energy balance is a fundamental biological process that is dependent on a complex interplay of calories consumed as macronutrients (carbohydrate, fat, and protein), and energy expended and stored. A dysregulation of the mechanisms that sense and signal dietary nutrients in the gut may predispose to obesity and metabolic complications. Among the macronutrients, the intake of protein is tightly regulated and dietary protein restriction is purported to extend lifespan, improve energy balance and cardiometabolic health (1); however, the underlying mechanisms remain poorly understood. Here we review the potential mechanisms by which dietary protein restriction modulates energy homeostasis to alter energy intake and energy expenditure (EE).

REGULATION OF FOOD INTAKE BY LOW PROTEIN DIETS

Effect of Dietary Protein Restriction on Food Intake

The "protein leverage" hypothesis posits that protein intake is tightly regulated in several species including rodents, small animal pets, birds and humans (2–4). When isocaloric high protein diets are fed, in order to keep the amount of protein consumed constant and avoid protein excess, the total caloric intake and hence the intake of carbohydrates and fats is reduced. As a corollary, when low protein diets are fed, in order to avoid protein deficiency and to meet the protein requirements,

the total food consumption is increased and hence the total caloric intake from carbohydrate and fat is also increased as a consequence (2, 3, 5). In humans, diets that are moderately deficient in protein were reported to increase food consumption in some (6–8), but not all (9, 10) studies. Notably, protein intake across 13 countries was found to be remarkably stable at ∼16% of total calories (11), and even partial protein leverage caused by a reduction in protein intake was predicted to contribute to at least one-third of weight gain and the obesity epidemic (12). In contrast to other species, previous studies were unable to detect a hyperphagic response to mild [<25% lower crude protein (CP) than requirements] and moderate (25–50% lower CP than requirement) protein restriction in pigs. We and others showed that moderate protein restriction (12–14% CP) reduced feed intake in pigs (13–15), but slightly low protein diets (25% lower CP than requirement) did not change their energy intake (14, 16, 17). Reduced food intake in response to low protein diets (22% metabolizable energy) has been also reported in cats (18). However, severely low protein diets (50% lower CP than requirements) have been shown to increase the energy intake in pigs (19–21). A caveat is that most swine studies have primarily focused on improving production efficiency by supplementing essential amino acids to low protein diets, which adds complexity in interpreting the energy intake data. The resistance of species such as pigs to mount a hyperphagic response to mild to moderate dietary protein restriction, but showing an increased energy intake in response to severe protein restriction, is suggestive of differences in protein dilution threshold sensing by different species, which warrants further studies. Thus, gaining insights into the mechanisms of food intake regulation by low protein diets is important for developing effective prevention strategies for weight gain and improving feed efficiency.

Mechanisms of Sensing Protein Insufficiency

The hepatic amino acid sensing and signaling mechanisms play an important role in detecting amino acid insufficiency to coordinate a systemic response to restore protein balance. A relative deficiency of dietary amino acids leads to accumulation of uncharged cytoplasmic tRNA that bind to general control non-derepressible (GCN2) which in turn phosphorylates eukaryotic translation initiation factorα (eIF2α) leading to activation of activating transcription factor 4 (ATF4) and CCAAT/Enhancer-binding protein homologous protein (CHOP) to inhibit protein synthesis and increase fibroblast growth factor-21 (FGF21) expression and secretion (22–25). Further, GCN2 through other intermediaries inactivates mTORC1 leading to dephosphorylation of 4E-binding protein (4EBP1) to inhibit protein synthesis (22, 26). Consistent with these studies that were mostly conducted *in vitro* and with amino acids, we (27) and others (28–30) showed that a similar pathway for sensing dietary protein deficiency also operates in the liver to upregulate hepatic FGF21 expression and secretion. Interestingly, we found that similar amino acid sensing pathways were also upregulated in the duodenum (31) suggesting that the intestine may detect protein deficiency prior to the liver, and/or that the intestinal sensing may serve to amplify the hepatic response to protein restriction. Independent of sensing by gut-associated tissues, amino acid deprivation causes a rapid anorexic response that is triggered by GCN2 signaling in the piriform cortex (32, 33).

Mechanisms of Regulation of Food Intake by Low Protein Diets

Accumulating evidence indicates that moderate protein restriction in rodents (5–8% protein kcal) stimulates FGF21 secretion from the liver which acts on β-klotho receptors in the brain to promote hyperphagia (34–36) (**Figure 1**). We have also shown that such hyperphagic responses to protein dilution are a consequence of increased meal size in rodents (31). The hyperphagic responses to moderate protein dilution are also associated with reduction in circulating concentrations of leptin and IGF-1, increased plasma ghrelin, and upregulation of orexigenic neuropeptide Y transcripts in the rodent hypothalamus (27, 31, 37, 38). Although the role of anorexigenic lower gut peptides such as peptide YY and glucagon-like peptide-1 (GLP-1) in high-protein induced hypophagia is well-documented in rodents (39–41) and humans (42), we were unable to detect a graded insufficiency in circulating concentrations of these and other anorectic gut hormones (e.g., gastric inhibitory polypeptide and amylin) in rats (27, 31) that might explain the hyperphagia with protein dilution. It is unknown whether sensitivity to hypophagic effects of these satiety hormones is impaired with dietary protein restriction. However, birds fed with low protein diets were found to have a higher occurrence of GLP-1-immunoreactive cells in the ileum (43). Though the hyperphagic responses to protein dilution are consistently observed in a no-choice condition (27–31, 36), when protein restricted rodents are given a choice of macronutrients, they tend to prefer protein (8, 34, 44) with reduced preference for carbohydrates (45, 46). Similarly, in humans, the fMRI BOLD responses to food cues were greater in reward areas such as orbitofrontal cortex and striatum under low protein conditions (7). In contrast to mild protein restriction, severe protein restriction or depletion (<5% protein kcal) leads to profound hypophagia with a rapid reduction in both meal size and number in rats (31). Such aversive responses are due to rapid sensing of the amino acid imbalance by the anterior piriform cortex where the GCN2–eIF2α pathway is activated to repress protein synthesis, together with activation of glutamatergic outputs to the hypothalamus to cause anorexia (47). The relative importance of peripheral and central sensing and signaling mechanisms that regulate food intake in response to varying degrees of dietary protein restriction across species remains to be resolved.

REGULATION OF ENERGY EXPENDITURE BY LOW PROTEIN DIETS

Effects of Low Protein Diets on Energy Expenditure

Protein or total amino acid restriction increases thermogenesis in rodents (27, 29, 31, 35, 36, 48–61). An increased EE was also

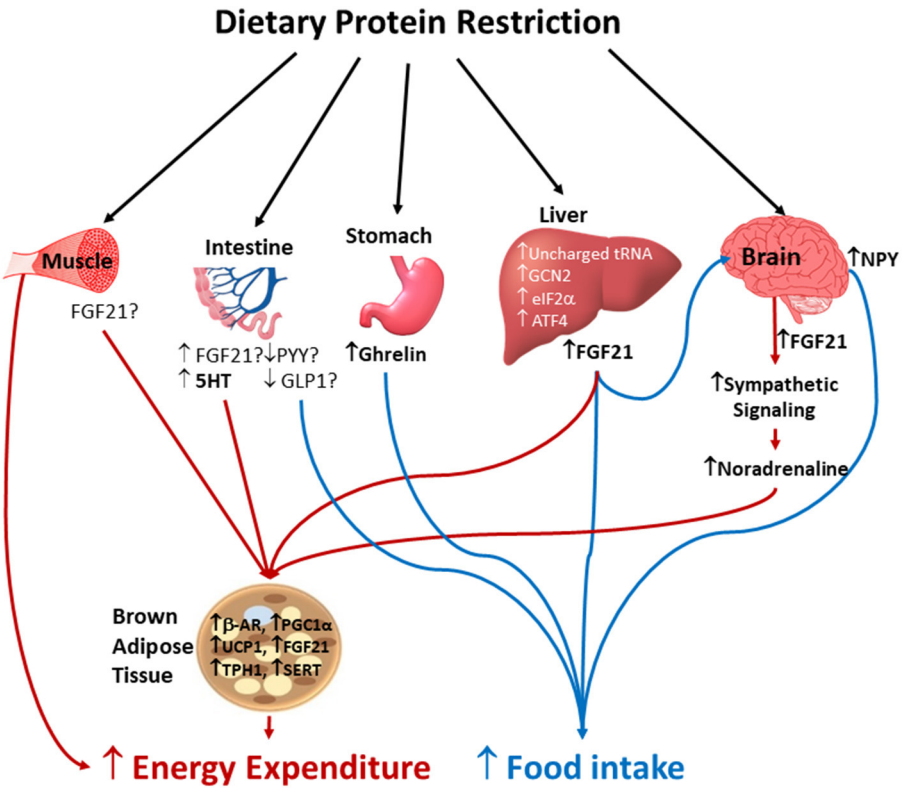

FIGURE 1 | Potential mechanisms by which protein dilution alters energy intake and energy expenditure. Protein restriction concurrently increases energy intake and energy expenditure. Amino acids insufficiency is sensed by liver, small intestine, and brain by the involvement of unchanged tRNA, non-derepressible (GCN2), eukaryotic translation initiation factorα (eIF2α) and activating transcription factor 4 (ATF4) which results in hyperphagia to restore protein balance. The low protein induced hyperphagia is possibly mediated by increased circulating concentrations of ghrelin and fibroblast growth factor-21 (FGF21), and neuropeptide Y (NPY) expression in the hypothalamus. Whether reduced secretion of anorexigenic gut peptides such as peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) contribute to increased energy intake following protein dilution is not completely known. The increased energy expenditure in response to protein restriction is mediated by multiple mechanisms including increased sympathetic flux and upregulation of thermogenic markers in brown adipose tissue (BAT), and muscle thermogenesis. Whether local FGF21 secreted by skeletal muscle, small intestine and BAT, and 5-hydroxytryptamine (5HT) signaling in BAT play a role in low protein induced thermogenesis via autocrine, paracrine and endocrine pathways remains unclear. B-AR, beta adrenergic receptors; UCP1, uncoupling protein-1; PGC-1α, proliferator-activated receptor gamma coactivator 1-alpha; TPH1, tryptophan hydroxylase 1; SERT, serotonin transporter.

reported in pigs fed with severely low protein diets (19–21). In one study, pigs maintained equal body weight when fed protein deficient diets with high or low energy content suggestive of enhanced EE in these groups (62). We also showed that moderate reduction of dietary protein results in an increased EE in early weeks of study in young pigs (13, 14). Similarly, protein restricted diets have also been shown to acutely increase EE in humans (63–65). Overall, increased EE and subsequently reduced food efficiency may contribute to reduced weight gain and lean mass during protein restriction (27, 36, 56, 58, 66).

Given the concurrent increase in EE and energy intake in response to protein restriction (27, 51, 56, 60), and as increased EE is generally considered a compensatory response to hyperphagia (67–69), one may question whether the enhanced EE with protein restriction is a consequence of increased energy intake. Leveraging effect of dietary protein content on energy intake has been reported previously (49) suggesting that low protein induced EE could be considered as a primary response to dietary protein content. Recently, we showed that protein restricted rats sustain an enhanced EE in the absence of hyperphagia, which is suggestive of an energy intake independent pathway for low protein induced thermogenesis (31). Likewise, others have shown that low protein induced EE occurs independent of hyperphagia (35) and that energy intake changes as a secondary response to compensate for the enhanced EE (70). Therefore, it appears that increased EE in response to protein restriction is partly related to enhanced basal metabolic rate component of total EE (53), although the contribution of basal metabolic rate and diet-induced thermogenesis was reported to be negligible (71). An increased spontaneous motor activity was reported in mice fed with low protein diets (71), which does not seem to be related with the overall activity level (69, 71). Further research is required to better understand the effect of low protein diets with variable dietary carbohydrate and fat contents on different components of total EE.

Due to high carbohydrate content of low protein diets (13, 14, 27, 48, 49, 51, 53–56, 60, 61) an increased EE in response to

such diets could be the result of either high carbohydrate or low protein content. In an independent study, using obesity-prone rats we showed that low protein diets with fixed carbohydrate, but variable fat contents increased EE (31), similar to low protein-high carbohydrate diets (27). A greater EE response to protein dilution was also observed in humans regardless of dietary carbohydrate and fat content (65). Altogether, these studies suggest that enhanced EE is likely a primary response to protein restriction rather than carbohydrate or lipid content, or energy intake; however, further studies are required to assess the contribution of dietary carbohydrate and fat content to enhanced EE under protein restriction.

Mechanisms of Regulation of Energy Expenditure by Low Protein Diets

Despite intense efforts to gain insights into the hyperphagic responses driven by protein dilution, the underlying mechanisms of changes in EE received less scrutiny. The thermogenic effects of low protein diets have been associated with (i) increased sympathetic flux to brown adipose tissue (BAT) via β-adrenergic receptor (β-AR) signaling (50, 51, 55–57, 72, 73), as well as stimulation of thermogenesis in white adipose tissue (74) and muscle (27), (ii) FGF21 and mitochondrial uncoupling protein-1 (UCP1) mediated mechanisms (27, 29, 31, 36, 70, 75) and (iii) serotonergic signaling (27, 76) (**Figure 1**).

The BAT plays an important role in diet-induced thermogenesis (77–79). An increased sympathetic influx to BAT appears to be essential for the thermogenic effects of low protein diets (50–52, 55, 56). This involves release of noradrenaline from postganglionic sympathetic nerve terminals and subsequent interaction of noradrenaline with β-AR, in particular β3-AR in BAT (80–83). We and others showed that the transcripts of adrenergic signaling and thermogenic markers including β2 and β3-AR, peroxisome proliferator-activated receptor gamma coactivator 1-alpha and UCP1 were increased in BAT of rats fed with protein deficient diets (27, 57). We also showed that low protein induced EE is attenuated following administration of propranolol, a β1 and β2-AR antagonist (27, 31). This suggests that low protein induced EE is mediated by sympathetic signaling.

Fibroblast growth factor-21 is released in response to nutrient deficiency and stimulates browning of white adipose tissue to regulate adaptive thermogenesis (53, 74). Infusion of FGF21 increases EE and core body temperature in rodent models (84–86). Using FGF21 deficient rodent models, the effect of protein restricted diets on basal and cold-induced EE have been shown to be FGF21 dependent (35, 61, 87, 88). Although hepatic FGF21 seems to stimulate BAT thermogenesis via an endocrine pathway (29, 30, 36, 74, 89), the FGF21 expression and secretion in BAT is also increased by sympathetic stimulation (72, 90). We and others showed that plasma FGF21 concentrations and transcripts in liver, small intestine, skeletal muscle and BAT were increased when rodents are fed with low protein diets (27, 31, 36, 53, 60, 75, 91). Further, methionine and leucine restriction increases FGF21 expression particularly in liver and circulation (92–94). Dietary protein restriction also increases circulating FGF21 concentrations in humans (36, 61, 63–65). FGF21 appears to be produced in variety of organs including skeletal muscle in response to cellular stress triggered by various stimuli (95–99). Whether FGF21 derived from BAT, muscle and other organs reaches the circulation and contributes to low protein induced thermogenesis via endocrine pathway remains unclear, but it appears that FGF21 most likely mediates the low protein induced EE through endocrine, paracrine and autocrine signaling. Further, FGF21 signaling in glutamatergic neurons of the ventromedial hypothalamus appears to be essential for the increase in EE with dietary protein dilution (45). The roles of peripheral and central FGF21 sensing and signaling mechanisms in the thermogenic effects of dietary protein restriction remains to be further delineated.

The role of serotonergic neurons in regulation of thermogenesis and BAT activity has been previously documented (100, 101). In particular, central serotonin signaling is crucial for the activity of BAT and thermoregulation (102–104). Serotonergic signaling also appears to be associated with low protein induced EE. We showed that the mRNA abundance of tryptophan hydroxylase 1, an enzyme involved in biosynthesis of serotonin, and serotonin transporter is increased in the BAT of rats fed with low protein diets which might suggest a paracrine or autocrine control of low protein enhanced EE by local 5-hydroxytryptamine (5-HT or serotonin). The VO_2 and EE were shown to be decreased by administration of a non-selective 5-HT receptor antagonist, metergoline (76) and 5-HT3 receptor antagonist, ondansetron (27) in rats fed low protein diets. This is suggestive of higher serotonergic tone in rats fed with protein restricted diets. Whether both central and peripheral serotonergic signaling are equally essential for the thermogenic effects of low protein diets remains to be studied.

Dietary protein restriction results in reduced concentration of most essential amino acids in the circulation, which play a role in metabolic adaptations to protein deficient diets. Among the amino acids studied, methionine (60, 105–108), tryptophan (41), and leucine (109) restriction have been shown to enhance EE. This is suggestive of the importance of amino acid profile and protein quality in regulation of thermogenesis. We showed that methionine restriction can partly recapitulate the total amino acid restriction-induced EE (48). This increase in EE in response to methionine restriction has been linked with greater secretion of hepatic FGF21 (92, 94, 110), upregulation of UCP1 in BAT (105, 107) and increased sympathetic signaling (48, 108). We and others using pharmacological (i.e., chemical sympathectomy and propranolol) and genetic (i.e., β3 receptor knockout mice) tools, showed that sympathetically driven enhanced EE in response to methionine restriction is mediated by β-AR (48, 108). Similarly, increased EE following tryptophan and leucine restrictions is mediated through sympathetic system and upregulation of UCP1 in BAT as well as FGF21 (41, 93, 111, 112). Whether deficiency of other essential amino acids play a role in the higher thermogenic effects of low protein diets, and the underlying pathways and organs involved, warrants further investigation.

CONCLUSIONS AND FUTURE IMPLICATIONS

Dietary protein restriction orchestrates a whole organismal physiological response to stimulate energy intake and EE. The low protein induced thermogenesis is largely driven by dietary protein content and less likely by the concurrent hyperphagia. Though the liver and brain appear to be the primary sites for sensing protein deficiency, the coordination of these tissues with intestinal sensing mechanisms to promote hyperphagia and thermogenesis remains poorly understood. Although liver driven FGF21 seems essential for stimulating EE in response to protein or amino acid restricted diets, the role of local FGF21 synthesized and released from BAT, skeletal muscle and gut in the thermogenic responses to low protein diets remains to be determined. Further, the roles of systemic and local amino acid concentrations, as well as sympathetic and serotonergic signaling in brain, adipose and muscle tissues to coordinate intake and expenditure responses to dietary protein restriction remains largely unexplored. A deeper understanding of the neuroendocrine mechanisms by which dietary protein dilution modulates energy balance may lead to the development of novel strategies for preventing and treating obesity and associated comorbidities in humans, as well as for improving production efficiency in domestic animals.

AUTHOR CONTRIBUTIONS

AP and PC wrote the article and agree to be accountable for the content of the work. All authors contributed to the article and approved the submitted version.

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A Muscle-Centric Perspective on Intermittent Fasting: A Suboptimal Dietary Strategy for Supporting Muscle Protein Remodeling and Muscle Mass?

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Muscle protein is constantly "turning over" through the breakdown of old/damaged proteins and the resynthesis of new functional proteins, the algebraic difference determining net muscle gain, maintenance, or loss. This turnover, which is sensitive to the nutritional environment, ultimately determines the mass, quality, and health of skeletal muscle over time. Intermittent fasting has become a topic of interest in the health community as an avenue to improve health and body composition primarily via caloric deficiency as well as enhanced lipolysis and fat oxidation secondary to attenuated daily insulin response. However, this approach belies the established anti-catabolic effect of insulin on skeletal muscle. More importantly, muscle protein synthesis, which is the primary regulated turnover variable in healthy humans, is stimulated by the consumption of dietary amino acids, a process that is saturated at a moderate protein intake. While limited research has explored the effect of intermittent fasting on muscle-related outcomes, we propose that infrequent meal feeding and periods of prolonged fasting characteristic of models of intermittent fasting may be counter-productive to optimizing muscle protein turnover and net muscle protein balance. The present commentary will discuss the regulation of muscle protein turnover across fasted and fed cycles and contrast it with studies exploring how dietary manipulation alters the partitioning of fat and lean body mass. It is our position that intermittent fasting likely represents a suboptimal dietary approach to remodel skeletal muscle, which could impact the ability to maintain or enhance muscle mass and quality, especially during periods of reduced energy availability.

Keywords: intermittent fasting, muscle protein metabolism, dietary protein, muscle mass, weight loss, muscle protein synthesis/breakdown, lean body mass, time-restricted eating

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INTRODUCTION

Skeletal muscle's central role is the production of contractile force. However, this tissue also serves as the primary site of postprandial glucose disposal (1) and is the largest contributor to resting energy expenditure (2), which collectively positions it as a vital tissue for the maintenance of health and function. Muscle is a dynamic tissue in a constant state of turnover as characterized by

rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB). These processes are responsive to nutrients and contractile activity with changes in MPS and MPB ultimately influencing muscle tissue mass, quality, and health, all of which can influence physical performance (3), injury prevalence (4), and disease risk and/or progression in clinical populations (5).

MPB, which is primarily influenced by the suppressive effect of insulin (6), serves to eliminate old, damaged, mutated and/or redundant proteins through breakdown into their constituent amino acids (AA) (7). These liberated AA enter the muscle's free intracellular pool whereby they may serve as a fuel source (e.g., oxidative phosphorylation) or precursors to be recycled back into protein synthesis. Intramuscular free AA can also be released into circulation to be used by other tissues for synthesis, oxidation or as substrates for gluconeogenesis or ketogenesis (e.g., in the liver), the latter of which is irreversible and contributes to net AA loss. The prevailing view is that MPB plays a relatively minor role in the regulation of muscle mass in healthy humans (7), although whole body protein breakdown that is influenced by higher turning over non-muscle protein pools may play a greater role in whole body net protein balance (8).

MPS is the sequencing of individual AA, made available through protein breakdown or exogenous sources (e.g., digestion and absorption of dietary protein/AA), into polypeptide chains that form the functional protein of muscle tissue. When MPS exceeds MPB, a positive muscle net protein balance and, by extension anabolic environment, occurs. In healthy adults, MPS is generally the more responsive variable and is the primary mediator of muscle net protein balance (7) and long-term changes in muscle mass (9). However, MPS is also important for replacing old, damaged, and mutated tissue proteins to maintain muscle quality (10). Thus, the optimal stimulation of MPS ultimately influences the mass and quality of skeletal muscle, which may impact a variety of health and/or performance related factors including glucose utilization (1), resting and activity energy expenditure (11), and disease risk and mortality (12).

The dietary strategy of intermittent fasting (IF) has become a topic of interest as an avenue to improve health (13, 14) and is often divided into three subclasses: alternate-day fasting, whole-day fasting, and time-restricted eating (TRE) (14). Alternate-day fasting involves alternating between *ad libitum* feeding days and very low energy intake (e.g., a single meal containing ∼25% of daily calorie needs) or complete fasting days. Whole-day fasting typically consists of 1–2 days of either complete abstinence from calories or severe restriction on fasting days plus *ad libitum* eating on the other days. Finally, TRE, which arguably is the "mildest" form of IF, consists of restricting one's eating window to a certain number of hours per day often ranging from 4 to 8 h (14) with a suggested frequency of 1–3 meals (13). Thus, these IF strategies ultimately have a marked influence on the availability of postprandial dietary AA to support MPS and insulin to attenuate MPB.

Many of the health promoting effects of IF are mediated by its effectiveness to induce weight loss (15). For example, when IF is compared to controls with no intervention it generally results in weight loss (16, 17), although when compared to continuous energy restriction it is not superior in this outcome (18). By first principles, this suggests that IF may be an elementary means of inducing energy deficiency with no further diet modifications, which may in the short term enhance dietary adherence (19). This proposition is supported by the observation that skipping meals for up to 12 weeks is not compensated for by an increase in energy intake at subsequent meals consumed *ad libitum* (20). Additionally, 18 h compared to 12 h fasting has demonstrated significantly lower ghrelin levels, which could contribute to the reported reduced desire to eat and increased fullness over a 24 h period (21). Thus, as reduced energy availability can influence MPS rates (22, 23), IF strategies would need to consider the impact of total energy intake as a potential confounder contributing to the postprandial regulation of muscle protein turnover.

The following discussion outlines the current understanding of muscle protein metabolism in relation to the anticipated effect of IF as a dietary strategy on muscle mass and remodeling.

NUTRITIONAL REGULATION OF MUSCLE PROTEIN BREAKDOWN

The breakdown and removal of muscle proteins is regulated by the ubiquitin-proteosome, calpain, and autophagy systems. While some benefits of IF are suggested to be mediated by increased autophagy (24), induction of this system with short term fasting (i.e., up to 36h) is not readily apparent in human skeletal muscle, unlike with exercise (25, 26). In contrast, the ubiquitin-proteosomal and calpain systems are the primary systems regulating nutrient and contraction-induced changes in MPB in humans (7) and therefore will be the primary focus of the present review. MPB is sensitive to feeding indirectly via the nutrient (i.e., carbohydrate and/or AA)-induced release of insulin from the pancreas (27). Maximal reductions in MPB require only modest elevations in plasma insulin concentrations (i.e., ∼15–30 mU/L) (6, 28), which can be stimulated with a modest carbohydrate or protein intake (i.e., ∼20–30g) (29, 30). Thus, the postabsorptive state when insulin is low is characterized by the highest rates of MPB to supply free AA, which are primarily "stored" in skeletal-muscle proteins (31), for other tissues (32–34) and as gluconeogenic precursors (31, 35, 36). This enhancement in MPB is demonstrated both with an overnight (∼10 h) fast (31, 35) and prolonged (60–72 h) fasting (36–38). Given that IF typically involves a relatively prolonged fasting period (i.e., ≥16 h) as a primary means to reduce systemic insulin and promote lipolysis, MPB would be greater over a 24 h period with IF as compared to more typical meal feeding (i.e., 3–5 meals over ∼16 h postprandrial period). With the contraction-induced anabolic stimulus of resistance exercise there is an increase in MPB, although this primarily serves to provide AA precursors to support MPS in the fasted state (39, 40). Thus, resistance exercise may help retain muscle mass with IF by attenuating the negative muscle protein balance of fasted, rested muscle. However, the exercise-induced increase in MPB is completely ablated with exogenous AA (41), highlighting an important role for dietary AA to support muscle anabolism via attenuated catabolism as well.

NUTRITIONAL REGULATION OF MUSCLE PROTEIN SYNTHESIS

Dietary AA are the primary stimulators of and precursors for the synthesis of new muscle proteins (42). The equivalent of ∼0.25 g/kg of leucine-enriched dietary protein in a single meal generally provides a saturating dose of AA for the postprandial stimulation of MPS (43–45), which persists for up to 6 h with the ingestion of whole foods (e.g., egg, beef and dairy proteins) (46–52). Importantly, after attainment of peak MPS (i.e., ∼1.5–3h after protein feeding) (46, 47, 49, 51, 53), MPS gradually reverts back to basal levels even in the presence of sustained plasma aminoacidemia (54, 55). This is referred to as the "muscle full" effect (56) and demonstrates that there is a refractory period following ingestion of a protein bolus with the MPS pathway not able to be stimulated sequentially for ∼3–5 h. Resistance exercise can prolong this postprandial muscle protein synthetic response (i.e., >5 h) (particularly of the myofibrillar fraction) (57, 58), although the maximal stimulatory protein dose is similar to what is sufficient at rest (i.e., ∼0.3 g/kg) (45). There is some evidence that energy deficiency may increase the acute meal protein intake required to maximize MPS (22, 59) with estimates of ∼0.4–0.5 g/kg being potentially sufficient (45). While protein and AA may have an insulinogenic effect (29), insulin only has a permissive effect for supporting maximal rates of MPS at rest and after exercise (29, 30, 60). Thus, manipulating the amount and timing of dietary AA ingestion represents the most important nutritional variable to optimize MPS.

NUTRITIONAL REGULATION OF AMINO ACID OXIDATION

AA oxidation is generally low after an overnight fast but can increase with the duration of the fast (i.e., up to 3 d) (37), which during a period of acute starvation would contribute to a negative whole body (61) and muscle protein balance (38). While meal protein ingestion initiates a normal postprandial increase in AA oxidation (62), dietary AA consumed in excess of their ability to be incorporated into new body (especially muscle) proteins are further irreversibly oxidized and their nitrogen excreted (43–45). It has been suggested that the protein dose required to enhance whole body anabolism may be substantially greater than that required at the level of the muscle (63, 64). Accordingly, it is theorized that AA may be sequestered in splanchnic tissue (primarily the gut) to be later broken down and made available for synthesis of other tissues including muscle (63), although this has yet to be demonstrated. Thus, it is arguably more beneficial to consume acute meal protein intakes that maximize MPS yet minimize AA oxidation in order to optimize the daily dietary protein efficiency. In support of this notion, a recent study (21) comparing a 6 h feeding window with 3 meals to a 12 h feeding window with three meals (protein intake of ∼0.3 g/kg per meal), the 6 h feeding window had significantly increased rates of 24 h protein oxidation by ∼13 g/d (∼85 vs. ∼71 g/d).

DISCUSSION

Research on IF is growing exponentially with ∼34 and ∼45% of the >600 and >200 references since 2010 occurring in the past calendar year for the search terms "intermittent fasting" and "time-restricted eating," respectively (source: Pubmed®; accessed December 11, 2020). A current limitation to the field of IF research is that no study, to the best of our knowledge, has measured muscle protein kinetics with alternate-day fasting or TRE. However, information may be gleaned from studies investigating the impact of daily feeding pattern on protein metabolism. For example, consuming a balanced pattern of moderate protein-containing meals (i.e., 3–4 meals at ∼0.25–0.3 g/kg per meal) supports greater rates of myofibrillar and mixed muscle protein synthesis (65, 66) as well as whole body net balance (67) at rest and during recovery from resistance exercise in energy balance as compared to larger less frequent meals or in a skewed distribution (i.e., majority of protein in a single meal). These longer acute trials (i.e., 12–24 h) support the "muscle full" concept (56) that is exemplified by a maximal muscle protein synthetic response to acute protein ingestion (68). Collectively these acute studies support the concept that meal feeding pattern, irrespective of total protein intake, can influence whole body and muscle protein remodeling with large protein-containing meals stimulating postprandial AA oxidization rather than muscle tissue synthesis (**Figure 1**). Thus, based on the acute research to date, we argue that the lost opportunity for AA-induced MPS with more feedings may not be compensated for with fewer feedings at higher doses, as what is likely to occur with IF.

To our knowledge, no studies have examined whether adaptations in MPB, MPS, and AA oxidation take place over time to an IF protocol. Available literature suggests that following an overnight fast, the first meal demonstrates a similar MPS response to other meals (65), including those preceded by a large protein containing mixed-meal 4 h prior (44). Also, no adaptation is observed in the MPS response after 7-days consuming a skewed distribution of daily protein intake (66), drawing into question whether adaptation may occur with prolonged fasting and/or a chronically altered dietary protein intake pattern such as with IF. Therefore, while we cannot discount that the MPS response may be greater with a meal that breaks a prolonged fasting window and/or that MPB may adapt to a lower set point with chronic IF, there is currently little evidence to support this thesis.

Randomized control trials analyzing the effect of IF on fat free mass (FFM) demonstrate similar (19, 69–81) outcomes compared to controls. As IF often results in negative energy balance and weight loss (16, 17), when IF is compared to continuous energy restriction some systematic reviews suggest similar (82) or enhanced (83) preservation of FFM. The divergence in some of these results may be due to the differences in the types of IF or the self-selected meal frequency by research participants. As discussed above, there is a broad range of IF protocols and those which result in fewer meals (e.g., whole-day) would have greater effects than those with more meals (e.g., TRE). It is also important to note that the length of the studies to date may not have been

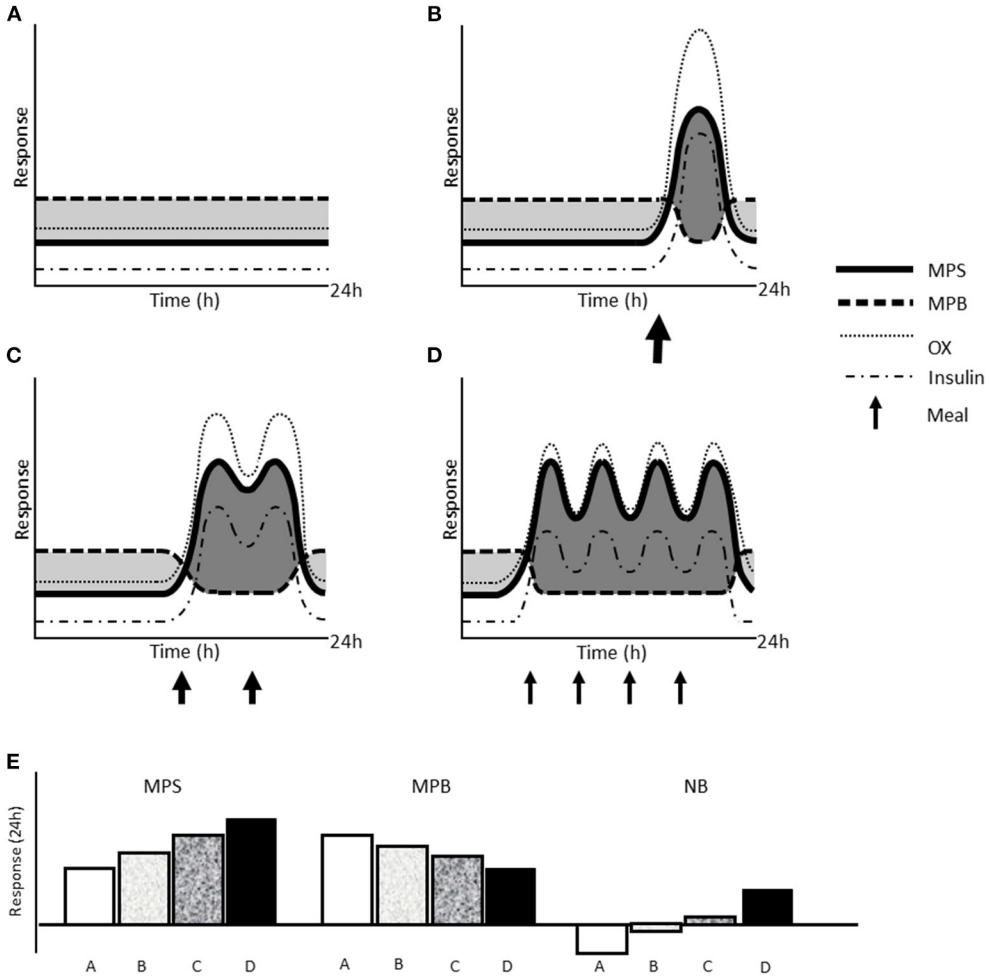

FIGURE 1 | Schematic representation of the effect of different types of IF on acute muscle protein kinetics. Changes in muscle protein synthesis (MPS) and breakdown, amino acid oxidation (OX), and circulating insulin concentration over 24 h at rest with whole day fasting **(A)**, TRE with one meal **(B)**, TRE with two meals over 6–8 h **(C)**, and an unrestricted eating window (∼16 h) with balanced meal pattern **(D)**. Size of arrows reflects the relative protein and energy content of each meal. Shaded dark gray areas indicate time in net positive protein balance and light gray areas indicate time in net negative protein balance, which are summarized as net 24 h response in MPS, MPB, and net muscle protein balance (NB; MPS—MPB) **(E)**.

sufficient to elucidate differences in FFM given the sensitivity of body composition measurement modalities used and their ability to detect changes over short (i.e., ≤12 weeks) interventions (84–86). Of note is a relative large recent study ($n = 116$ adult participants) that reported reductions in appendicular FFM by dual-energy X-ray absorptiometry with TRE over 12 weeks (87), which may be more representative of skeletal muscle mass than total FFM (88). Many of the studies mentioned above prescribe variations of IF as the independent variable but do not explicitly control dietary intake (19, 69, 70, 72–78, 81, 89, 90) and/or physical activity (19, 69–72, 75–78, 80, 89, 90), the latter of which is important to consider given that spontaneous physical activity may be modified by restricted eating (91) and can also influence the sensitivity of skeletal muscle to dietary AA (92). When IF is coupled with the potent anabolic stimulus of resistance exercise, a systematic review (93) observed no significant differences in FFM outcomes when compared to those resistance training with a normal diet. However, given the normal diet group also did not experience gains in FFM, as would be expected, the length (i.e., 4–8 weeks) of the included studies may also not have been adequate to reliably measure changes in FFM. It has been proposed that interventions >8 weeks are required for reliable FFM differences to become apparent with resistance training (94). In fact, a recent study suggests that resistance training-induced gains in FFM over 12 weeks are enhanced with a balanced as compared to a skewed daily protein distribution in healthy young men despite consuming a moderate (i.e., 1.3–1.45 g/kg/d) protein intake (95), which could be lower than that which would maximize growth (96, 97). Collectively, research to date evaluating the impact of IF on changes in body composition in young adults with and without prescribed exercise is equivocal. Therefore, it is important to acknowledge that the hypothesis of IF having consequences for muscle mass in particular may be complex. Based on our current understanding of acute muscle protein

metabolism, the potential effect of IF may be small relative to other lifestyle related variables (e.g., total protein intake and exercise) but could be meaningful when extrapolated over time. However, we acknowledge that acute measures of muscle protein metabolism in laboratory settings may be oversimplified and their relationship to muscle mass and/or muscle quality need further investigation (9).

A limitation in evaluating the impact of IF on muscle mass and function is the overreliance on whole body estimates of FFM, which have been questioned as to their ability to specifically delineate skeletal muscle mass given they include substantial organ and non-muscle lean tissue (98, 99). While including additional outcomes such as appendicular lean mass, muscle thickness, or cross-sectional area, and/or fiber characteristics would help address the consequences of IF on muscle mass, characterizing changes in muscle protein turnover has been suggested to be an effective means to "predict" the direction of change in muscle mass over time, especially if measured over days (100, 101). Therefore, future research should include muscle specific outcomes (e.g., measures of mass and/or function) in chronic, controlled diet trials and/or measures of muscle protein turnover in acute trials to more clearly establish the impact of IF on skeletal muscle quality.

If the hypothesis of more protein feedings per day being optimal for mass and remodeling based on the acute literature is true, IF may represent a dietary conundrum for some populations. While IF is often employed to reduce feeding intakes, restrict total energy intake, and maintain a low insulin profile to help mobilize and metabolize endogenous fat (13, 14), based on our current understanding of the acute, nutritional regulation of muscle protein turnover it seems antithetical to what would presumably optimize muscle protein synthesis and net muscle protein balance (as summarized in **Figure 1**). Critically, populations who may experience a level of "anabolic resistance" to dietary protein, such as sedentary obese (102) and/or older adults (103), may be further susceptible to the suboptimal muscle protein turnover and anabolic environment borne of IF. For example, older adults who consume a balanced daily protein intake and/or consume a greater number of meals containing adequate protein ingestion generally have greater leg lean mass and muscle strength (104). There is also evidence that reduced energy availability, which often occurs in tandem with IF (16, 17, 20), increases the per meal protein intake required to maximize muscle protein synthesis (22, 23). Thus, while this would ostensibly favor larger protein meals that may be characteristic of TRE in particular, it does not preclude the need to consume protein more frequently, which would ultimately also help meet the higher recommended daily protein intakes that enhance muscle and FFM retention with weight loss (59, 105). Finally, performance populations such as athletes and military personnel may also be concerned with the quality of retained muscle/FFM with or without targeted weight loss (59), which would be important considerations for future research.

If the acute effects of IF lead to detrimental long-term outcomes for muscle, whole-day, and alternate-day fasting would have the greatest consequential effect on muscle mass and remodeling. This is due to the prolonged period with greater MPB and lower MPS compounded by the greater energy deficient state likely to occur (107) relative to TRE (108). In consideration of TRE, fewer meals would likely have a greater negative impact on muscle protein turnover (**Figure 1**). If TRE were to be employed, the hypothesis to improve muscle mass and remodeling suggests that protein intake should be consumed at a daily intake of at least 1.6 g/kg and into the number of meals that the feeding window allows separated by 3–5 h.

In conclusion, while IF may represent an option for a variety of populations to promote fat loss and improve aspects of metabolic health, additional research needs to focus on the impact of meal frequency on the quantity and quality of muscle mass. Inasmuch as IF may be purported as the enemy of body fat, future research must ensure this is not also the case for muscle. From our current understanding of muscle protein metabolism and taking a "muscle-centric" view for diet, we highlight that current acute evidence suggests IF may represent a counterproductive strategy to optimize muscle mass and, as far as protein turnover can remodel old/damaged proteins, muscle quality. Thus, studies that concurrently measure muscle protein metabolism and muscle mass and function will be instrumental in resolving these issues.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

EW and DM wrote and revised the manuscript. Both authors read and approved the final version.

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SYSTEMATIC REVIEW
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Evaluating the Leucine Trigger Hypothesis to Explain the Post-prandial Regulation of Muscle Protein Synthesis in Young and Older Adults: A Systematic Review

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Background: The "leucine trigger" hypothesis was originally conceived to explain the post-prandial regulation of muscle protein synthesis (MPS). This hypothesis implicates the magnitude (amplitude and rate) of post-prandial increase in blood leucine concentrations for regulation of the magnitude of MPS response to an ingested protein source. Recent evidence from experimental studies has challenged this theory, with reports of a disconnect between blood leucine concentration profiles and post-prandial rates of MPS in response to protein ingestion.

Aim: The primary aim of this systematic review was to qualitatively evaluate the leucine trigger hypothesis to explain the post-prandial regulation of MPS in response to ingested protein at rest and post-exercise in young and older adults. We hypothesized that experimental support for the leucine trigger hypothesis will depend on age, exercise status (rest vs. post-exercise), and type of ingested protein (i.e., isolated proteins vs. protein-rich whole food sources).

Methods: This qualitative systematic review extracted data from studies that combined measurements of post-prandial blood leucine concentrations and rates of MPS following ingested protein at rest and following exercise in young and older adults. Data relating to blood leucine concentration profiles and post-prandial MPS rates were extracted from all studies, and reported as providing sufficient or insufficient evidence for the leucine trigger hypothesis.

Results: Overall, 16 of the 29 eligible studies provided sufficient evidence to support the leucine trigger hypothesis for explaining divergent post-prandial rates of MPS in response to different ingested protein sources. Of these 16 studies, 13 were conducted in older adults (eight of which conducted measurements post-exercise) and 14 studies included the administration of isolated proteins.

Conclusion: This systematic review underscores the merits of the leucine trigger hypothesis for the explanation of the regulation of MPS. However, our data indicate that the leucine trigger hypothesis confers most application in regulating the post-prandial response of MPS to ingested proteins in older adults. Consistent with our hypothesis, we provide data to support the idea that the leucine trigger hypothesis is more relevant within the context of ingesting isolated protein sources rather than protein-rich whole foods. Future mechanistic studies are warranted to understand the complex series of modulatory factors beyond blood leucine concentration profiles within a food matrix that regulate post-prandial rates of MPS.

Keywords: blood leucine kinetics, leucine threshold, intact proteins, protein-rich whole foods, skeletal muscle, aging, exercise, muscle hypertrophy

INTRODUCTION

Dietary protein is widely regarded as crucial for skeletal muscle health and performance across the lifespan. While muscle hypertrophy is a common goal and pre-requisite to success for strength/power-based athletes and exercise enthusiasts, the maintenance of muscle mass and quality also provides a fundamental hallmark of healthy aging. At the metabolic level, muscle mass and quality are dependent on the continuous remodeling of skeletal muscle proteins via temporal fluctuations in rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) (1). Over time, the relationship between rates of MPS and MPB dictate the net gain or loss of skeletal muscle protein. Both MPS and MPB are responsive to diet, specifically protein feeding and the subsequent aminoacidemia (2), and mechanical loading including resistance (3), endurance (4), and concurrent (5) exercise modalities. However, of these two metabolic processes, the fold change in MPS with protein feeding or exercise is 4–5 times greater than MPB (6), meaning that MPS is the primary locus of control for muscle protein mass, at least in healthy individuals. Accordingly, understanding the regulation of MPS with protein/amino acid feeding and exercise is fundamental to optimizing protein nutrition recommendations for muscle health and performance, both from athletic and clinical perspectives.

The magnitude of the muscle protein synthetic response to an ingested protein source is regulated on multiple levels of physiology that include, but may not be limited to, (i) the systemic availability of amino acids, (ii) the transport and uptake of amino acids into skeletal muscle, and (iii) the activity of intramuscular cell signaling proteins known to modulate MPS (7). Accordingly, it has been proposed that the anabolic potential of a protein source is dependent on factors related to protein digestibility and amino acid kinetics, and amino acid composition. A longstanding debate within the field of muscle protein metabolism relates to whether MPS is regulated by changes in the intracellular (8) or extracellular (9) availability of amino acids. Mechanistic studies support the notion that a more rapid appearance of dietary protein derived amino acids (10), specifically the essential amino acids (EAA) (11), into the circulation is stimulatory for MPS during post-exercise recovery, albeit not under resting conditions (12). Moreover, of all EAA, the branched-chain amino acid, leucine, has been shown to independently upregulate the muscle protein synthetic machinery by activating the mechanistic target of rapamycin complex 1 (mTORC1) which is an intracellular signaling cascade that switches on the translation initiation process of MPS (13, 14). As a result, the "leucine trigger" hypothesis has been proposed. This hypothesis predicts that the magnitude (amplitude and rate) of post-prandial increase in blood leucine concentrations, termed leucinemia, serves to regulate the magnitude of post-prandial MPS response to an ingested protein source (15–17).

Experimental support for the leucine trigger hypothesis primarily stems from studies of isolated protein sources such as intact whey, micellar casein, and soy protein fractions (15, 16). In this regard, the amplitude of peak post-prandial leucinemia was highest for whey, intermediate for soy and lowest for casein. This hierarchy corresponded to the differential post-prandial response of MPS to each protein source at rest and during exercise recovery (15). Accordingly, this relationship was used as the basis to develop the leucine trigger hypothesis (18). Interestingly, the leucine trigger hypothesis has recently been challenged following observations from a series of experimental studies that revealed an apparent disconnect between blood leucine concentration profiles (i.e., the amplitude and rate of leucinemia) and the MPS response to ingested protein in both young and older adults (19, 20). Moreover, recent studies reported that protein-rich whole food sources also are potent in stimulating MPS, despite not facilitating a rapid rise in leucine concentrations during exercise recovery (19). Therefore, the primary aim of this qualitative systematic review was to examine the influence of blood leucine concentration profiles on the post-prandial regulation of MPS in response to protein ingestion at rest and post-exercise in young and older adults. We hypothesize that experimental support for the leucine trigger hypothesis will depend on several factors, including (i) the demographic characteristics of participants (i.e., age), (ii) exercise status (i.e., rest vs. exercise recovery), and (iii) the dose and source of ingested protein (i.e., isolated proteins vs. protein-rich whole food sources).

METHODS

The methodology for this systematic review is based on the PRISMA 2009 guidelines and a PICOS framework was used to determine the search strategy and study characteristics. Consistent with Shad et al. (21), we chose to qualitatively synthesize data from included studies given the heterogenous methodology used to measure MPS between laboratories, meaning that quantitative analysis across studies was not feasible.

Search Strategy

A systematic literature search was conducted in PubMed, Scopus, Cochrane, Google Scholar databases, with the final literature search completed on 1st February 2021. These databases were selected to capture the wide range of content in the field of protein nutrition and muscle protein metabolism. A MeSH (Medical Subject Headings) tree method was used to determine the following search terms: (Healthy old adults OR healthy elderly OR older OR elderly OR healthy young adults OR young adults) AND (rest OR exercise OR resistance exercise OR endurance exercise) AND (protein feeding OR protein digestion kinetics OR amino acid ingestion OR protein supplementation OR whey protein OR soy protein OR casein protein OR wheat protein OR milk protein OR whey OR casein OR soy OR wheat OR milk OR leucine OR leucine trigger) AND (protein turnover OR MPS OR muscle protein synthesis OR FSR OR fractional synthetic rate OR protein synthesis OR myofibrillar protein synthesis OR plasma amino acid concentrations OR plasma leucine concentrations OR dietary protein OR protein-rich). Further studies were identified through the reference lists of relevant original articles and review articles.

Eligibility Criteria

Types of Studies

Randomized controlled trials (RCT), non-randomized clinical trials and comparative studies that combined measurements of blood (plasma) leucine concentrations and post-prandial rates of MPS in response to the oral ingestion of two or more different sources of isolated intact proteins or protein-rich whole foods were eligible for inclusion. Only original manuscripts (not abstracts or reviews) written in English were selected and no limitations on publication date were applied.

Types of Participants

All studies included in this systematic review were conducted in accordance with ethical standards. Studies that recruited healthy young, middle-aged, or older males or females were included in this systematic review. Young adults were defined in the range of 18–35 y, middle-aged in the range of 35–60 y and older adults in the age range of >60 y. Studies of participants diagnosed with compromised metabolic or genetic health issues were excluded from review, e.g., individuals with diabetes, cardiovascular conditions, cancer cachexia, arthritis osteoporosis or any distinct chronic illness. This decision was taken because such conditions may impact post-prandial rates of MPS. Likewise, studies that included participants on any medications (e.g., diabetes medications), which may produce hypo- or hyper anabolic stimuli, were excluded.

Types of Interventions

This systematic review was limited to interventions that administered protein in a single oral bolus, and compared post-prandial blood leucine concentration profiles and rates of MPS between two or more protein-based interventions. Dietary protein could be provided in supplement form (isolated whey, micellar casein, soy, wheat, collagen) or in food form (milk and beef), but interventions had to be matched for protein dose. Studies that included an exercise (resistance, aerobic, or concurrent) stimulus also were included.

Types of Outcome Measurements

The primary outcome measurement from eligible studies was a qualitative appraisal of the leucine trigger hypothesis, i.e., sufficient evidence that blood leucine concentration profiles correspond with post-prandial rates of MPS, or insufficient evidence that blood leucine concentration profiles correspond with post-prandial rates of MPS. This approach was based on the statistical outcomes for measurements of post-prandial blood leucine concentrations and MPS when compared between protein conditions within the same study. Hence, if "protein condition A" resulted in both a greater blood leucine response and MPS response than "protein condition B," the study was classified as "yes," e.g., providing support for the leucine trigger hypothesis. In contrast, the study was classified as "no" if "protein condition A" resulted in a greater blood leucine response than "protein condition B," but the MPS response was not statistically different between conditions or a greater MPS response was observed in "protein condition B." Blood leucine concentration profiles were determined by measurements of plasma leucine concentrations, expressed as peak values during the post-prandial period or as area under the curve (AUC) to represent the "overall" leucine response over the entire post-prandial period. Post-prandial rates of MPS were measured over the same time period, thus enabling us to determine the correspondence between blood leucine concentration profiles and post-prandial rates of MPS in response to an ingested protein source. All included studies assessed MPS by calculating the fractional synthesis rate (FSR) of muscle proteins using the gold standard precursor-product approach. Included studies assessed either mixed-muscle or myofibrillar protein synthesis rates.

Data Collection and Analysis

Selection of Studies

The eligibility of study titles and abstracts generated by the literature search was performed by two reviewers (G Zaromskyte and T Ioannidis). Studies that matched the criteria were reserved and full texts obtained for further screening. Full texts were subsequently screened by two independent reviewers (K Prokopidis and O Witard) based on the eligibility criteria detailed above. Any disagreements between reviewers were resolved by consensus. All records generated by the literature search on PubMed, Scopus, and Ovid MEDLINE and EMBASE were

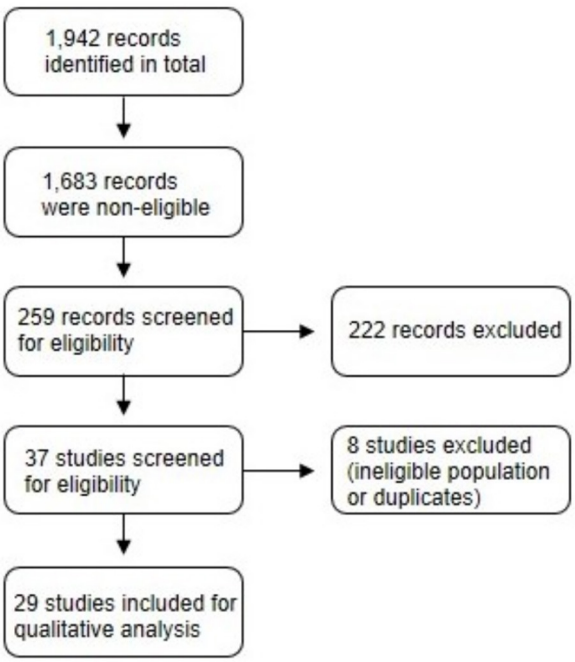

FIGURE 1 | A flow diagram of the screening process for selecting eligible studies.

managed using the reference management software EndNote (Thomson Reuters, version X7).

Data Extraction and Management

Two reviewers (G Zaromskyte and K Prokopidis) extracted all data (i.e., participant characteristics, blood leucine concentration profiles, post-prandial rates of MPS) from included studies using a customized table. Data were organized based on study participant age and whether post-prandial rates of MPS were measured in the rested or post-exercise state. Categories of data extracted included descriptive information on participant characteristics (age, sex, and physical activity status), study design/intervention (i.e., details of protein sources), methodological details regarding measurement of MPS (mixed or myofibrillar muscle protein fraction, tracer incorporation period), and details of data outcomes (i.e., qualitative appraisal supporting or refuting the leucine trigger hypothesis (yes or no) and main findings.

Method of Data Synthesis

Data from included studies were synthesized qualitatively as a quantitative analysis was not appropriate given the heterogenous nature of between laboratory assessments of MPS (21). As part of the data extraction process, reviewers were required to synthesize datasets for each study to determine whether there was sufficient evidence to support the leucine trigger hypothesis. Sufficient evidence of the leucine trigger included a data set whereby a greater blood leucine concentration profile corresponded with higher rates of MPS during the post-prandial period. Following extraction, data were synthesized based on the age of participant studied and whether post-prandial rates of MPS were measured under resting or post-exercise conditions.

RESULTS

Literature Search

Figure 1 displays the screening process for selecting eligible studies. A total of 1,942 records were produced by the literature search. Of this total, 1,683 records were removed because they were either conducted in animals or in human subjects with a pre-existing health condition. A total of 37 studies were screened and 8 were excluded due to ineligible population characteristics or duplicates. A final sample of 29 studies were included for qualitative analysis.

Included Studies

Tables 1–**4** detail all studies included in the systematic review. Among the selected studies, a large heterogeneity across studies was identified in terms of participant characteristics, type of ingested protein, and exercise modality (resistance/aerobic/concurrent exercise). **Tables 1**, **2** display the summary of findings from studies that measured blood leucine concentration profiles and post-prandial rates of MPS in older and young adults at rest, whereas **Tables 3**, **4** display the summary of findings from studies that measured blood leucine concentration profiles and post-prandial rates of MPS in older and young adults following exercise.

Participants

All participants across studies were healthy, as defined by the absence of metabolic conditions, no prescription medication, no smoking or excessive alcohol use and a BMI <30. Overall, 18 studies recruited males, five studies recruited females, and six studies recruited both males and females. At rest, 10 studies recruited older adults (1 middle-aged; 8 in males only, 2 females) and two studies recruited younger adults (1 in males only; 1 in males and females combined). Of the eight studies in young adults, six recruited exercise trained individuals. Following exercise, 11 studies recruited older adults (5 females; 4 males; 2 females and males) and eight studies recruited young adults (6 males; 2 males and females). Two studies (23, 24) were included in both **Tables 1**, **3** since the measurement of MPS (and blood leucine concentrations) was conducted under both rested and exercised conditions in older adults.

Details of Anabolic Interventions

Of the 28 studies, 18 measured post-prandial rates of MPS in response to ingested protein plus exercise, 11 studies measured post-prandial rates of MPS in a rested state, while two measured post-prandial rates of MPS in both resting and post-exercise states. Only interventions that included the oral administration of protein (physiologically relevant) were included in the systematic review, as opposed to studies that administered an amino acid source intravenously (not relevant to leucine trigger hypothesis since a square wave in amino acid appearance is clamped without fluctuation of magnitude). The anabolic

TABLE 1 | Summary of findings from studies that measured blood leucine concentration profiles and post-prandial rates of muscle protein synthesis at rest in older adults.

References	Participants	Study design/Intervention	Muscle fraction for post-prandial MPS measurement	Evidence supporting "leucine trigger" hypothesis	Blood leucine concentration profile	Post-prandial rates of MPS profile
Fuchs et al. (22)	Healthy, untrained males (71 ± 1 yr)	Double-blinded, Parallel RCT 6 g BCAA ($n = 15$) 6 g BCKA ($n = 15$) 30 g milk protein ($n = 15$)	Myofibrillar 0–5 h	No	Peak plasma leucine concentrations: BCAA (45–60 min) > Milk (75–90 min) > BCKA (45–60 min). Overall plasma leucine concentrations: BCAA > Milk > BCKA.	Milk (0.022 ± 0.002%/h) = BCAA (0.022 ± 0.002%/h) = BCKA (0.021 ± 0.001%/h) at 0–2 h from BL. Milk (0.039 ± 0.004 %/h) > BCAA (0.024 ± 0.005 %/h) = BCKA (0.024 ± 0.005%/h) at 2–5 h.
Devries et al. (23)	Healthy, untrained females (69 ± 1 yr)	Single-blinded, parallel RCT 15 g milk (4.2 g LEU) ($n = 11$) 15 g milk + soy (1.3 g LEU) ($n = 11$)	Myofibrillar 0–4 h	Yes	Peak and overall plasma leucine concentrations: Milk > milk + soy.	Milk>milk + soy; +53% from BL vs. +13% from BL Correlation between peak plasma leucine concentrations and MPS: ($r = 0.57$, $P = 0.01$)
Reitelseder et al. (24)	Healthy, moderately active males (69 ± 1 yr)	Single-blinded, RCT 0.45 g/kg LBM WH ($n = 10$) 0.45 g/kg LBM CAS ($n = 9$)	Myofibrillar 0–3 h	No	Overall plasma leucine concentrations: WH > CAS at 15–90 min.	CAS (0.045 ± 0.003%/h) => WH (0.043 ± 0.004%/h)
Kouw et al. (25)	Healthy, untrained males (72 ± 1 yr)	Double-blinded, parallel RCT PLA ($n = 12$) PRO20 [20 g casein ($n = 12$)] PRO20 + LEU [20 g casein and 1.5 g LEU ($n = 12$)] PRO40 [40 g casein ($n = 12$)]	Myofibrillar 0–7.5 h	Yes	Peak plasma leucine concentrations: PRO20 + LEU (396 ± 20 μM) > PRO40 (316 ± 19 μM) > PRO20 (269 ± 10 μM) at 30–180 min. PRO40 > PRO20 + LEU at 180–480 min.	L-[ring-2H5]-phenylalanine) PRO40 (0.044 ± 0.003%/h) > PRO20 + LEU (0.039 ± 0.002%/h) > PRO20 (0.037 ± 0.003%/h) > PLA (0.033 ± 0.002%/h). L-[1-13C]-leucine PRO40: (0.058 ± 0.003%/h) > PRO20 + LEU (0.056 ± 0.002%/h) > PRO20 (0.046 ± 0.004%/h) = PLA (0.047% ± 0.004%/h).
Gorissen et al. (26)	Healthy, untrained males (71 ± 1 yr)	Double-blinded, parallel RCT 35 g wheat ($n = 12$) (2.5 g LEU) 35 g WPH ($n = 12$) (2.5 LEU) 35 g micellar casein ($n = 12$) (3.2 g LEU) 35 g whey ($n = 12$) (4.4 g LEU) 60 g WPH ($n = 12$) (4.4 g LEU)	Myofibrillar 0–4 h	No	Peak plasma leucine concentrations: 35g whey (580 ± 18 μM) > 60g wheat (378 ± 10 μM).	35 g micellar casein > Whey > Wheat at 0–4h 60 g WPH > 35 g whey at 2–4 h. Micellar casein (0.050% ± 0.005%/h) > 60 g WPH (0.049% ± 0.007%/h) > 35 g WPH (0.032% ± 0.004%/h).
Churchward-Venne et al. (27)	Healthy, untrained males (71 ± 1 yr)	Parallel RCT $n = 32$ 25 g bovine milk serum casein 25 g casein protein	Myofibrillar 0–5 h	No	Overall plasma leucine concentrations: Casein > Bovine milk serum casein at 30–180 min.	Bovine milk serum casein = casein at 0–2 h. (0.038 ± 0.005 vs. 0.031 ± 0.007%/h). Casein > Bovine milk serum casein at 2–5 h. (0.067 ± 0.005 vs. 0.052 ± 0.004 %/h).

(Continued)

TABLE 1 | Continued

References	Participants	Study design/Intervention	Muscle fraction for post-prandial MPS measurement	Evidence supporting "leucine trigger" hypothesis	Blood leucine concentration profile	Post-prandial rates of MPS profile
Mitchell et al. (28)	Healthy, middle-aged sedentary to recreationally active males WPC (52.6 ± 3.9 yr) MPC (52.1 ± 6.4 yr)	Double-blinded RCT 20 g WPC (2.3 g LEU; $n = 8$) 20 g MPC (2.1 g LEU; $n = 8$)	Myofibrillar 0–3.5 h	No	Plasma leucine concentrations: WPC > MPC at 45 and 75 min	WPC (0.021 ± 0.018 %/h) > MPC (0.019 ± 0.009 %/h) at 0–210 min MPC (0.057 ± 0.018 %/h) > WPC (0.052 ± 0.024 %/h) at 0–90 min
Wall et al. (29)	Healthy, untrained males (74 ± 1 yr)	Parallel RCT CAS + LEU [20 g casein ($n = 12$)] CAS [20 g casein and 2.5 g LEU ($n = 12$)]	Mixed 0–6 h	Yes	Overall plasma leucine concentrations: CAS + LEU > CAS at 30–180 min.	CAS + LEU (0.0078 ± 0.001 %/h) > CAS (0046 ± 0.001 %/h) at 0–2 h. CAS + LEU (0.023 ± 0.002 %/h) > CAS (0.019 ± 0.001 %/h) at 2–6 h. CAS + LEU (0.049 ± 0.003 %/h) > CAS (0.040 ± 0.003 %/h) at 0–6 h.
Pennings et al. (30)	Healthy, untrained males (74 ± 1 yr)	Parallel RCT 20 g whey ($n = 16$) 20 g casein ($n = 16$) 20 g casein hydrolysate ($n = 16$)	Mixed 0–6 h	Yes	Peak plasma leucine concentrations: Whey (526 ± 21 μM) > casein hydrolysate (381 ± 14 μM) > casein (282 ± 13 μM).	Whey (0.15 ± 0.02%/h) > Casein hydrolysate (0.10 ± 0.01%/h) > Casein (0.08 ± 0.01%/h). Strong positive ($r = 0.66$) correlation between plasma leucine concentrations and mixed MPS.
Koopman et al. (31)	Healthy, untrained males (64 ± 1 yr)	Crossover, Double-blinded trial $n = 10$ CAS (35 g intact casein) or CASH (35 g hydrolyzed casein)	Mixed 0–6 h	Yes	Overall plasma leucine concentrations: CASH: 42.7 ± 2.3 > CAS: 32.6 ± 1.8 μmol·6 h/kg (AUC).	CASH (0.068 ± 0.006 %/h) > CAS (0.054 ± 0.004 %/h).

AUC, area under curve; BCAA, branched-chain amino acids; BCKA, branched-chain keto acids; BL, baseline; LEU, leucine; MPC, milk protein concentrate; MPS, muscle protein synthesis; PLA, placebo; RCT, randomized controlled trial; WPC, whey protein concentrate; WPH, wheat protein hydrolysate.
Values are presented as means ± SE.

TABLE 2 | Summary of findings from studies that measured blood leucine concentration profiles and post-prandial rates of muscle protein synthesis at rest in younger adults.

References	Participants	Study Design/ Intervention	Muscle fraction for MPS measurement	Evidence supporting "leucine trigger" hypothesis	Blood leucine concentration profile	Post-prandial rates of MPS profile
Pinckaers et al. (32)	Healthy, recreationally active males (23 ± 3 yr)	Double-blind, Parallel RCT 30 g milk protein (n = 12) 30 g wheat + milk protein (n = 12) 30 g wheat (n = 12)	Myofibrillar 0–5h	No	Peak plasma leucine concentrations: Milk (353 ± 45 μM) > wheat + milk (301 ± 44 μM) > wheat (280 ± 37 μM). Overall plasma leucine concentrations (AUC): Milk (36 ± 7 mmol·300 min/L) > wheat + milk (25 ± 9 mmol·300 min/L) > wheat (22 ± 3 mmol·300 min/L).	Wheat + milk (0.067 ± 0.032 %/h) > milk (0.059 ± 0.024 %/h) > wheat (0.053 ± 0.025 %/h) at 0–2 h. Wheat (0.058 ± 0.013 %/h) > Wheat + milk (0.054 ± 0.036 %/h) > milk (0.049 ± 0.017 %/h) at 2–5 h. Wheat + milk (0.059 ± 0.025 %/h) > wheat (0.056 ± 0.012 %/h) > milk (0.053 ± 0.013 %/h) at 0–5 h.
Luiking et al. (33)	Healthy, untrained males and females (23 ± 1 yr)	Single-blinded, RCT 0.21 g/kg/bw casein (n = 12) 0.21g/kg/bw soy (n = 10)	Mixed 0–4h	Yes	Overall plasma leucine concentrations: Soy (128 ± 13 μM) > casein (95 ± 7 μM) at 0 h. Soy (117 ± 9 μM) < casein (121 ± 5 μM) at 4 h.	Soy > casein.

AUC, area under curve; MPS, muscle protein synthesis; RCT, randomized controlled trial.
Values are presented as means ± SE.

interventions were isolated proteins, including whey (16 study arms), casein (14 study arms), soy (4 study arms), wheat (2 study arms), and collagen (1 study arm), as well as protein-rich foods including milk (10 study arms), milk + soy (1 study arm), wheat + milk (1 study arm), and beef (1 study arm).

Experimental Methodology and Quality Assessment

Of the 29 studies, 14 were double-blinded, four were single-blinded and 11 were unblinded. Moreover, two studies utilized a cross-over research design, whereas 16 studies were parallel in design with participants either in experimental or control groups. Similarly, physical activity prior to the experiment was monitored across studies, mostly for 2 d by requesting that participants refrain from exercise for this period. With regards to the measurement of MPS, 14 studies measured MPS at the mixed protein level and 15 studies measured MPS in the myofibrillar fraction. Muscle biopsies for measurement of MPS were obtained from the *vastus lateralis* in all studies. Finally, the incorporation period for assessment of MPS ranged from 0 to 3 h post feeding to 0–7.5 h post feeding.

Data Synthesis

The leucine trigger hypothesis was examined in 29 eligible studies, comprising 31 study arms, under resting and post-exercise conditions in young and older adults (**Figure 2**). Two studies (23, 24) conducted measurements of plasma leucine concentrations and post-prandial rates of MPS under both resting and post-exercise conditions (**Tables 1**, **3**). Data from five studies in older adults at rest provide evidence to support the leucine trigger hypothesis for stimulating MPS, whereas five studies did not support the leucine trigger hypothesis. In the post-exercise state, eight studies of older adults support the hypothesis, whereas three studies (one in middle-aged adults) reported a greater MPS response when blood leucine concentrations were lower during the post-prandial period. In young adults at rest, data from one study support the leucine trigger hypothesis, whereas a disconnect between blood leucine concentration profiles and post-prandial rates of MPS also was observed in one study. In the post-exercise state, two studies of young adults supported the leucine trigger hypothesis, whereas six studies reported a greater MPS response when blood leucine concentrations were lower during the post-prandial period.

DISCUSSION

The primary aim of this systematic review was to evaluate the role of the leucine trigger hypothesis to explain post-prandial rates of MPS in response to protein ingestion in young and older adults.

Overall, this systematic review revealed that 16 study arms support the leucine trigger hypothesis to explain the post-prandial regulation of MPS, whereas 15 study arms refute the hypothesis that a more rapid rate of appearance (magnitude and/or duration) of leucine into the circulation is stimulatory for increasing post-prandial rates of MPS. Indeed, two study arms observed a more modest profile of blood leucine concentrations to correspond with greater rates of MPS. We have identified four

TABLE 3 | Summary of findings from studies that measured blood leucine concentration profiles and post-prandial rates of muscle protein synthesis following exercise in older adults.

References	Participants	Study Design/Intervention	Muscle fraction for MPS measurement	Evidence supporting "leucine trigger" hypothesis	Blood leucine concentration profile	Post-prandial rates of MPS profile
Oikawa et al. (34)	Healthy, untrained females (69 ± 3 yr)	Double-blinded, parallel RCT 30 g whey (4.3 g LEU) (n = 11) 30 g collagen (0.9 g LEU) (n = 11) Unilateral leg extension, 4 × 8–10 reps @ 60% 1RM	Mixed 0–4 h	Yes	Overall plasma leucine concentrations (AUC): Whey (103,800 ± 17,000 μmol min/L) > collagen (43,600 ± 10,100 μmol ·min/L) Peak plasma leucine concentrations: Whey (645±206 μM) > collagen (223±117 μM)	Whey>Collagen Whey: 0.017 ± 0.008%/h (rest); 0.032 ± 0.012%/h from BL Collagen: 0.009 ± 0.014%/h (rest); 0.012 ± 0.013%/h from BL
Hamarsland et al. (35)	Healthy, trained males and females (74 ± 3.5 yr)	Double-blinded, partial crossover, RCT 20 g milk (2 g LEU) (n = 10) 20 g native whey (2.7 g LEU) or 20 g WPC (2.2 g LEU) (n = 11) 4 × 8 reps on leg press and leg extension @ 50–80% 1RM	Mixed 0–5 h	Yes	Overall plasma leucine concentrations: Native whey 45% > WPC (AUC). Native whey 130% > milk (AUC). WPC 60% > milk (AUC).	Native whey > WPC > milk.
Holwerda et al. (36)	Healthy, untrained males (67 ± 1 yr)	Double-blinded, RCT 15 g milk (milk) (n = 12) 15 g milk + 1.5g LEU (milk+LEU) (n = 12) 5 × 10 reps on horizontal leg press 2 × 10 reps on latissimus dorsi pulldown 2 × 10 reps on chest press 5 × 10 reps on leg extension @ 50–80% 1RM	Mixed 0–6 h	Yes	Overall plasma leucine concentrations: Milk + LEU > milk at 0–2 h. Peak plasma leucine concentrations: Milk + LEU (407 ± 23 μM) > milk (234 ± 16 μM), at 30 min	(L-[*ring*-2H5]phenylalanine) Milk + LEU (0.0575 ± 0.0032%/h) > milk (0.0495 ± 0.0021%/h) (L-[1-13C]leucine) Milk + LEU (0.0710 ± 0.0048 %/h) > milk (0.0598 ± 0.0030 %/h)
Devries et al. (23)	Healthy, untrained females (69 ± 1 yr)	Single-blinded, parallel RCT 15 g milk (4.2 g LEU) (n = 11) 15 g milk + soy (1.3 g LEU) (n = 11) Unilateral leg extension exercise (2 sets @ 50% 1RM; 2 sets @ 60% 1RM)	Myofibrillar 0–4 h	Yes	Peak and overall plasma leucine concentrations: Milk > milk + soy.	Milk +87% > milk + soy + 30% from BL Correlation between peak plasma leucine concentrations and MPS: (r = 056, P = 0.01)
Devries et al. (37)	Healthy, untrained females (69 ± 1 yr)	Single-blinded, parallel RCT 24.9 g WPI (3 g LEU) (n = 11) 10.0 g milk (3 g LEU) (n = 11) Unilateral leg extension exercise (4 sets @ 50–60% 1RM)	Myofibrillar 0–4 h	Yes	Overall plasma leucine concentrations: Milk > WPI at 0–45 min. Milk < WPI at 120–240 min. Peak plasma leucine concentrations: Milk>WPI.	WPI > milk, +63% from BL vs. +58% from BL (rest) WPI = milk, +9% from BL for WPI and milk (post-exercise)

(Continued)

TABLE 3 | Continued

References	Participants	Study Design/Intervention	Muscle fraction for MPS measurement	Evidence supporting "leucine trigger" hypothesis	Blood leucine concentration profile	Post-prandial rates of MPS profile
Reitelseder et al. (24)	Healthy, moderately active males (69 ± 1 yr)	Single-blinded, RCT 0.45 g/kg LBM WH ($n = 10$) 0.45 g/kg LBM CAS ($n = 9$) Unilateral leg extensions (10 sets × 8 reps @ 70% 1RM)	Myofibrillar 0–3 h	No	Overall plasma leucine concentrations: WH > CAS at 15–90 min.	CAS (0.043 ± 0.004%/h) = WH (0.041 ± 0.004%/h)
Wilkinson et al. (38)	Healthy, untrained females (65 ± 1 yr)	Parallel RCT LEAA_1.5 (0.6 g LEU; 1.5 g EAA) ($n = 8$) LEAA_6 (2.4 g LEU; 6 g EAA) ($n = 8$) Whey [40 g whey (4 g LEU) ($n = 8$)] Unilateral knee extensions (6 sets × 8 reps @ 75% 1RM)	Myofibrillar 0–7 h	No	Overall plasma leucine concentrations: Whey > LEAA_6 > LEAA_1.5 at 60–240 min.	LEAA_6=LEAA_1.5=Whey (rest). Whey > LEAA_6 > LEAA_1.5 (post-exercise).
Borack et al. (39)	Healthy, recreationally active males WPI (69.3 ± 2.1 yr) PB (62.2 ± 1.5 yr)	Double-blinded, RCT 30.4 g WHPI (3.26 g LEU; $n = 10$) 30.5 g PB (2.8 g LEU; $n = 9$) Leg extensions 8 × 10 reps (sets 4–8 @70% 1RM)	Mixed 0–4 h	Yes	Overall plasma leucine concentrations (AUC): WPI = PB	WPI (0.09 ± 0.01%) = PB (0.09 ± 0.01%)
Bukhari et al. (40)	Healthy, untrained females (66 ± 3 yr)	Parallel RCT LEAA [3 g EAA (1.2 g LEU)] ($n = 8$) WP [20 g whey (2 g LEU)] ($n = 8$) Unilateral leg extension, 6 × 8 reps @ 75% 1RM	Myofibrillar 0–4 h	Yes	Overall plasma leucine concentrations: WP > LEAA at 60–220 min.	WP (0.016 ± 0.003 %/h) = LEAA (0.018 ± 0.004 %/h) (rest) WP (0.029 ± 0.007 %/h) > LEAA (0.014 ± 0.010 %/h) (post-exercise)
Burd et al. (16)	Healthy, active males (72 ± 1 yr)	Parallel RCT 20 g micellar casein ($n = 7$) 20 g whey ($n = 7$) Unilateral leg extension, 3 sets @ 10 RM	Myofibrillar 0–4 h	Yes	Overall plasma leucine concentrations (mean): Whey (193 ± 17 μM) > micellar casein (175 ± 17 μM). Peak plasma leucine concentrations: Whey (296 ± 20 μM) > micellar casein (202 ± 21 μM) at 60 min.	Whey > micellar casein
Dideriksen et al. (41)	Healthy, moderately active males and females (68 ± 1 yr)	Parallel RCT $n = 24$ 50 g whey (11.8 g LEU) 46.5 g caseinate (8.8 g LEU) 5 × 8 on leg press and knee extensions @ 80% 1RM	Myofibrillar 0–6.5 h	No	Peak plasma leucine concentrations: Whey (490 ± 32 μmol/L) > Caseinate (282 ± 17 μmol/L)	Whey (0.09 ± 0.005%/h) = Caseinate (0.09 ± 0.003%/h)

AUC, area under curve; BL, baseline; CAS, caseinate protein; EAA_HL, essential amino acids with high leucine; EAA_LL, essential amino acids with low leucine; LEAA, leucine-enriched essential amino acids; LEU, leucine; MPS, muscle protein synthesis; PB, soy-dairy protein blend; RCT, randomized controlled trial; RM, repetition maximum; WH, whey hydrolysate; WPC, whey protein concentrate; WPI, whey protein isolate.
Values are presented as means ± SE.

TABLE 4 | Summary of findings from studies that measured blood leucine concentration profiles and post-prandial rates of muscle protein synthesis following exercise in young adults.

References	Participants	Study Design/Intervention	Muscle fraction for MPS measurement	Evidence supporting "leucine trigger" hypothesis	Blood leucine concentration profile	Post-prandial rates of MPS profile
Churchward-Venne et al. (42)	Healthy, recreationally active males (23 ± 0.4 yr)	Double-blinded, parallel RCT 20 g whey (2.6 g LEU) (n = 12) 20 g soy (1.44 g LEU) (n = 12) 20 g soy + LEU (2.6 g LEU) (n = 12) 4 × 8 reps on leg press and leg extension machine (80% 1RM) and 30 min static cycling (60% Wmax)	Mixed 0–6 h	No	Overall plasma leucine concentrations: Whey > Soy + LEU > Soy (AUC) Peak plasma leucine concentrations: Soy + LEU (328 ± 14 μM; +165% from BL) > Whey group (322 ± 10 μM; +152% from BL) > Soy (216 ± 6 μM; +75% from BL, at 30–180 min.	Whey (0.054 ± 0.002%/h) = Soy (0.053 ± 0.004%/h) = Soy +Leu (0.056 ± 0.004%/h)
Churchward-Venne et al. (43)	Healthy, recreationally active males (23 ± 0.3 yr)	Double-blinded, parallel RCT 20 g milk (1.7 g LEU) (n = 12) 20 g whey (2.6 g LEU) (n = 12) 20 g micellar casein (2 g LEU) (n = 12) 4 × 8 reps on leg press (80% 1RM) and 30 min cycling (60% VO2max)	Myofibrillar 0–6 h	No	Peak plasma leucine concentrations: whey (322 ± 10 μmol/L) > micellar casein (245 ± 5 μmol/L) > milk (242 ± 8 μmol/L)	Milk (0.059 ± 0.003%/h) = Casein (0.059 ± 0.005%h) > Whey (0.054 ± 0.002%/h)
Chan et al. (44)	Healthy, untrained males (22.5 ± 3.0 yr)	Parallel RCT 25 g MPC (2.6 g LEU) (n = 10) 25 g mMPC (2.6 g LEU) (n = 10) 25 g CAS (2.35 g LEU) (n = 10) 3 sets on leg press (80% 1RM) and 3 sets on leg extensions (80% 1RM)	Myofibrillar 0–4 h	Yes	Overall plasma leucine concentrations: mMPC > CAS by 58% from BL mMPC > MPC by 54% from BL, both at 30–90 min.	CAS by (140.6 ± 52.4%) > mMPC by (137.8 ± 72.1%) > MPC by (82.6 ± 64.8%) from BL
Trommelen et al. (45)	Healthy, recreationally active males (24 ± 1 yr)	Double-blinded, RCT PRO (30 g casein) (n = 12) PRO + LEU (30 g casein protein + 2 g LEU) (n = 12) 6 × 10 reps on horizontal leg press and leg extension	Mixed 0–7.5 h	No	Overall plasma leucine concentrations: PRO + LEU > PRO at 30–300 min.	(L-[*ring*-$^2H_5$]phenylalanine) PRO + LEU (0.055 ± 0.004%/h) = PRO (0.055 ± 0.002 %/h) (L-[1-13_C]leucine) PRO + LEU (0.083 ± 0.006%/h) > PRO (0.073 ± 0.004 %/h)
Burd et al. (19)	Healthy, recreationally active males (22 ± 1 yr)	Crossover RCT n = 12 30 g skimmed milk protein (2.7 g LEU) or 30 g minced beef protein (2.5 g LEU) 4 × 8-10 reps on leg press and extension	Mixed 0–5 h	No	Overall plasma leucine concentrations: Milk > beef at 30 min. Beef > milk at 60–120 min. Peak plasma leucine concentrations: Beef (277 ± 12 μM @ 115 min) > Milk (231 ± 11 μM @ 135 min).	Milk by (128% ± 23%) > Beef by (91% ± 15%) at 0–2 h from BL. Milk (0.071 ± 0.005%/h) > Beef (0.057 ± 0.006 %/h) at 0–5 h.

(Continued)

TABLE 4 | Continued

References	Participants	Study Design/Intervention	Muscle fraction for MPS measurement	Evidence supporting "leucine trigger" hypothesis	Blood leucine concentration profile	Post-prandial rates of MPS profile
Reidy et al. (46)	Healthy, recreationally active males and females (WPI; 23.1 ± 1.0 yr; PB; 25.1 ± 1.2 yr)	Double-blinded, RCT $n = 19$ 19 g PB (1.8 g LEU; 8.7 g EAA) 18 g WPI (1.9 g LEU; 8.9 g EAA) 8 × 10 on leg extension (55–70% 1RM)	Mixed 0–4 h	No	Overall plasma leucine concentrations: WPI > PB at 20–120 min.	PB (0.088 ± 0.007%/h) = WPI (0.078 ± 0.009%/h) at 0–2 h; similar increase from BL PB (0.087 ± 0.003%/h) > WPI (0.074 ± 0.010%/h) at 2–4 h.
Tang et al. (15)	Healthy, resistance-trained males (22.8 ± 3.9 yr)	Parallel RCT 21.4 g whey (10 g EAA; 2.3 g LEU) ($n = 6$) 21.9 g casein (10 g EAA; 1.8 g LEU) ($n = 6$) 22.2 g soy (10 g EAA; 1.8 g LEU) ($n = 6$) Unilateral leg press and leg extension 4 × 10–12 RM	Mixed 0–3 h	Yes	Overall plasma leucine concentrations: Whey > soy by 74% from BL Whey > casein by 200% from BL	Whey (0.091 ± 0.015 %/h) > soy (0.078 ± 0.014 %/h) > casein (0.047 ± 0.008%/h) (rest) Whey > soy by 31% (post-exercise) from BL Whey > casein by 122% (post-exercise) from BL
Wilkinson et al. (47)	Healthy, resistance-trained males (23.1 ± 0.3 yr)	Single-blinded, RCT $n = 8$ 18 g protein; calorie-matched milk or soy beverages 4 × 10 reps on leg press, hamstring curl and knee extension @ 80% 1RM	Mixed 0–3 h	No	Overall whole-blood total amino acid concentrations: Soy ~ Milk at 60–180 min Peak whole-blood total amino acid concentrations: Soy (25 μmol/L) > Milk (14 μmol/L) at 30 min Soy ↓ 9 μmol/min; Milk ↓ 0.8 μmol/min at 30–60 min Muscle leucine concentrations: Milk (0.69 ± 0.06 mmol/kg) > Soy (0.59 ± 0.04 mmol/kg) at 60 min Milk (0.55 ± 0.03 mmol/kg) > Soy (0.54 ± 0.04 mmol/kg) at 120 min Milk (0.54 ± 0.04 mmol/kg) > Soy (0.44 ± 0.02 mmol/kg) at 180 min	Milk (0.10 ± 0.01 %/h) > Soy (0.07 ± 0.01 %/h)

AUC, area under curve; BL, baseline; CAS, calcium caseinate; EAA, essential amino acids; LEU, leucine; mMPC, modified milk protein concentrate; MPC, milk protein concentrate; MPS, muscle protein synthesis; PB, protein blend; RCT, randomized controlled trial; VO_2max, maximal oxygen uptake; Wmax, maximal power output in watts; WPI, whey protein isolate.
Values are presented as means ± SE.

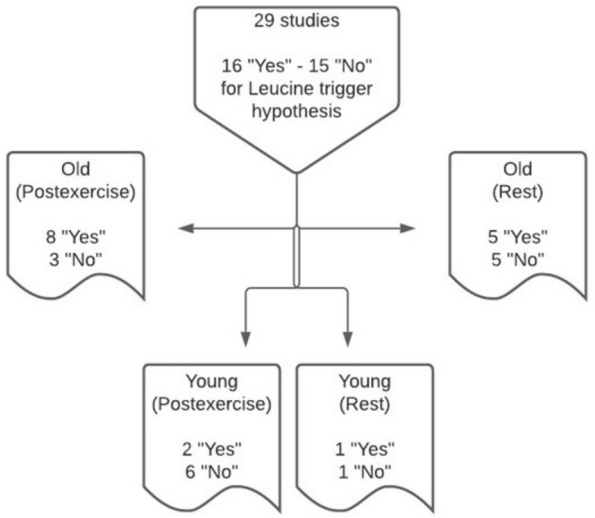

FIGURE 2 | Synthesis of findings from 29 studies that support or refute the leucine trigger hypothesis to explain the post-prandial regulation of muscle protein synthesis in young and older adults at rest and following exercise, using 31 study arms overall.

key factors that contribute to the discrepant findings, namely (i) the dose of protein, (ii) exercise status, (iii) the type/source of ingested protein, and (iv) methodological considerations primarily related to the measurement of MPS.

Protein Dose

The equivocal findings regarding the application of the leucine trigger hypothesis to explain differential post-prandial rates of MPS in response to ingested protein may be related, at least in part, to the age of studied participants. Perhaps surprisingly, our findings indicate that the strength of evidence supporting the leucine trigger hypothesis is greater in older vs. young adults. In this regard, only three study arms in young adults provide evidence supporting the leucine trigger hypothesis (15, 33, 44), while seven studies refute the hypothesis (19, 32, 42, 43, 45–47). In contrast, the preponderance of evidence in older adults supports the leucine trigger hypothesis, with 13 studies reporting a greater post-prandial leucinemia following protein ingestion to correspond with an increased stimulation of MPS, and only seven studies reported a disconnect between the blood leucine concentration profile and post-prandial rates of MPS. As such, this observation may have age-specific implications for optimizing protein-based nutrition recommendations for the maximal stimulation of MPS in young and older adults.

The phenomenon of muscle anabolic resistance describes the impaired stimulation of MPS in response to key anabolic stimuli (i.e., muscle loading and/or amino acid/protein provision) and is generally accepted to be a fundamental mechanism underpinning the age-related decline in skeletal muscle mass (21, 48). However, recent evidence highlights comparable post-prandial rates of MPS between young and older adults when the dose of ingested protein, and constituent leucine profile, exceeds a certain (leucine) "threshold" in older adults (21, 27). In this systematic review, a 20 g protein dose was typically administered in studies of older adults to mimic the protein content of a typical meal. Assuming a constituent amino acid profile of ∼10% leucine, the total leucine content of ingested protein in these studies was equivalent to ∼2 g of leucine that is below the 3 g leucine threshold proposed for the maximal stimulation of MPS in older adults (49). Hence, in the context of a meal-like dose of protein, our data support the notion that the amplitude of peak post-prandial leucinemia serves as key factor in regulating post-prandial rates of MPS in older adults. In contrast, the regulatory role of blood leucine availability in stimulating MPS becomes less apparent if the protein dose and leucine content is sufficient to stimulate a maximal post-prandial response of MPS (50, 51), as was the case in most studies of young adults included in this systematic review. By virtue of this age-related anabolic resistance phenomenon, alongside the inevitable decline in appetite and oral health associated with advancing age (52, 53), the administration of an "optimal" protein dose for maximal stimulation of MPS is more challenging in older adults. Accordingly, we present evidence that the leucine trigger hypothesis appears to confer greater application in explaining differences in post-prandial rates of MPS in older vs. young adults.

Rest Vs. Exercise

The leucine trigger hypothesis was originally conceived, at least in humans, to explain divergent post-prandial rates of MPS in response to ingesting different isolated protein sources (i.e., whey, micellar casein and soy fractions) following exercise in healthy, trained, young men who engaged in whole-body resistance training at least 2 times per week (15, 54). Thereafter, this hypothesis has been extrapolated to encompass the post-prandial regulation of MPS at rest and following exercise in both young and older adult cohorts of both trained and untrained status. When pooling data for young and older adults, the findings from this systematic review indicate that the strength of evidence supporting the leucine trigger hypothesis is similar under post-exercise conditions (10/19 or 53% of studies support the hypothesis; **Figure 2**) and resting conditions (6/12 or 50% of studies support the hypothesis). However, when stratified by age, support for the hypothesis is stronger in older (8/11 or 73% of studies support the hypothesis) vs. young (2/8 or 25% of studies support the hypothesis) adults when assessed under post-exercise conditions. Taken together, these data suggest an interaction exists between age and exercise status with regards to supporting the leucine trigger hypothesis as an explanation for the regulation of MPS.

The notion that the leucine trigger applies only to exercise conditions has previously been challenged by two studies that manipulated the leucine content of a low dose of EAA (3 g) or whey protein (6.25 g) and measured post-prandial rates of MPS at rest and following exercise in young (55) and older (40) adults. In these studies, ingesting a leucine-enriched amino acid source elicited a robust increase in blood leucine concentrations and stimulated a similar response of MPS to the bolus ingestion of 20–25 g of whey protein at rest in both young and older adults

(40, 55). Interestingly however, whereas ingesting the low dose leucine-rich EAA source stimulated similar post-exercise rates of MPS compared with 20 g of ingested whey protein in older adults (40), fortifying a low dose of whey protein with leucine failed to stimulate an equivalent post-exercise response of MPS to ingesting 25 g of whey protein in young adults, particularly during the later (3–5 h) exercise recovery period (55).

Intuitively, the authors reasoned that the capacity for a protein source to sustain an exercise mediated increase in MPS is not only dependent on extracellular leucine availability. Instead, an abundant supply of EAA (and potentially non-essential amino acids) also are required to provide additional substrate for the synthesis of new muscle proteins under conditions of higher "anabolic drive" stimulated by resistance exercise compared with feeding alone. The apparent disconnect between this thesis (55) and our observation that the leucine trigger hypothesis confers greater application during post-exercise conditions is difficult to reconcile, but may be explained by the range of different ingested protein sources included in this systematic review, particularly with regards to the potential interactive role of other nutrients (carbohydrate, lipids, fiber, and other bioactive constituents) within a food matrix in regulating post-prandial rates of MPS following the ingestion of protein-rich whole foods such as milk (19, 56), beef (19, 57), or pork (58). Unfortunately, a limited number of the studies included in this systematic review recruited previously trained individuals. Hence, the impact of training status on the role of the leucine trigger in modulating MPS warrants future investigation. This additional analysis is particularly interesting given the complex relationship between acute measurements of MPS and chronic changes in muscle mass (59). In this regard, the predictive value of acute measurements of MPS for chronic changes in muscle mass appears to be greater in trained vs. untrained individuals (60), suggesting that the leucine trigger hypothesis may be most relevant in trained individuals.

Amino Acid/Protein Source

Burd et al. (20) recently proposed the idea that the leucine trigger hypothesis is more relevant within the context of ingesting isolated protein sources rather than protein-rich whole foods. This idea stems from the observation that ingesting protein-rich whole foods, such as skimmed milk or minced beef, are effective in stimulating a robust post-prandial increase in MPS, albeit in the absence of a rapid rise in leucinemia during post-exercise recovery in trained young men (19). This apparent disconnect between blood leucine concentration profiles and post-prandial rates of MPS in response to protein-rich foods contrasts with studies that administered isolated whey, soy and micellar casein fractions as fast, intermediate and slow proteins, respectively (15, 16). In these studies, the post-prandial response of MPS corresponded with the magnitude of leucinemia (as well as higher plasma EAA and BCAA concentrations), resulting in higher, intermediate and lower rates of MPS for whey, soy, and casein, respectively. The reason(s) behind these discrepant findings are yet to be fully elucidated, but may be related to the notion that other, non-protein, components within the whole food matrix are modulatory in regulating MPS.

The food matrix refers to the overall chemical dynamics of food, including how various food components are structured and interact (50). Consistent with this idea, a recent study demonstrated a greater post-prandial stimulation of MPS after ingesting whole eggs (egg white and yolk remained intact) than egg whites (egg yolk removed) during exercise recovery, despite a similar profile of blood leucine concentrations between egg conditions (51). Moreover, Elliot et al. (56) demonstrated that ingesting whole milk after exercise stimulated a greater amino acid uptake across the leg than fat-free milk when either matched for carbohydrate or energy content. Ultimately, this systematic review fails to provide additional insight into this theory given the limited number of studies that directly compare post-prandial rates of MPS in response to ingesting different whole protein foods. Hence, future mechanistic studies are warranted to elucidate the nutrient-nutrient interactions within the food matrix that may contribute to differential post-prandial rates of MPS following the ingestion of protein-rich food sources.

Methodological Considerations

We cannot discount the possibility that methodological differences between studies, specifically in the measurement of MPS, may contribute to the mixed findings presented in this systematic review regarding the leucine trigger hypothesis. Such methodological considerations include, but may not be limited to, the duration of measurement for post-prandial rates of MPS, selection of muscle sub-fraction (i.e., mixed or myofibrillar) extracted for measurement of MPS, choice of isotopic tracer (i.e., $^{13}C_6$ phenylalanine, 1- ^{13}C leucine) and choice of precursor amino acid pool (plasma or intracellular) used to calculate fractional synthesis rates as the unit measurement for MPS (61). With regards to the duration over which post-prandial rates of MPS were measured, the tracer incorporation period ranged from 3 to 7.5 h within this systematic review, thus representing a wide range of measurement durations. Previous work demonstrates a transient post-prandial response of MPS that peaks ∼3 h following protein ingestion (62). In theory, it follows that the leucine trigger may be more relevant within the early 0–3 h post-prandial period. Consistent with this notion, 10 of the 17 study arms that provide evidence to support the leucine trigger hypothesis measured MPS over a relatively short incorporation period, i.e., <4 h. Hence, in our hands, a link appears to exist between the duration of MPS assessment and support for the leucine trigger hypothesis. Moreover, previous studies have reported a differential response of MPS to exercise and/or nutritional stimuli dependent on whether mixed muscle or myofibrillar protein synthesis rates were measured (63). Whereas, the muscle intracellular amino acid pool arguably serves as a more accurate surrogate precursor for the calculation of MPS, for practical reasons (i.e., low tissue yield from biopsy) several studies, including some presented in this systematic review, used tracer enrichments in the blood amino acid pool as a more accessible precursor. Finally, discrepant findings have been reported for measurements of muscle protein metabolism within the same study based on choice of tracer infused (64). Taken together, it is feasible that these technical differences in methodology may contribute to

the mixed findings regarding the regulatory role of the leucine trigger hypothesis.

CONCLUSION

This systematic review is the first, to our knowledge, to qualitatively evaluate the leucine trigger hypothesis to explain the post-prandial regulation of MPS at rest and following exercise in young and older adults. In this systematic review, overall, 16 study arms (13 in older adults) provide evidence to support the hypothesis that the magnitude (amplitude and rate) of post-prandial increase in blood leucine concentrations, termed leucinemia, serves to regulate the magnitude of post-prandial MPS response to an ingested protein source. In contrast, 13 study arms refute the hypothesis. To conclude, these data underscore the merits of the leucine trigger hypothesis with greatest application in predicting the post-prandial response of MPS to ingested proteins in older adults. Moreover, and consistent with previous reports (20), we provide data to support the idea that the leucine trigger hypothesis is more relevant within the context of ingesting isolated protein sources rather than protein-rich whole foods. Moving forward, future studies should report more complete datasets that include basal measurements of MPS, thus allowing for the quantification of relative changes in MPS between conditions in follow-up systematic reviews and meta-analyses on this increasingly controversial topic of the leucine trigger hypothesis. Follow-up mechanistic studies also are warranted to understand the complex series of modulatory factors within a food matrix that regulate post-prandial rates of MPS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

GZ, TI, KT, and OW conceived and designed the research. GZ, TI, KP, and OW assisted with data analysis and result interpretation. GZ and KP prepared figures and tables. GZ, KP, and TI drafted manuscript. KP, KT, and OW revised manuscript. All authors approved final version of manuscript.

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BRIEF RESEARCH REPORT
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Brief Research Report: Estimation of the Protein Digestibility-Corrected Amino Acid Score of Defatted Walnuts

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Introduction: Walnuts are considered a good source of essential fatty acids, which is unique among tree nuts. Walnuts are also composed of about 10–15% protein, but the quality of this protein has not been evaluated. Pistachios and almonds have been evaluated for their protein content using a protein digestibility-corrected amino acid score (PDCAAS), but it is unclear how the quality of protein in walnuts relates to that in other commonly consumed tree nuts. The objective of this study was to substantiate the protein quality of walnuts by determining their PDCAAS.

Methods: A small, 10-day dietary intervention trial was conducted using male Sprague-Dawley rats (n = 8, 4 per group) with two diets: a nitrogen-free diet and a diet containing protein exclusively from defatted walnuts. Feed intake and fecal output of nitrogen were measured to estimate the true protein digestibility, and the amino acid compositions of walnuts compared to child and adult populations were used to calculate amino acid scores (AAS) and PDCAAS.

Results: The true protein digestibility score of raw walnuts was calculated to be 86.22%. Raw walnuts contained 15.6 g protein/g walnut with AAS of 0.45 and 0.63 for children aged 6 months to 3 years and 3–10 years, respectively. For each population, a PDCAAS of 39 and 46% was calculated, respectively, using a protein conversion constant of 5.30. Using a protein constant of 6.25, a PDCAAS of 39% (6 months - 3 years) or 46% (3-10 years) was calculated.

Conclusions: This is the first known assessment of the PDCAAS of walnuts. Like almonds, they appear to have a low-to-moderate score, indicating they are not a quality source of protein.

Keywords: tree nuts, PDCAAS, protein, digestibility, walnuts

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INTRODUCTION

Among tree nuts, walnuts represent a high-quality source of polyunsaturated fatty acids and are particularly high in α-linolenic and linoleic acid (1). Though walnuts may be unique in their lipid content, 10–15% of the composition of walnut is made up of protein (2), although the quality of this protein is unclear.

There are 20 amino acids that comprise human proteins; of these, nine are considered essential due to their required provision from dietary sources, rather than an endogenous source (3). Besides soy, high-quality protein sources tend to be found in animal products (4).

However, it is widely recognized that plant sources of protein, including nuts and seeds, are also valuable sources of protein. With respect to tree nuts, lysine (Brazil nuts, cashews, hazelnuts, pine nuts, and walnuts), methionine and cysteine (almonds), and tryptophan (macadamias, pecans) are the limiting amino acids (5). To measure the quality of a protein source, a protein digestibility-corrected amino acid score (PDCAAS) can be calculated, which measures the quality of a protein-based on the amino acid requirements adjusted for digestibility as compared to a given reference population (e.g., children or adults).

The objective of this study was to determine the PDCAAS for walnuts such that the protein quality could be objectively evaluated against other tree nuts (namely, almonds and pistachios) and other sources of protein. To do this, a small, 10-day dietary intervention trial in rats was completed to estimate the true digestibility of nitrogen from walnuts for use in calculating the PDCAAS of walnuts. In this study, we estimated four PDCAAS using two conversion factors (5.30 and 6.25, discussed below) and for two reference populations {young children [Food and Agriculture Organization of the United Nations (FAO) scoring patterns for 6 months to 3 years of age] and older children and adults (FAO scoring pattern for 3–10 years of age)} such that the appropriate PDCAAS is available for multiple populations.

METHODS

Animals

All animal care and study procedures complied with The Animal Welfare Act and were approved by the internal International Animal Care and Use Committee at Covance, Inc. (Princeton, NJ; protocol #8379737). The study was conducted at Covance test facilities in Greenfield, IN.

Eight male Sprague Dawley rats (21–28 days of age) were acquired from Envigo RMS, Inc. ("Envigo," Indianapolis, IN) and allowed to acclimate to the test facility for 2 days prior to the start of the study. Rats were housed singly in polycarbonate caging with woodchip bedding and allowed *ad libitum* access to water and a standard rodent diet (Teklad Global Diet—Rodent 2014C; Envigo). Environmental conditions were controlled at a temperature of 20–26°C and relative humidity of 40–70% with 12-h light/dark cycles. Each animal was identified using a tail mark, microchip, and cage card. Animals were provided with cage enrichment devices. Animals were checked two times daily for mortality, clinical abnormalities, signs of pain, or distress. No clinical observations of adverse effects were noted throughout the trial; thus, all animals met the criteria for inclusion throughout the trial.

Experimental Diets

Two experimental diets were formulated by Envigo. The nitrogen-free diet was an extremely low nitrogen modification of AIN-76A with no casein, additional starch to replace sucrose, and increased lipid (corn oil) content. The test diet (test) was formulated to contain 10% defatted walnuts as a protein source and to match the nitrogen-free diet with a 3.9 kcal metabolizable energy/g diet, 10% fat, 5% fiber, 3.5% AIN-76 mineral mix, 1% AIN-76A vitamin mix, and 0.2% choline bitartrate. Details of the composition of both diets can be found in **Table 1**. The diets were stored between 2 and 8°C until 2 h before administration each day. All diets and raw defatted walnut were analyzed for crude protein (AOCS Ac 4-91), fat (AOAC 948.22), total fiber (AOAC 985.29), and dry matter (DM) (AOAC 925.09).

TABLE 1 | Composition of the experimental diets and defatted walnut.

Formulated ingredients, g/kg	Nitrogen-free	Test	
Defatted walnut	0	211.0	
Corn starch	653.0	484.41	
Maltodextrin	150.0	150.0	
Corn oil	100.0	94.15	
Cellulose	50.0	13.08	
Mineral mix (AIN-76 170915)	35.0	35.0	
Vitamin mix (AIN-76A 40077)	10.0	10.0	
Choline bitartrate	2.0	2.0	
Analyzed nutrient composition, as-is basis			
Metabolizable energy, g/kg	3.9	3.9	**Defatted walnut**
Crude protein ($N \times 5.30$), g/100 g	0.29	10.41	47.45
Crude protein ($N \times 6.25$), g/100 g	0.34	12.28	55.96
Nitrogen, g/100 g	0.05	1.97	8.95
Fat, g/100 g	9.3	9.0	2.6
Total dietary fiber, g/100 g	5.09	5.17	17.5
Dry matter, g/100 g	89.6	89.6	98.0

Study Design

The study design was chosen to be in line with recommendations made by the World Health Organization (WHO) for performing an *in vivo* rat assay for true protein digestibility (6). After a 2-day acclimation period, the 10-day trial started. Rats were randomly assigned to either the nitrogen-free or test groups ($n = 4$ rats per group) based on body weight such that the group mean body weight did not vary by more than 5 g. Detailed observations were conducted for each animal one time during the acclimation phase and then one time daily during the trial. During the 10-day trial period, 15 g/d of each diet was offered so that feed intake could be measured. Full feeder weights were collected on days 5–9 of the trial, empty feeder weights were collected on days 6–10. Spilled feed was also collected and composited individually per animal for days 5–9. At the end of the study, spilled feed was air-dried for at least 3 days and weighed. The weights of uneaten and spilled feed were deducted from the weight of the feed offered to determine the total feed intake. Body weights were measured on days 1, 5, and 10 of the trial; feces were collected on days 6–10. Instances of coprophagy or urinary contamination were not measured.

Fecal Analyses and True Protein Digestibility Calculation

Fresh feces were collected each day (days 6–10) and held at ambient temperature before being dried in a vacuum oven at 100°C. Feces were weighed, ground, and combined into a single

sample per animal prior to the analysis of nitrogen content (AOCS Ac 4-91) and DM content (AOAC 925.09). For true protein digestibility, all variables were divided by DM intake to control for differing amounts of feed intake and DM between groups. The true protein digestibility score was calculated as:

$$T = \frac{I - (F - Fk)}{I} x\ 100$$

where I is equal to the nitrogen intake of the test group, F is equal to fecal nitrogen of the test group, and Fk (endogenous nitrogen) is equal to fecal nitrogen of the nitrogen-free group.

Amino Acid Score and PDCAAS Calculations

Crude protein [AOAC (7) 968.06], tryptophan (AOAC 988.15), and other amino acids (8–11) were determined on raw walnuts (not defatted, **Table 2**). Amino acid scores (AAS) were estimated as:

$$AAS = \frac{mg\ amino\ acid\ in\ 1\ g\ of\ test\ protein}{mg\ of\ same\ amino\ acid\ in\ 1\ g\ of\ reference\ pattern} x\ 100$$

where the scoring patterns for the "child" (6 months to 3 years of age) and "older child, adolescent, and adult" (3–10 years of age) groups from Table 5 of the 2013 FAO Expert Consultation on Protein Quality Evaluation in Human Nutrition (12) were used as the reference pattern. PDCAAS was estimated by multiplying the lowest amino acid score by true protein digestibility. All scores were estimated using nitrogen conversion factors of 5.30 and 6.25.

RESULTS

All animals completed the study as described and were transferred to a stock colony upon study completion. No adverse events or remarkable observations were reported. As expected, feed intake differed between groups with the test group consuming more feed (**Table 3**), resulting in the nitrogen-free group losing 14.5 ± 1.7 g (mean ± SD) and the test group gaining 5.5 ± 1.7 g throughout the trial (a mean difference of 20 g, $P < 0.01$). Using the mean and SD of metabolic nitrogen, it was observed that an effect size (Cohen's d) of 7.49 was achieved with a power (1-β) of 0.99 and an α of 0.01.

True protein digestibility of the test diet was estimated to be 86.22 ± 1.83% (**Table 3**). Four AAS and PDCAAS were generated using protein conversion factors of $N \times 5.30$ and $N \times 6.25$, and two reference patterns (**Table 4**). The AAS ratios were determined to be: Child ($N \times 5.30$), 0.53; Child ($N \times 6.25$), 0.45; Adult ($N \times 5.30$), 0.63; Adult ($N \times 6.25$), 0.54. The PDCAAS were determined to be: Child ($N \times 5.30$), 46%; Child ($N \times 6.25$), 39%; Adult ($N \times 5.30$), 55%; Adult ($N \times 6.25$), 46%. Lysine was the limiting amino acid in all cases.

TABLE 2 | Analyzed amino acid composition of raw walnuts.

		Crude protein estimate, g protein/100 g walnut	
		***N* × 5.30** **13.2**	***N* × 6.25** **15.6**
Amino acid	**mg AA/g walnut**	**mg AA/g protein**	**mg AA/g protein**
Indispensable			
Arginine	22.30	168.94	143.22
Histidine	2.87	21.74	18.43
Isoleucine	5.81	44.02	37.32
Leucine	10.50	79.55	67.44
Lysine	4.02	30.45	25.82
Methionine	2.38	18.03	15.29
Phenylalanine	6.40	48.48	41.10
Threonine	4.96	37.58	31.86
Tryptophan	1.41	10.68	9.06
Valine	6.47	49.02	41.55
Dispensable			
Alanine	6.24	47.27	40.08
Aspartic acid	14.00	106.06	89.92
Cystine	2.12	16.06	13.62
Glutamic acid	27.30	206.82	175.34
Glycine	7.13	54.02	45.79
Proline	5.39	40.83	34.62
Serine	7.57	14.01	48.62
Tyrosine	5.06	38.33	32.50
Sulfur amino acids (Met + Cys)	43.98	333.18	282.47
Aromatic AA (Phe, Tyr, Trp)	12.87	97.50	82.66

N, nitrogen; AA, amino acid; Met, methionine; Cys, cystine; Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan.

DISCUSSION

In this study, we report the results of a dietary intervention trial in male Sprague Dawley rats consuming either a nitrogen-free diet or a diet composed of 12.28% protein ($N \times 6.25$ on an as-is basis) from defatted walnuts to determine the PDCAAS for walnuts. The PDCAAS of walnuts for adults was 46 or 55%, and for children 39 or 46%, depending on the conversion factor. Regardless, this suggests that walnuts are not a quality source of protein, specifically limited by the concentration of lysine.

The FAO, WHO, and the US Food and Drug Administration (FDA) first published PDCAAS scoring in 1991 (6). PDCAAS is designed to allow for comparisons of whole-food protein source quality, where a score of 1 is indicative of a quality protein source, while a score of 0 is indicative of a poor-quality protein source (the absence of one or more essential amino acids). The quality of protein is typically higher in meat and dairy products (4, 13). However, some plant sources including soy and pea protein also have high PDCAAS (4). Among the nine types of tree nuts, a PDCAAS previously existed for only two of them: almonds and pistachios. While pistachios have high PDCAAS of 0.73 (raw) and 0.81 (roasted) (14), almonds have a lower score between 0.22 and 0.48 (depending on varietal), indicating that they are a low-quality source of protein (15, 16).

The provision of multiple PDCAAS in this report may be a point of confusion. The FDA Code of Federal Regulations

TABLE 3 | True protein digestibility of defatted walnuts[a].

Group	Subject	Feed intake, g DM	Nitrogen intake, mg	mg Nitrogen intake/g DM intake	Calculated Fecal Nitrogen, mg	Metabolic Nitrogen, mg Fecal N/g DM intake	Corrected Metabolic Nitrogen, mg Fecal N/g DM intake
Nitrogen-free	1	21.5	13.1	0.6	13.2	0.61	
Nitrogen-free	2	18.8	11.4	0.6	10.1	0.54	
Nitrogen-free	3	21.5	13.1	0.6	18.8	0.88	
Nitrogen-free	4	20.6	12.5	0.6	12.7	0.62	
Test	5	41.2	903.9	21.9	145.8	3.53	2.87
Test	6	43.0	943.2	21.9	192.7	4.48	3.82
Test	7	39.4	864.6	21.9	137.3	3.48	2.82
Test	8	43.0	943.2	21.9	138.8	3.23	2.57
Nitrogen-free (Mean ± SD)		20.6 ± 1.3	12.5 ± 0.8	0.6 ± 0.0	13.7 ± 3.7	**0.66** ± 0.15	
Test (Mean ± SD)		41.7 ± 1.7	913.7 ± 37.6	**21.9** ± 0.0	153.7 ± 26.3	**3.68** ± 0.55	3.02 ± 0.55
True protein digestibility estimate							
I		**21.9** ± 0.0					
F		**3.68** ± 0.55					
Fk		**0.66** ± 0.15					
TD		**86.22%** ± 1.83%					

[a] *True protein digestibility was estimated using the equation* $TD = \frac{I-(F-Fk)}{I}$, *where I is equal to nitrogen intake, F is equal to fecal nitrogen of the test diet, and Fk is equal to fecal nitrogen of the nitrogen-free diet. Estimates were corrected for dry matter intake. Bolded numbers indicate values used in the calculation of true protein digestibility.*
N, nitrogen; DM, dry matter.

TABLE 4 | Protein digestibility-corrected amino acid score (PDCAAS) of raw walnuts.

			AAS ratios			
	Reference scoring pattern mg/g protein requirement		Child		Older child, adolescent, adult	
Amino acid	**Child (6 months to 3 years)[a]**	**Older child, adolescent, adult[b] (3–10 years)**	***N**5.30**	***N**6.25**	***N**5.30**	***N**6.25**
Histidine	20	16	1.09	0.92	1.36	1.15
Isoleucine	32	30	1.38	1.17	1.47	1.24
Leucine	66	61	1.21	1.02	1.30	1.11
Lysine	57	48	**0.53**	**0.45**	**0.63**	**0.54**
Sulfur Amino Acids (Met + Cys)	27	23	12.34	10.46	14.49	12.28
Aromatic AA (Phe, Tyr, Trp)	52	41	1.88	1.59	2.38	2.02
Threonine	31	25	1.21	1.03	1.50	1.27
Tryptophan	8.5	7	1.26	1.07	1.62	1.37
Valine	43	40	1.14	0.97	1.23	1.04
Population	**Protein conversion**	**AAS**	**PDCAAS**			
Child	*N**5.30	0.53	46%			
	*N**6.25	0.45	39%			
Older child, adolescent, adult	*N**5.30	0.63	55%			
	*N**6.25	0.54	46%			

[a,b] *Scoring patterns for the child and older child, adolescent, and adult groups were taken from Table 5 of the 2013 FAO Expert Consultation on Protein Quality Evaluation in Human Nutrition. Bolded numbers indicate the AAS ratios used in the estimation of PDCAAS.*
PDCAAS, protein digestibility corrected amino acid score; AAS, amino acid score; N, nitrogen; Met, methionine; Cys, cystine; Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan.

21 C.F.R. § 101.9 states that "Protein content may be calculated based on the factor 6.25 times the nitrogen content of the food as determined by the appropriate method of analysis as given in the 'Official Methods of Analysis of the AOAC International,' except when official AOAC procedures described in this paragraph (c) (7) require a specific factor other than 6.25, that specific factor shall be used." Bailey and Stein (14) provide the digestible indispensable amino acid score (DIAAS) of pistachios using

conversion factors of both 5.30 and 6.25, noting that the AOAC recommends a conversion factor of 5.30 for "other nuts" (not peanuts, Brazil nuts, or almonds) to calculate crude protein on food labels. The FAO, on the other hand, suggests a conversion of 6.25 is used as a standard across all proteins (12). Furthermore, the FAO recommends that for regulatory purposes, reference amino acid scoring patterns for young children should be taken from children aged 6 months to 3 years, whereas for older children, adolescents, and adults, the pattern for 3- to 10-year-old children should be used. Hence, we estimated four PDCAAS using both conversion factors and two reference populations such that readers could use the appropriate score for their use case.

This study represents a step toward better understanding the protein quality of tree nuts. However, better metrics to evaluate protein quality do exist, such as the DIAAS, calculated from ileal digesta (12). Determining a DIAAS for walnuts may be pertinent, as some studies have shown that PDCAAS may overestimate the quality of some proteins, especially those of poor quality (17, 18). Currently, pistachios are the only tree nuts with an estimated DIAAS (14). The study in pistachios also highlights the differences in protein quality based on preparation. In this study, only defatted walnuts were evaluated. However, the method of preparation may impact the protein quality scores (PDCAAS or DIAAS); this could be a focus of future study. Additionally, the PDCAAS works presented in this study and on almonds (15, 16) were performed in rats, while the pistachio feeding trial was conducted in pigs, a model that better estimates human nutrition (19). There are notable limitations to using PDCAAS, including the sample collection site and the artificial truncation of scores. Specifically, estimated nitrogen in fecal samples includes that from microbial fermentation of protein, whereas the DIAAS uses true ileal digestibility using samples collected from the terminal ileum. Additionally, PDCAAS is limited to an artificial truncation of 1.0, underestimating the value of high-quality proteins where scores might be above 1.0. To date, many foods have been evaluated using PDCAAS, while limited (but increasing) numbers of foods have been assessed using DIAAS. Despite these limitations, the PDCAAS of walnuts has not been demonstrated previously, and future research should determine a DIAAS value for walnuts and other tree nuts to increase the comparability of these nutrient-dense foods.

CONCLUSIONS

In this study, we have determined a PDCAAS for walnuts, as has been previously reported for pistachios and almonds. The true digestibility score for walnuts was determined to be 86.22 ± 1.83% and PDCAAS of 39 or 46% for children and 46 or 55% for adults, depending on the conversion factor used. Raw walnuts are not a good quality source of protein.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by International Animal Care and Use Committee at Covance, Inc. (Princeton, NJ; protocol #8379737).

AUTHOR CONTRIBUTIONS

SF performed the analysis. KL wrote the first manuscript draft. Both authors contributed to manuscript revisions, have read, and approved the final manuscript.

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Older Adults' Knowledge and Perceptions of Whole Foods as an Exercise Recovery Strategy

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Resistance exercise is a widely advocated treatment for improving muscle strength and performance in older adults. Maximizing the benefit of resistance exercise by ensuring optimal recovery is an important aim and studies are now seeking interventions to expedite exercise recovery in older people. A recovery strategy that has acquired considerable interest is the consumption of protein, and more recently, the consumption of protein-rich whole foods. This study aimed to understand the perspectives of community-dwelling older adults, and determine their knowledge of exercise recovery strategies, their preferences for recovery strategies, and their attitudes toward using whole foods, such as milk as a post-exercise recovery aid. Two hundred ninety-one older adults (74 ± 4 years) were recruited to complete a self-administered online survey. A mixed methods approach was used to gather in-depth data from the cohort. Participants were asked to complete a combination of free-text (open-ended) and multiple-choice questions. Content analysis was conducted on responses to open-ended questions through a systematic classification process of coding. The most common recovery strategies reported were heat treatment, rest, and massage. Nutrition was rarely cited as a recovery strategy. Less than 2% of respondents mentioned nutrition, of these, only half mentioned a protein source. Forty-nine percent expressed negative opinions toward recovery supplements (e.g., "waste of money") compared to 7% expressing positive opinions. Whole foods such as milk, meat, fish, and fruit, were deemed to be a more acceptable recovery strategy than supplements by 80% of respondents. Those that found whole foods to be equally as acceptable (18%), cited efficacy as their main concern, and those that declared whole foods less acceptable (2%) had no common reason. Despite the high acceptability of whole foods, only 35% were aware that these foods could aid recovery. When asked about milk specifically, the majority of older adults (73%) said this would, or might, be an acceptable exercise recovery strategy. Those that found milk an unacceptable recovery strategy (27%) often cited disliking milk or an allergy/intolerance. In conclusion, whilst whole foods represented an acceptable recovery intervention for older adults, the majority were unaware of the potential benefits of nutrition for post-exercise recovery.

Keywords: exercise recovery, whole foods, dietary protein, older adults, online survey

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INTRODUCTION

Resistance exercise is a widely advocated treatment for improving muscle health in older adults (1). However, despite a considerable body of literature demonstrating the benefits of resistance exercise in older adults (2, 3), the process of recovery from resistance exercise is largely understudied. Similarly, the identification of efficacious and acceptable exercise recovery strategies in an older population has not yet been achieved.

Maximizing the potential benefit of resistance exercise programs by ensuring optimal recovery is an important aim. Indeed, any transient decrements in physical functioning as a result of resistance exercise could be detrimental to an older individual when performing habitual daily activities (4, 5). Similarly, rapid and effective recovery from exercise underpins both improvements in physical performance and body punctuation, Therefore, many studies are now seeking to identify interventions to expedite exercise recovery in older people (6). Acute recovery from resistance exercise is a multifaceted process, and hence, any recovery strategy must consider the underlying physiological processes that occur. These processes include, but are not limited to, increasing rates of muscle protein synthesis and muscle protein breakdown, a coordinated cytokine response, rehydration, and glycogen resynthesis (7, 8). A recovery strategy that has therefore acquired considerable interest is the consumption of protein, and more recently, the consumption of protein-rich whole foods.

The beneficial effects of post-exercise protein supplementation on exercise recovery has been well-documented in younger adults (9, 10). In a recent meta-analysis, post-exercise intake of whey protein, which is rich in the amino acid leucine, was shown to expedite the recovery of muscle function in young adults (10). A potential whole food nutritional intervention that has both a high whey and leucine content is cow's milk. Semi-skimmed milk contains approximately 3.6 g of protein per 100 ml, of which approximately 80% is whey protein, and the remaining 20% casein. Milk also contains several essential micronutrients such as calcium and sodium, alongside carbohydrates (4.7–5.0 g/100 ml) and varying amounts of fats (0.3–3.7 g/100 ml), and is also approximately isotonic (11). This unique nutritional composition aids rehydration, glycogen synthesis, and restores energy balance, alongside optimizing muscle protein turnover and reducing muscle soreness (11). Hence, milk could be considered an optimal post-exercise recovery beverage. Indeed, it has already been shown that the ingestion of milk following muscle damaging exercise can attenuate muscle soreness and decrements in physical performance in younger adults (12–14). As a readily available and cheap food source, cow's milk may therefore potentially be a useful recovery strategy for older adults. However, despite evidence for the role of nutrition in enhancing exercise recovery in both younger (14, 15) and older (6) adults, current evidence of its acceptability amongst older adults is scarce—only one recent study has explored the feasibility and acceptability of milk as a post-exercise beverage (15). Similarly, the authors are unaware of any literature that has sought to define the perspectives of older adults, or determine their knowledge of, exercise recovery strategies, or exercise recovery supplements. To inform the direction of future research in this area, it is important that researchers and practitioners alike understand what exercise recovery strategies are currently used by older adults, their attitudes toward recovery supplements, and the acceptability of whole foods as recovery interventions. As such, this study aimed to understand the perspectives of community-dwelling older adults, and determine their current knowledge of exercise recovery strategies, their preferences for recovery strategies, and their attitudes toward using whole foods, such as milk as a post-exercise recovery aid.

MATERIALS AND METHODS

Design and Procedures

An online survey was developed that sought to understand older adults' knowledge in several categories. This was a broad survey, and hence, only one section of the data is reported in this paper. This section included (1) knowledge of exercise recovery strategies, (2) preferences for recovery strategies, and (3) attitudes toward using whole foods, such as milk as a post-exercise recovery aid. Questions pertaining to participant characteristics were included at the start of the survey. In total, the survey featured 32 questions (**Appendix A** in **Supplementary Material**). This paper focusses on the findings from question 22 onwards. The survey was administered over a 7-week period during July–August 2020 via *OnlineSurveys* (www.onlinesurveys.ac.uk), and was only available in English. An overview of the methodology can be found in **Figure 1**.

This survey used a mixed-methods approach. Participants were asked to complete a combination of free-text (open ended) and multiple-choice questions. A vignette was provided within the section "attitudes toward exercise recovery interventions" to help with contextualization of the following questions. For some multiple-choice questions, participants were prompted to answer further questions, or give a free-text response to provide additional information. For example, if participants answered "Yes" to the question "Have you ever purchased an exercise recovery supplement for yourself?" they were directed to answer the follow-up question "What supplement(s) did you purchase?"

Piloting

In the first instance, the survey was sent electronically to all members of the research team to ensure attainment of research aims and some minor amendments to wording and answer order were made. To assess the clarity of the questions and the usability of the survey platform, the survey was piloted by a small sample (∼five individuals) who provided feedback and submitted responses to the survey. This data was exported to ensure that the questions and requested answer type were correctly formatted. Finally, the survey was sent to three older adults for further feedback. Only minor amendments were made following this process.

Ethics

Ethical approval for this study was granted by Newcastle University Ethics Committee (Ref: 3648/2020). Before

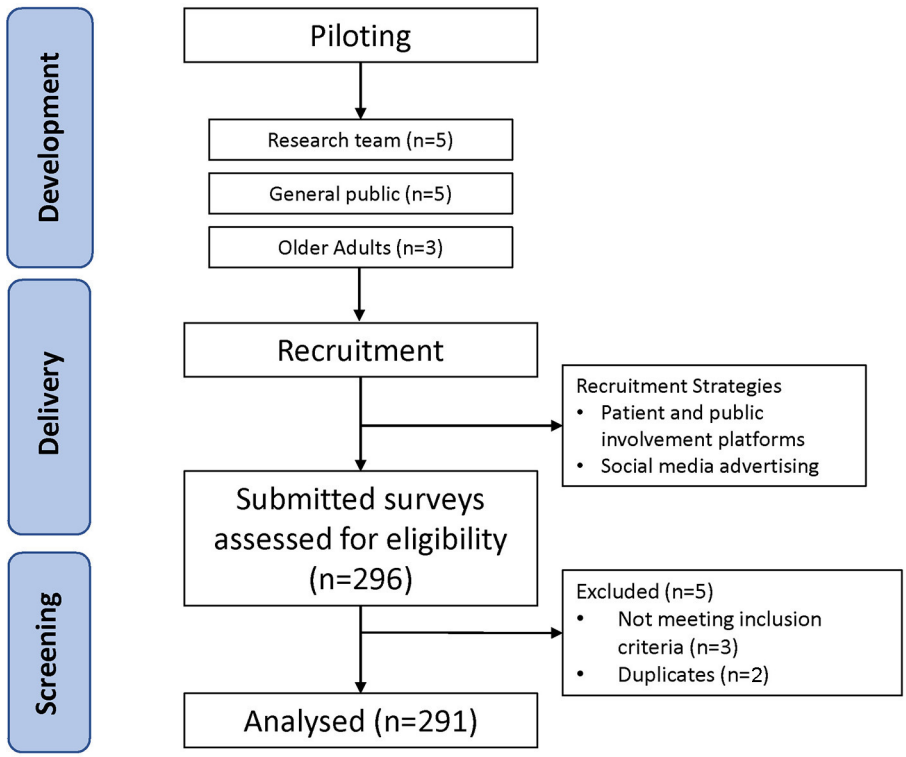

FIGURE 1 | Schematic of methodology.

completing the survey, participants were given a brief overview of the purpose and aims of the study and were provided with contact details of the research team should they have had any questions. Participants were informed that by submitting their responses they were providing consent to participate in the questionnaire. Participants were able to withdraw at any time before submitting their responses by simply closing the tab on their web browser. If participants withdrew by closing the tab, no data was collected, but they could choose to participate again at any time by re-opening the survey on their browser.

Participants

Participants were recruited through social media advertising (Facebook and Twitter), and patient and public involvement platforms (www.voice-global.org). Any individual over the age of 70 years with internet access could complete the survey—no other inclusion or exclusion criteria were applied.

Data Analysis

Three pre-defined categories were established to meet the aims of the project including; knowledge of exercise recovery strategies, attitudes toward exercise recovery supplements, and attitudes toward cow's milk as an exercise recovery strategy. A convergent parallel design was used to analyse both the quantitative and qualitative data. Answers given to multiple choice questions are reported as a fraction followed by a percentage. Manifest content analysis was conducted on responses to open-ended questions through a systematic classification process of coding to quantify the identification of words or concepts (16). Data were read and re-read to ensure familiarization and understanding, and interesting aspects of the data were highlighted that captured key concepts in relation to the pre-defined categories. Where more than one code could be derived from a singular free-text answer, codes are reported as a frequency. Where codes are mutually exclusive, data are reported as a frequency followed by a percentage. All percentages are rounded to the nearest whole integer. Where an answer could not be designated to a code, for example, if there were no other similar answers or it was not deemed to be answering the question, it was denoted as "'unassigned." If data are missing, the results are reported as a frequency and/or percentage of the sum of responses for that specific question, as opposed to the total number of participants. Similarly, if the question was such that an answer could be assigned more than one code (for example, if an answer mentioned several recovery strategies), then results are reported as frequencies only, and do not include a percentage.

RESULTS

Participant Characteristics

At the time of survey closure, 296 responses to the survey had been submitted. After initial screening, five responses were removed (Duplicate participant $n = 2$; Under 70 years of age $n = 3$) leaving 291 older adults (74 ± 4 years; mean ± SD) included

in the final analysis. Participants were largely independent, with 95% of respondents requiring no help to perform any activities of daily living, and 89% self-reporting at least "good" physical health. Twenty-three percent of participants that reported their gender were male, and 77% were female. Participants were also moderately active. Forty-two percent reported that they regularly participate in aerobic training (e.g., cycling, jogging, spinning classes, dancing, swimming), and 25% reported participating in resistance exercise. Fewer than 32% of respondents said that they perform <1 h of physical activity per day.

Knowledge of Exercise Recovery Strategies

The most common recovery strategy that older adults' reported using was heat treatment, including both hot baths/showers and cold treatments ($n = 107$). Other common exercise recovery strategies included rest ($n = 77$), stretching ($n = 41$), gentle exercise ($n = 32$), painkillers ($n = 31$), massage ($n = 28$), and topical ointments ($n = 22$) (**Table 1**). The use of nutrition as a recovery strategy was mentioned by only five (<2%) individuals, of these, only two reported using a high protein food.

Attitudes Toward Exercise Recovery Supplements

Ninety-five percent of respondents had never bought an exercise recovery supplement, and <1% had ever bought a protein supplement. Only 7% of older adults responded positively when asked their views on exercise recovery supplements, and 49% expressed negative views such as "waste of money" (**Table 2**). Eighty percent of respondents said that the use of whole foods (e.g., berries, fruit, meat, milk, and fish) was more acceptable than supplements as an exercise recovery strategy for reasons such as "food is more natural" and it being a "healthy diet" (**Table 3**). Those that were unsure if whole foods were more acceptable (18%) generally reported concerns of efficacy (**Table 3**), and those that found whole foods less acceptable (2%) generally referenced allergies/intolerances or ethical concerns. Despite whole foods being deemed more acceptable than supplements by a large majority of older adults, only 35% were aware that these foods could aid recovery.

Attitudes Toward Cow's Milk as an Exercise Recovery Strategy

When asked specifically if they thought milk was an acceptable recovery strategy, 43% said "yes" due to already liking milk, or believing in milk's health benefits (**Figure 2**); for example, one participant stated "Milk is a protein and would help building the damaged muscle" (**Supplementary Table 4**). Those that said milk was not acceptable (27%) generally disliked milk or had allergies/intolerances (**Figure 2**). Thirty-one percent said that milk "may be" be an acceptable recovery strategy, with the most common reasons for hesitancy being that they disliked milk ($n = 25$), or had not heard of milk as a recovery aid before ($n = 20$) (**Figure 2**). Others that were unsure were also unconvinced of the efficacy of milk for recovery (e.g., "If you could show me the science behind this I would be willing to try"). Just 46% of respondents said they thought they would be able to drink the suggested 500 ml of milk, and only 44% said they would be willing to drink 500 ml of milk after exercise.

TABLE 1 | Exercise recovery strategies reported by older adults[a].

Code	*n*	Examples
Heat treatment	107	• Used to take a long soak in a warm bath after a day walking • Hot water bottle • Use cold compress if necessary • Cold water shower over the muscles
Rest	77	• Have a day's rest then exercise muscles again. To get rid of lactic acid • Rested for a while until it eased
Stretching	41	• Stretched the area if possible • Gentle yoga
Gentle exercise	32	• Keep going with daily activities but avoid strenuous exercise for a few days and gradually increase • Took gentle walking exercise. Tried not to sit for long periods
Painkillers	31	• Two paracetamol often relieves the soreness and allows me to continue exercising • Take ibuprofen tablets or topical gel
Massage	28	• Massage the muscles • Used foam roller
Topical ointments	22	• Used over the counter pain relief rubs • 2 × Arnica 30c every 2 h
Drank water	16	• Drink plenty of water
Nothing	23	• Nothing, just carried on as normal • Usually gone in a couple of days without any intervention
Other	9	• A cup of coffee • I warmed up before exercise and stretched the muscles afterwards • I know about milk assisting recovery, but usually I don't do anything special
Change exercise routine	5	• Made sure I didn't push my body all out once start off slowly

[a] *"You may have experienced muscle soreness in the past after heavy gardening or DIY, jogging or exercising in a gym. If so, what did you do to ease muscle soreness and improve exercise recovery?" (291 responses).*

DISCUSSION

This study aimed to understand the perspectives of community-dwelling older adults, and determine their current knowledge of exercise recovery strategies, their preferences for recovery strategies, and their attitudes toward using whole foods, such as milk as a post-exercise recovery aid. To our knowledge this is the first study to explore these concepts in adults over 70 years of age using an online survey platform. Our main finding is that

TABLE 2 | Older adults' views on exercise recovery supplements[a].

Code	*n* (%)	Example
Positive	20 (7)	• Would be willing to give it a try • Some are beneficial to well-being
Neutral	43 (15)	• I'd prefer not to take any myself but think it's ok to do so • So-so some work, some don't • Proof they worked and how effective their use was to other older people, would be my view
Negative	144 (49)	• I would always be reluctant to take a supplement • Waste of money • Rubbish-a market encouragement to make money
No Opinion	84 (29)	• I know nothing about them • No idea, didn't know there was such a thing

[a] *"What are your views on exercise recovery supplements for older adults?" (291 responses).*

knowledge of nutritional strategies for exercise recovery was poor amongst older individuals, and despite not being aware of their benefits, whole foods were considered to be more acceptable than supplements for exercise recovery (see **Figure 3**).

Knowledge of Exercise Recovery Strategies

To determine what older adults currently know about exercise recovery strategies, participants were asked what exercise recovery strategies they had used in the past. The most common recovery strategy was the use of heat treatment by approximately a third of older adults, which included both hot (e.g., using a hot water bottle) and cold (e.g., using a cold compress) treatments. As the use of heat treatments is widely used for muscular complaints and injuries, this is perhaps unsurprising. Interestingly, rest was only the second most common stated recovery strategy, but this could be due to rest being a passive strategy, rather than being thought of as an active recovery strategy. Gentle exercise, stretching, massage, and topical ointments such as heat rub, were also popular. Drinking water was mentioned on 16 occasions as a recovery strategy. However, other than drinking water, nutrition was mentioned by only five individuals. Considering the research to date demonstrating the potential effectiveness of nutrition as a recovery aid for both younger (8, 17) and older adults (6), it is surprising that few older adults in this survey reported nutrition as a strategy to help muscle soreness. This suggests a discord between the knowledge of researchers and older members of the general public population, and displays a need for improved education and patient and public involvement when identifying suitable exercise recovery strategies for older adults.

Similarly, protein as an exercise recovery aid was mentioned by only two individuals (<1% of the sample). The low reported incidence of dietary protein as a recovery aid in the current study may have been exaggerated as a result of a general lack of understanding of exercise recovery and muscle soreness as a whole. However, our findings may demonstrate a lack of awareness, or understanding, of the benefits of dietary protein amongst older adults. Although not directly investigating protein for exercise recovery, a recent study has also found poor knowledge of dietary protein amongst 1,825 community-dwelling European older adults. Using an online survey, it was determined that 35.3% of the sample did not know what dietary protein was, and low protein knowledge was observed in 902 (49.4%) participants of the total study sample (18). Of more relevance to the current study is that amongst individuals with low protein knowledge, only 65% responded correctly to the statement that "You need protein in the diet for repairing bones and muscles." Similarly, a recent feasibility study has shown that giving older adults dietary advice and protein rich food products increased their dietary protein intake over 4 weeks, and most reported that they would continue following the advice on cessation of the study (19). If these results are translatable to a post-exercise setting, it is possible that there would be good compliance with protein-rich foods as an exercise recovery

TABLE 3 | Older adults' reasons for the acceptability of whole foods for exercise recovery compared to supplements[a].

Code	*n*	Examples
More Acceptable		
Food is more natural	92	• I would rather rely on natural food than supplements • It seems like a more natural process
It is a healthy diet	47	• Eating those food items would be good for me, regardless of the reason for taking them.
Dislike of supplements	24	• I don't like taking supplements or medication if I can avoid them • I am not a great pill popper so doing things through diet makes more sense.
I eat these foods already	19	• They are what I would eat normally anyway
Cost/Accessibility	11	• They carry no additional cost and no profit for charlatan's • Easier to implement and probably cheaper
Know what is in food	10	• I prefer to know what I am eating • Don't like not knowing what supplements are made of
Enjoyment of food	9	• More pleasant to consume
Unassigned	24	
Equally Acceptable		
Unsure of efficacy	12	• Will it seriously make a difference?
Already eat these foods	6	• I try to have a diet including such items already
Open to anything	5	• I'm quite flexible in other views to relieving post exercise pain
Food is a better option	5	• Fresh fruit and meat are always a better option
Supplements are easier	3	• Sometimes supplements are easier to take
Unassigned	11	

[a] *"Are whole foods more or less acceptable to you than supplements as an exercise recovery intervention? What is your reason for this?" (291 responses).*

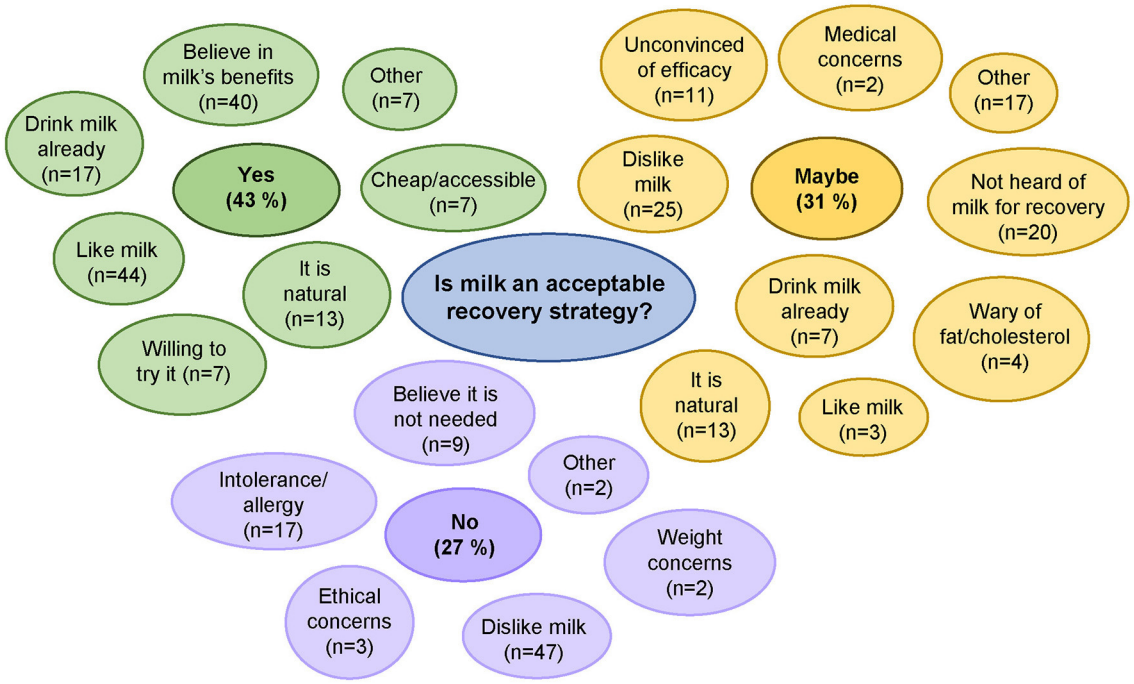

FIGURE 2 | Participant responses to; "We are specifically interested in milk as an exercise recovery beverage in older adults. Would this be an acceptable strategy to you? Please explain your answer?"

FIGURE 3 | Summary of main findings.

strategy. As a whole, these findings demonstrate a clear need to educate older adults on the benefits of protein, both for health and as an exercise recovery strategy. However, this is not necessarily exclusive to dietary protein. As mentioned previously, very few individuals named nutrition as an exercise recovery strategy and hence, education of several areas of nutrition may be warranted for older adults.

Attitudes Toward Exercise Recovery Supplements

Exercise recovery supplements have been shown to be efficacious (20) and are widely used (21, 22) in younger adults. However, the vast majority of older adults in this study had never bought an exercise recovery supplement, and <1% had ever bought a protein supplement. Given the previous finding that older adults' knowledge of dietary protein for exercise recovery is limited, it is perhaps unsurprising that so few had purchased exercise recovery supplements. Older adults' views on exercise recovery supplements is likely also a factor in how few had purchased a supplement in the past. Indeed, only 7% of older adults in this sample expressed a positive opinion of exercise recovery supplements, and almost half voiced a negative opinion (e.g., "*Rubbish—A market encouragement to make money*"). It is therefore unlikely that there would be good compliance to any recommended exercise recovery supplement for older adults. Instead, research may wish to focus on the use of commonplace foods that older adults are familiar with, and can access easily at their supermarket or food supplier.

In support of this, the current study found evidence that whole foods (e.g., berries, fruit, meat, milk, and fish) were a more acceptable recovery strategy than supplements by a large majority (80%) of older adults, despite over a third of older adults not being aware that these foods could aid recovery. The most common reason for this is that food was considered more natural or healthy. However, another common reason for finding whole foods more acceptable was simply a strong dislike of supplements. As mentioned by a number of participants, a whole food approach is also often cheaper than supplements, and hence could be more accessible to a greater proportion of older adults.

Emerging literature also suggests that a food-first approach could be more effective in enhancing post-exercise muscle remodeling when compared to isolated protein supplements in younger adults (23). This is because whole foods often contain other non-protein nutritive components (e.g., carbohydrates, lipids, micronutrients) that may interact to increase muscle protein synthesis rates beyond those expected from isolated proteins alone (23). Interestingly, such foods that may be useful for exercise recovery in older adults (e.g., meats, fish, eggs, legumes, nuts, and dairy) have also been shown to be myoprotective (24, 25), and so their increased consumption would likely complement resistance exercise to further preserve muscle mass and strength. Therefore, future research aiming to identify exercise recovery strategies for older adults could focus on a food-based approach to ensure uptake and adherence, whilst also considering the overall effectiveness of the food-stuff in a post-exercise setting.

Attitudes Toward Cow's Milk as an Exercise Recovery Strategy

The high-protein content of milk (3.6 g/100 ml) alongside its nutritional composition of carbohydrates, fats, and micronutrients suggests it could be useful for aiding exercise recovery (11). Indeed, milk has previously been shown to be an effective exercise recovery strategy in younger adults (14, 26), and has been shown to be beneficial for skeletal muscle health (25), but its acceptability to older adults is uncertain (15, 27). We explored older adult's attitudes toward milk as a high-protein exercise recovery strategy. In our survey, nearly half of older adults considered milk to be an acceptable recovery strategy, whilst approximately a quarter of older adults thought milk was unacceptable, and gave reasons such as dislike of milk, allergies, intolerances, and ethical concerns. Those that thought milk might be acceptable either disliked milk by itself, or had not heard of the benefits of milk as a post-exercise recovery strategy. This suggests that milk may be more acceptable to a larger proportion of older adults if they were educated on the benefits of milk, but there is currently no evidence to support this.

We have identified one recent study which explored older adults' attitudes and barriers to engaging in a resistance training program and consuming a recovery drink (bovine milk) after exercise (27). The study conducted semi-structured interviews after a 6-week exercise and nutrition intervention aiming to understand older adults' barriers and motivations for engagement. The study concluded that older adults considered milk to be an acceptable post-exercise recovery drink. Only one participant struggled with the volume of milk (2 × 500 ml), but overall post the taste and volume of liquid were viewed as acceptable. Of interest is that some participants did not think they would find milk acceptable before the intervention began, but began to look forward to consuming their drink (27). The authors concede that there is currently no evidence examining the efficacy of cow's milk for post-exercise recovery in older adults, but there is a number of studies investigating its use in younger adults (11, 13, 14), and for enhancing muscle protein turnover (28, 29). However, data from a pilot study of community-dwelling older adults suggests that cow's milk is both an acceptable and feasible post-exercise intervention for this group (15). (15) reported compliance to consuming 2 × 500 ml of milk after resistance exercise twice per week for 6 weeks of 97.1 and 98.3% for whole milk and skimmed milk, respectively, in a group of 29 older adults. Additionally, no participants reported finding the milk intervention difficult, or saw the volume of milk as a barrier. Only two participants reported minor changes to their usual diet as a result of the milk intervention (15). This suggests that should milk be found to be beneficial in older adults, it would be a suitable and accepted strategy for post-exercise recovery. It should be recognized that this study had a small sample size, and participation was likely dependent on the individuals having a positive predisposition toward milk consumption, and hence, the acceptability of this intervention amongst the general population is still unclear. This is promising for milk as an exercise recovery strategy for older adults, but further research must first be conducted to determine the efficacy of milk for recovery in an older population, and to explore other potential high-protein recovery strategies that may be more acceptable.

STRENGTHS AND LIMITATIONS

To our knowledge, this is the first study that has attempted to understand older adults' knowledge of, and attitudes toward, exercise recovery strategies. The results of this study will help to direct future research to identify effective and acceptable exercise recovery strategies for older adults. The use of an online platform to gather these data allowed a high number of participants to be recruited from across the United Kingdom.

However, this study has several limitations. A main concern of the current methodology is that the questionnaire was not validated, and it is therefore possible that some of the questions posed lead to a bias in the response of participants or did not gather the data that was intended. Additionally, some groups, likely those of lower socio-economic status, minority ethnic groups, or the very old, may not have been represented due to a lack of internet access. Unfortunately, to limit participant burden, detailed demographic information and participant characteristics were not collected, and hence, it is not understood to whom the results of the current study are most relevant. The literature would benefit from a similar study that uses semi-structured interviews to address our aims, as this will allow researchers to probe for further information, which was not possible with our survey. Interviews conducted within the community may also

allow access to individuals whose views have not been captured by the current study.

SUMMARY OF FINDINGS

Older adult's knowledge of exercise recovery was limited, and there is a clear need for education, especially concerning the role of nutrition for aiding exercise recovery. Older adults were much more accepting of whole foods as a recovery strategy than supplements, although knowledge of their benefits is poor. More work needs to be done to (a) provide education on exercise recovery foods, (b) identify other high protein, acceptable strategies for older adults other than milk, and (c) identify whole foods to aid recovery for those that cannot consume animal-based products.

CONCLUSION

In conclusion, whilst whole foods represented an acceptable exercise recovery intervention for community-dwelling older adults, the majority were unaware of the potential benefits of nutrition for post-exercise recovery. Older adults may benefit from education about beneficial interventions such as nutrition for exercise recovery, and further research should be conducted to determine the efficacy of whole foods such as milk for improving exercise recovery.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EH, AG, CH, LD, ES, and AS contributed to the conception and development of the survey. EH, AG, and ES were responsible for the piloting of the survey. EH distributed the survey and completed data analysis. EH authored the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.748882/full#supplementary-material

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Different Effects of Soy and Whey on Linear Bone Growth and Growth Pattern in Young Male Sprague-Dawley Rats

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The aim of this investigation was to determine the better protein for supporting optimal linear growth, as the exact composition and benefits of specific dietary proteins in supporting linear growth is unknown. In the current study, we compared the effect of soy and whey proteins, both proteins contain all essential amino acids and are considered the best proteins in their categories. Young male rats were subjected to multiple feeding protocols using iso-energetic diets containing soy or whey as the sole protein source. The rats were allowed to eat *ad libitum* for 11, 24, or 74 days in the first set of experiments, and the soy group was pair-fed to the whey group in the second set. The differences in weight gain, food consumption, and humeri length of the soy group that were greater at the beginning of the *ad libitum* experiments lessened over time. Pair-fed experiments revealed that the increased weight and humeri length resulted from the differences in food consumption. However, other parameters were protein specific. Bone quality, which was better in the soy group at 24 days, was matched by the whey group and even surpassed that of the soy group in the long-term experiment, with a significantly greater bone mineral density, cortical thickness, and growth plate. Although in the short term the levels of insulin like growth factor (IGF)-I were similar between the groups, IGF-I increased with age in the whey group, and the levels at the long-term experiment were significantly higher compared to the soy group. Furthermore, using the pair fed setup made it clear that when the difference in food consumption were no longer playing part, whey was more efficient in increasing IGF-I. There were no indications of metabolic sequelae. Although the use of soy is gaining in popularity as a sustainable protein, our findings indicate a better effect of whey on linear growth by leading to slower growth with better-organized epiphyseal growth plates and bone quality.

Keywords: soy, growth plate, whey, catch up, micro CT, IGF-I, BMD

INTRODUCTION

Linear growth is a result of a complex system of interactions between genetics, epigenetics, and environmental factors (especially stress and nutrition). The association between nutrition and linear growth in children is well-recognized and documented in studies performed in underprivileged countries as well as in studies on the secular trend in height in European countries (1, 2). A study designed to explore the main correlates of male height in 105 countries (Europe & overseas, Asia, North Africa, and Oceania) with an average consumption of 28 protein sources and seven socioeconomic indicators concluded that nutrition and genetics are the strongest correlates of adult height (3). Intake of protein from milk products (dairy proteins), followed by total protein and animal protein (meat and eggs) consumption emerged as the most significant nutritional correlates of stature in most countries examined (3). The results of that study indicated that plant-based diets are not able to provide the optimal stimuli for physical growth, even if the intake of total protein and total energy are adequate. In fact, a difference of 10 cm in average male height (174 vs. 184 cm) was identified between nations relying upon the surplus of plant and animal proteins, respectively, pointing to the importance of protein quality (3).

Plant-based diets are sometimes considered a dietary strategy for maintaining good health, for protecting against inflammatory conditions, and for managing pathological conditions ranging from metabolic syndrome (including obesity, diabetes, and cardiovascular risk) to cancer. The use of plant-based protein isolates in food formulations has become a focus of interest due to greater sustainability, lower production costs, and a lower ecological footprint compared to animal-based ones. Although the average male height was found to correlate most negatively with proteins from rice and legumes (including soy) (3), the evidence of the differences in the effect of plant-based diets compared to animal-based diets on linear growth in children is mostly correlative, and the effect of plant-based diets on linear growth has not been tested in depth.

Proper growth and development in children are considered as markers for good health. Linear growth, driven by chondrocytes, is subject to regulation by numerous local and systemic factors, many of which are responsive to nutritional cues. The process of linear growth involves the sequential replacement of chondrocytes located in the cartilaginous growth center of the long bones [the epiphyseal growth plate (EGP)] by osteoblasts, a process regulated by complex interactions among hormones, local growth factors, and components of the extracellular matrix (ECM). Endochondral ossification begins with the proliferation of early chondrocytes (in the reserve zone), followed by their alignment in columns (in the proliferation zone), and, finally, their maturation into hypertrophic chondrocytes (in the hypertrophic zone). The hypertrophic cells then cease dividing, increase in volume by 5- to 10-fold, and boost the deposition of ECM components, mostly collagens and proteoglycans, and the secretion of matrix vesicles that serve as centers of mineralization. Thereafter, the chondrocytes undergo either programmed cell death, with calcification of the ECM, enabling the invasion of blood vessels and osteoblasts or transdifferentiating to endochondral osteoblasts. Bone tissue replaces the cartilage scaffold as a result of this chain of events. Proper elongation of the skeleton requires that endochondral ossification and bone modeling be tightly synchronized.

Although it is well-known that fasting and food restriction impair the rate of longitudinal bone growth and reduce the height of the EGP (2), the best food for supporting linear growth remains uncertain. Apart from the role in providing building blocks for cellular growth, proteins may also act as regulatory agents by affecting insulin like growth factor (IGF)-I and calcium absorbance and by altering the gut microbiome. Indeed, our previous studies have shown that the source of the consumed protein may affect proper growth (4). Our comparisons between two high-quality proteins of milk origin (whey and casein) revealed significant differences in linear growth and microbiome composition (4, 5).

In the current study, we compared the effect of soy and whey proteins on linear growth and bone strength in young fast-growing male rats. Both proteins contain all essential amino acids (EAA) and are considered the best proteins in their categories according to the protein digestibility-corrected amino acids score (PDCAAS). Whey is especially rich in branched amino acids (BCAA) as well as sulfur-containing amino acids (6) and is graded as the best protein source according to its essential amino acid score and PDCAAS (7). Soy ranks second after whey as a complete food protein and is the most popular plant proteins utilized in the production of newborn formulas and dietary supplements. Given that soy-based formulas are the formulas of choice for children with food allergies and for children whose parents opt to avoid food from animal-derived products for various reasons, it becomes all that more important to compare the effect of soy and whey proteins on linear growth.

METHODS

Animals

All experiments were performed on pre-pubertal 26-d-old male Sprague-Dawley rats (Envigo Laboratories Ltd., Jerusalem, Israel). The approval of the Tel Aviv University Institutional Animal Care and Use Committee to which the Felsenstein Medical Research Center (FMRC) is affiliated was obtained before the experiments were initiated (committee protocol approval number 01-20-062). All of the animals were kept under the same experimental conditions: mean ambient temperature 23 (±1) °C, mean relative humidity 50 (±2) %, 12 h light/dark cycle, and lights off at 19:00 h. They all had free access to unfiltered regular tap water and were fed one of the custom-made commercial diets (**Supplementary Table 1**). The animals were kept two in a cage at the animal care facility of the FMRC or in single cages to allow monitoring of food intake during the catch-up and the pair-fed experiments. The animals were observed daily, and none showed signs of disease throughout the study apart from restlessness and slight aggressiveness during the food-restriction period.

Feeding Regimens

The diets were iso-energetic and contained either soy protein (TD190912) or whey (TD190911; Teklad, Envigo Diets, Madison WI, USA) as the sole protein source (**Supplementary Table 1**). All other ingredients (cornstarch, carbohydrate, cellulose, fat, vitamins, and minerals) were identical. In the first *ad libitum* (AL) set of experiments, the rats were given free access to food and water for 11, 24, or 74 days. There were six animals per group in the short-term experiments, and eight animals per group in the 74-day experiments. In the second set of experiment (Pair-fed experiment) ($n = 8$), the amount that animals from whey group consumed was measured each day (during the 24 days of the study) and the same amount of food was then given to the pair-fed soy group the following day. To allow precise matching of food intake, the pair-fed group was started 1 day after the whey group. In the catch-up experiment, one group was fed AL with regular rat chow (TD 2918) (AL group, $n = 6$), and the restricted group was fed 60% of the normal daily intake of the same regular rat chow for 10 days (4). On day 10, the restricted group was further divided into one group that was fed the soy diet and the other group that was fed the whey diet for an additional 1 or 14 days ($n = 6$ in each group) with no restrictions. Experimental design is depicted in **Supplementary Figure 1**. All of the rats were euthanized by CO_2 inhalation at the end of the experiments.

Glucose Measurement and Serum Analysis

An intraperitoneal (i.p.) glucose tolerance test (GTT) was performed several days before the termination of the 74-day experiment. Animals were fasted for 6 h, and a glucose solution (1 g glucose/kg) was injected i.p. Blood glucose was measured by a portable glucometer (Contour plus, Ascensia Diabetes Care Holdings AG, Switzerland) in blood samples drawn by a needle puncture from a tail vein before and at 15, 30, 60, and 120 min post-glucose injection.

Fasting glucose levels were measured on a portable glucometer and assayed at the last day of the long-term experiment, in animals that were fasted for 12 h. The rats were euthanized by CO_2 inhalation at the end of this experiment, and blood was collected by cardiac puncture. Serum was separated by centrifugation at 1,500 RPM (239*g) in a Rotina 46R centrifuge (Hettich Zentrifugen, Apeldoorn, the Netherlands) for 10 min at 4°C and stored at −70°C. Chemical analysis of the samples was performed by American Medical Laboratories, Israel (AML), and the results were compared to the control values supplied by AML. Serum levels of insulin-like growth factor-I (IGF-I), were determined using a commercial kit according to the manufacturer's recommendations (Quantikine Mouse/Rat IGF-I assay kit, detection limit 8.4 pg/ml [cat. no. MG100, R&D Systems, Minneapolis, MN, USA]).

Histological Staining and Measurement of Growth Plate Height

The humeri of the euthanized animals were carefully removed, cleaned, and measured for length with a digital caliper. They were then fixed in 4% neutral buffered formalin for 48 h at room temperature, decalcified with Surgipath Decalcifier II (Leica Biosystems Richmond, Inc. USA) for several hours (depending upon the age of the animal), dehydrated through graded ethanol series (70, 95, and 100%), and stabilized by two sequential changes of chloroform for paraffin embedding. Histological studies and EGP height measurements were performed on deparaffinized sections of 6 μm thickness that had been stained with hematoxylin-eosin and Alcian blue. The height of the EGP was measured by drawing a straight line from the apical border of the reserve zone cells to the lower border of the mineralized cartilage. The findings presented here represent the average of at least five measurements per each section. The slides were photographed under an Olympus BX40 microscope equipped with an Olympus DP71 camera (Olympus Optical Co. GmbH, Hamburg, Germany), and analyzed with Image-Pro software (version 4.5.1.22, Media Cybernetics, Inc., Rockville, MD, USA).

μCT Analysis

The humeri were kept in 4% neutral buffered formalin for 48 h at room temperature and then stored in 70% ethanol. The entire right humerus was scanned with a micro-computerized tomographic (μCT) system (μCT50, Scanco Medical AG, Switzerland). The scans were acquired at 90 kVp, 200 μA, and 1,000 ms for energy, intensity, and integration time, respectively, generating images with an isotropic nominal resolution of 17.2 μm. Two-dimensional (2D) CT images were reconstructed in 2,048 × 2,048 pixel matrices by means of a standard convolution-backprojection procedure (Scanco uct_reconstruction v6.1). A 3D Gaussian filter was used to attenuate the background noise in the volumes ($\sigma = 0.8$; support = 1). The scans were segmented by a global thresholding procedure (trabecular attenuation = 130; cortical attenuation = 200 in permille of the total gray value range). Morphometric parameters were determined with a direct 3D approach (8) in three different pre-selected analysis regions by means of customized software developed on the proprietary Image Processing Language v5.15 (Scanco Medical). We measured humerus length and bone volume fraction (BV/TV, %) (9) along the entire bone. In the cortical bone, we used a 1-mm-height diaphyseal segment starting at the 6th tenth of the total length (slightly distal to the midshaft). Cortical measurements included total area (Tt.Ar, mm^2), cortical area (Ct.Ar, mm^2), cortical area fraction (Ct.Ar/Tt.Ar, %), and cortical thickness (Ct.Th, mm). To analyze the trabecular bone, we used the secondary spongiosa of the proximal metaphysis of the humerus that had been manually separated from the cortical bone by tracing the endosteal surface on the axial 2D tomographic slices. The measurements included trabecular bone volume fraction (BV/TV, %), trabecular number (Tb.N, mm^{-1}), trabecular thickness (Tb.Th, mm), and trabecular separation (Tb.Sp, mm).

Statistical Analysis

Data are presented as mean ± standard deviation (SD). We used the One-Sample Kolmogorov-Smirnov Test to test the null hypothesis that distribution of the parameters is normal; all *P*-value were non-significant ($p > 0.05$), therefore all parameters have normal distribution and the significance of differences between experimental groups was determined using Student's *T*-test. Levene's test for equality of variance was used to check equal

variance and we used the *P*-value accordingly. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Effect of Soy vs. Whey on Linear Growth (Short-Term AL Feeding)

Young male Sprague-Dawley rats were fed AL for either 11 or 24 days in order to investigate whether there is any difference between the effects of the soy and whey diets on growth parameters (**Figure 1A**). The weight gain of the soy-fed rats was greater compared to the whey-fed rats from the beginning to the end of the study in both experiments. Food consumption of the soy group was greater until day 16 of the experiment after which the difference between the two groups diminished considerably (**Figure 1B**). The humeri of the soy-fed rats were significantly longer in both experiments (**Figure 1C**), however, the EGP height was greater in sections taken from the whey-fed animals ($p < 0.05$; **Figures 1D,E**).

Effect of Soy vs. Whey on Linear Growth (Pair-Fed Feeding)

We performed a pair-fed study in which the amount of food provided to the soy-fed group was matched to that of the whey-fed group on the day before in order to determine if the different effect on growth was due solely to the difference in food consumption. It emerged that the weight gain in both groups was similar (**Figure 2A**). While the humerus length at the end of the experiment was not significantly different between groups (**Figure 2B**), the height of the EGP was significantly greater in the whey group compared to the soy group ($p < 0.05$; **Figures 2C,D**).

Effect of Soy vs. Whey on Linear Growth (After Food Restriction)

After a period of growth attenuation, the removal of the growth inhibitory factor is usually followed by spontaneous catch-up growth (10, 11). A permanent growth deficit occurs when recovery is incomplete, leading to short stature. In view of the different effects of the soy and whey diets on linear growth when fed AL, we further examined whether the type of protein ingested during the re-feeding period will affect the efficiency of the catch-up growth process. Specifically, the animals were food restricted for 10 days and then re-fed for 1 or 14 days with either the soy or the whey diets. Soy led to more rapid weight gain, while whey led to a greater EGP (**Figures 3A–H**).

Effect of Soy vs. Whey on Linear Growth (AL Feeding, Long-Term Follow Up)

We performed an additional AL study to check whether the different growth patterns that we had observed in both the AL and catch-up models will translate into differences in bone length when reaching adult size. The rats were randomized to eat one of the two diets AL after which they were followed up to the age of 100 days, an age at which, according to previous publications, bone length reaches its final length and the subsequent changes in length are minimal (12). The results (**Figures 4A,B**) showed that the differences in weight and food consumption that were apparent at the beginning of the study (and which matched those in the short-term experiments), were no longer apparent after 59 and 23 days, respectively. The weight, food consumption, and the length of the humeri (**Figure 4C**) were indistinguishable at the age of 100 days. However, EGP height was still significantly greater in the whey-fed group (**Figures 4D,E**). Moreover, EGP seemed to be better organized (**Figure 4E**), and the cell density in columns was greater in the whey group.

Serum Analysis

We checked to see if there had been any effect of diet consumed on metabolic parameters in the long-term after having observed that the growth pattern, especially with regard to weight gain, was more robust in the soy-fed animals at the beginning of the study. GTT performed several days before sacrifice showed no significant differences between the groups (**Supplementary Figure 2**). The fasting glucose assessment, at the last day of the long-term experiment showed no significant differences between the groups (soy group 81 ± 4.5 mg/dL, whey group 82.5 ± 2.9 mg/dL; $p = 0.44$). Analysis of the blood samples taken at sacrifice showed that all values were within the normal range for Sprague-Dawley rats (**Table 1**; normal range provided by AML Israel Ltd.), and there was no evidence of interference in either kidney or liver activity. Interestingly, the soy-fed animals showed a lower level of total cholesterol (by 15%), with no comparable effect on triglyceride levels.

IGF-I Levels

IGF-I levels were not significantly different between the groups at the end of the short-term experiment, however when the differences in food consumption were excluded (pair-fed and long-term experiments) the serum level of IGF-I were significantly greater in the whey group (**Table 2**). IGF-I increased over time (i.e., with age) by 55% in the whey group ($p = 0.001$) and by 15% in the soy group ($p = 0.03$).

μCT Analysis

The long bones consist mainly of two different types of structures: the cortical bone, which forms the hard outer layer of the long bone, and the trabecular bone (spongy bone), which is less dense and less stiff, and has a higher surface area enabling high vascularization. μCT analysis, the gold standard for determining bone microstructure in animal models, was used to study the effect of the diets on humeri from both the 24-day and the 74-day experiments (**Table 3**). The humerus length of the soy group was greater in the short-term experiment, and the μCT analysis showed that the diaphyseal diameter (Dia.Dia) of the cortical bone was also greater, suggesting an accelerated radial growth. This likelihood is also supported by the greater moment of inertia (MOI) parameters, which predict the resistance of the bone to shear forces, in the soy-fed rats (**Table 3** and **Figures 5A,B**). At age 100 days, after 74 days of feeding, we found that all the parameters that showed improved values at 24 days in the soy group were no longer different between the diet groups. Indeed, the whey group reached the same values for bone length, Dia.Dia and all MOI values, as those of the soy group at 74 days.

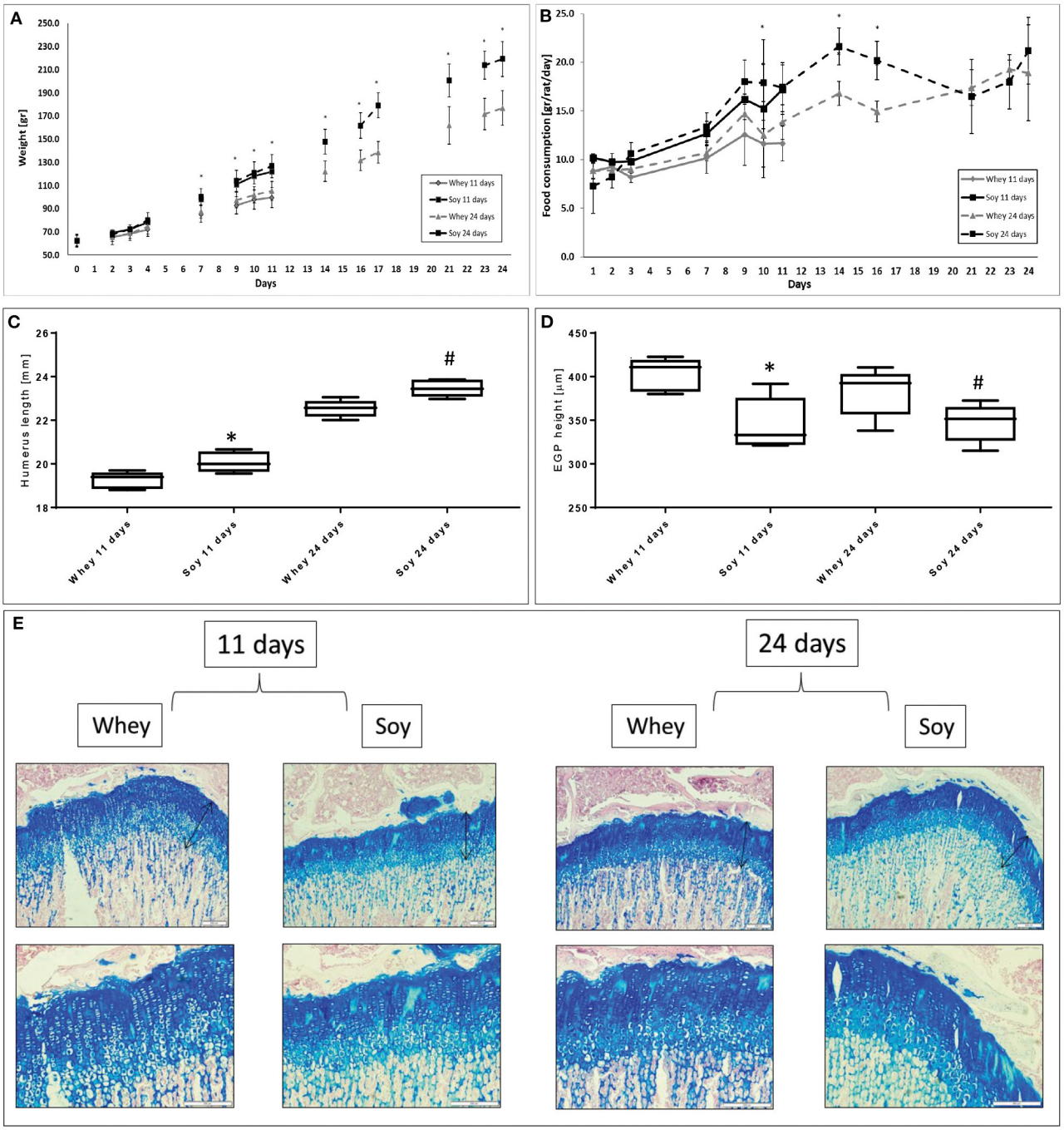

FIGURE 1 | Differential effect of soy and whey diets on growth during 11 or 24 days of ad libitum feeding. **(A)** Body weight, **(B)** food consumption, **(C)** humerus length, **(D)** EGP height, and **(E)** representative stained sections of the EGP (upper panel-magnification ×10; lower panel -magnification X 20). Statistical analysis was done by Student's *T*-test. The asterisk (*) within the graphs designates significant differences at $p < 0.05$ for whey vs. soy at 11 days; The pound sign (#) within the graphs designates significant differences at $p < 0.05$ for whey vs. soy at 24 days. The black arrows indicate the height of the growth plates. **(C,D)** The box plot shows the minimum, first quartile, median, third quartile, and maximum in humerus length **(C)** and EGP height **(D)** after 11 and 24 days of free feeding. EGP, epiphyseal growth plate.

Moreover, the whey-fed animals had superior micro architectural parameters in the full humerus (%BV/TV, vBMD), mainly due to improved cortical parameters. The Ct.Ar/Tt.Ar was greater in the whey group because of the thicker cortex (Ct.Th) at the expense of the medullary cavity diameter (Med.Dia) (**Table 3** and **Figures 5A,B**).

The differences in growth pattern were better exemplified by analysis of age-induced changes in the µCT parameters

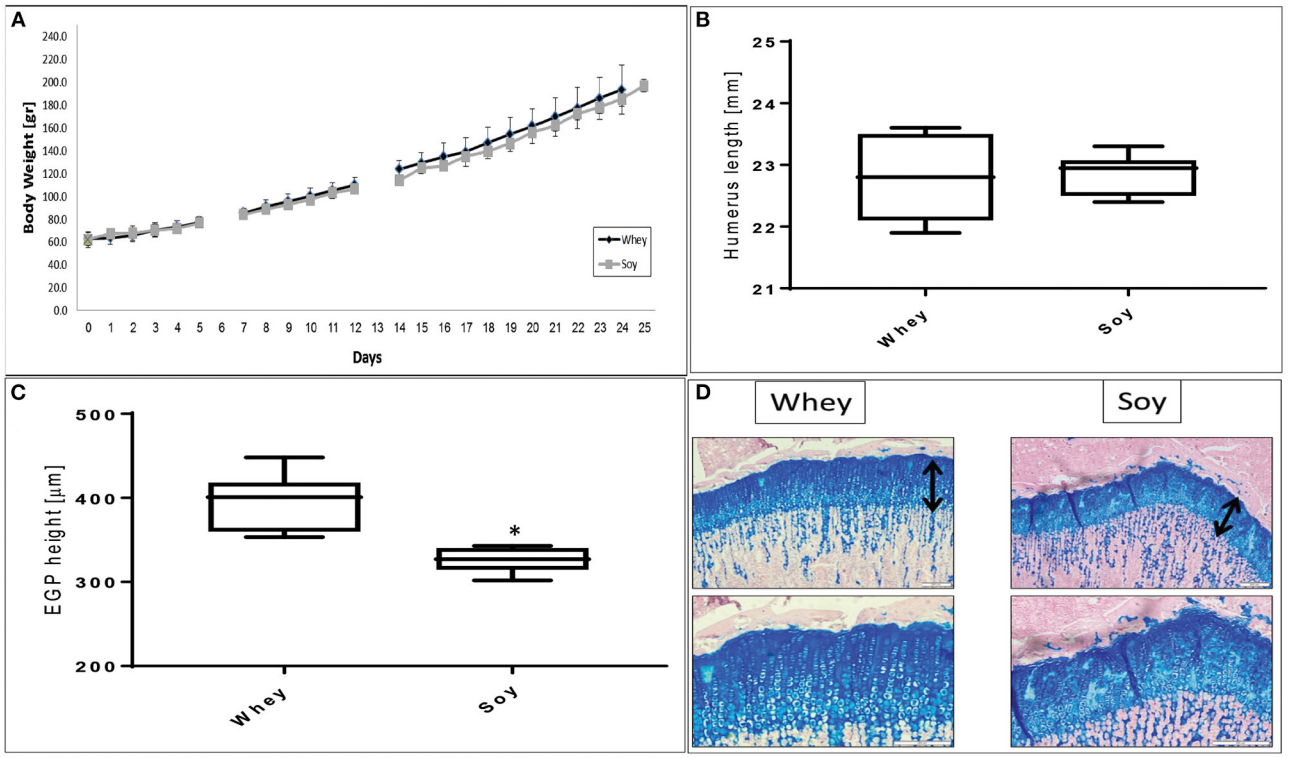

FIGURE 2 | Pair-fed experiment. **(A)** Animal body weight, **(B)** humerus length, **(C)** EGP height, and **(D)** representative stained sections of the EGP (upper panel-magnification ×10; lower panel magnification X 20). Values are means ± SD. Statistical analysis was done by Student's *T*-test. The asterisk (*) within the graphs designates significant differences at $p < 0.05$. The black arrows indicate the height of the growth plates. **(B,C)** The box plot shows the minimum, first quartile, median, third quartile, and maximum of humeru length **(B)** and EGP height **(C)** after 24 days of pair-fed feeding. EGP, epiphyseal growth plate.

(**Table 4**). Changes over time clearly showed that while many bone parameters were better in the soy group at the end of the short-term experiment, the whey group corrected most parameters of bone structure over time, leading to the same length and partially better cortical bone parameters.

In the humeral proximal metaphysis, the trabecular bone showed no statistically significant differences between the groups at 24 and 74 days (**Table 3**). However, there was a distinct pattern of time-related changes in the trabecular parameters (**Table 4**). While the connectivity density (Conn.D) tended to decrease in the soy groups between 24 and 74 days of the diet, it tended to increase in the whey group ($p = 0.045$). There was a similar pattern for the trabecular BV/TV, although the difference in time-related changes was of borderline significant ($p = 0.08$).

These data showed that the skeletal response to the type of protein in the diet had a time/age dependency, with increased growth in the soy group during the first 3.5 weeks and increased growth in the whey group during the following 10.5 weeks.

DISCUSSION

The aim of this investigation was to determine the better protein for supporting optimal linear growth. The most interesting observation of our current study was the different effect of the two proteins on the growth pattern and humerus bone quality in an animal model. The differences in weight gain observed after 24 days of feeding were no longer apparent after 74 days of feeding. Bone quality, which seemed to be better in the soy group after 24 days of feeding was matched and even surpassed, by the whey group after an additional 50 days of feeding. In the long-term experiment, µCT analysis revealed a significant difference in bone mineralization between the groups, suggesting better biomechanical parameters in the whey group. We also observed a higher and better-organized EGP in the whey groups, with no significant differences in the trabecular compartment throughout the study. The effect on growth was similar in all of the study setups: the soy diet led to a more rapid weight gain and bone growth, while the whey diet led to slower growth with better outcomes. The effect of whey on growth was slower, maintaining a higher EGP for a longer time, similar to what we had found in a previous study in which we compared casein- and whey-based diets (4). Both serum analysis and GTT showed that in spite of the greater food consumption and weight gain at the beginning of the study, there were no indications for metabolic disease in either group: there was no effect on kidney or liver function, and all metabolic values were within the normal range for rats.

There is an increasing interest in plant-based foods due to both ecological and financial reasons. Production of plant-based

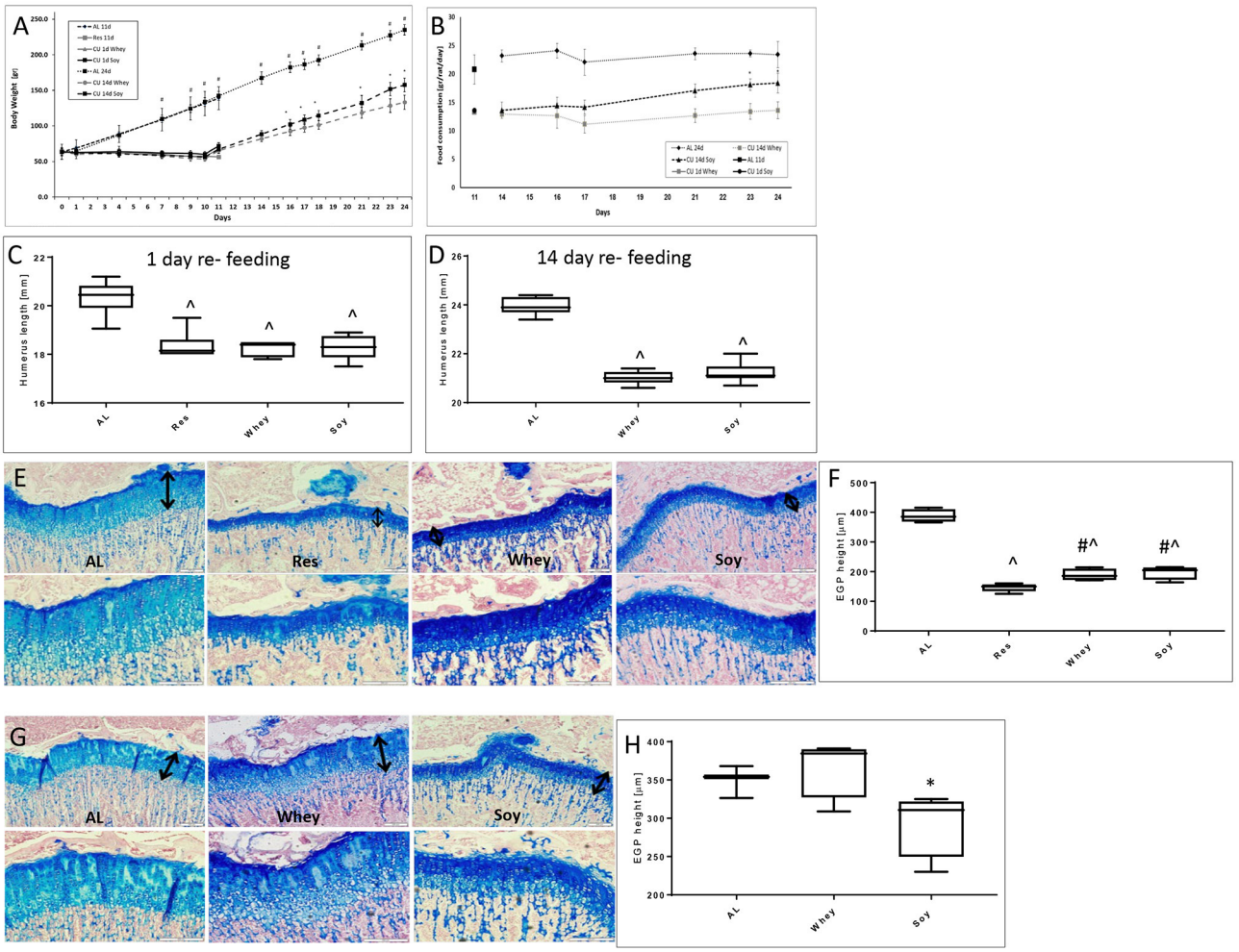

FIGURE 3 | Catch-up growth experiment. **(A)** Animal body weight, **(B)** food consumption, **(C)** humerus length 1 day refeeding, **(D)** humerus length 14 days refeeding, and **(E)** representative stained sections of the EGP at 1 day refeeding (upper panel magnification ×10; lower panel magnification X 20); **(F)** EGP height at 1 day of refeeding. **(G)** Representative stained sections of the epiphyseal growth plate (EGP) at 14 days refeeding (upper panel magnification ×10; lower panel magnification X 20). The black arrows indicate the height of the growth plates. **(H)** EGP height at 14 days refeeding. Statistical analysis was done by Student's *T*-test. The asterisk (*) within the graphs designates significant differences at $p < 0.05$ for soy vs. whey. The pound sign (#) within the graphs designates significant differences at $p < 0.05$ for Res vs. Whey/Soy; The caret sign (∧) within the graphs designates significant differences at $p < 0.05$ for AL vs. Res/Whey/Soy. **(C,D,F,H)** The box plots show the minimum, first quartile, median, third quartile, and maximum of humerus length after 1 **(C)** or 14 days refeeding **(D)** and EGP height at 1 **(F)** or 14 days of refeeding **(H)**. EGP, epiphyseal growth plate; AL, *ad libitum*; RES, food restriction; CU, re-fed group, showing catch up growth.

foods requires less land and water and is associated with lower greenhouse gas emissions compared with animal-based foods. At the same time, however, plant-based proteins are considered as being of lesser quality with respect to their ability to increase both post-prandial muscle protein synthesis rates (13) and linear growth, as exemplified by the differences in male adult height (3). However, there is large variability in amino acid composition among different plant-based protein sources (14), and soy protein is among the few plant-based proteins that meet the requirements for EAA content and is therefore considered the best vegetarian protein (15). Given that the quality of soy proteins is considered as being superior to that of other plant proteins, we decided to compare it to whey in fast-growing young rats.

Soy consumption has historically been associated with Asian countries, however, the popularity of soy foods in the United States increased significantly after the approval by the Food and Drug Administration that soy protein has the ability to protect against cardiovascular diseases (16). Indeed, in the current study, cholesterol levels were significantly lower in the soy-fed rats (by 15%), although no effect on triglyceride levels was noted. This is in agreement with previous studies that showed that the intake of soy products resulted in a significant reduction in serum cholesterol concentration (by about 5%) in both humans (17) and rodents (18–20). A possible mechanism of the cholesterol-lowering effect of soy protein is its ability to modulate low-density lipoprotein receptor levels in the liver (20).

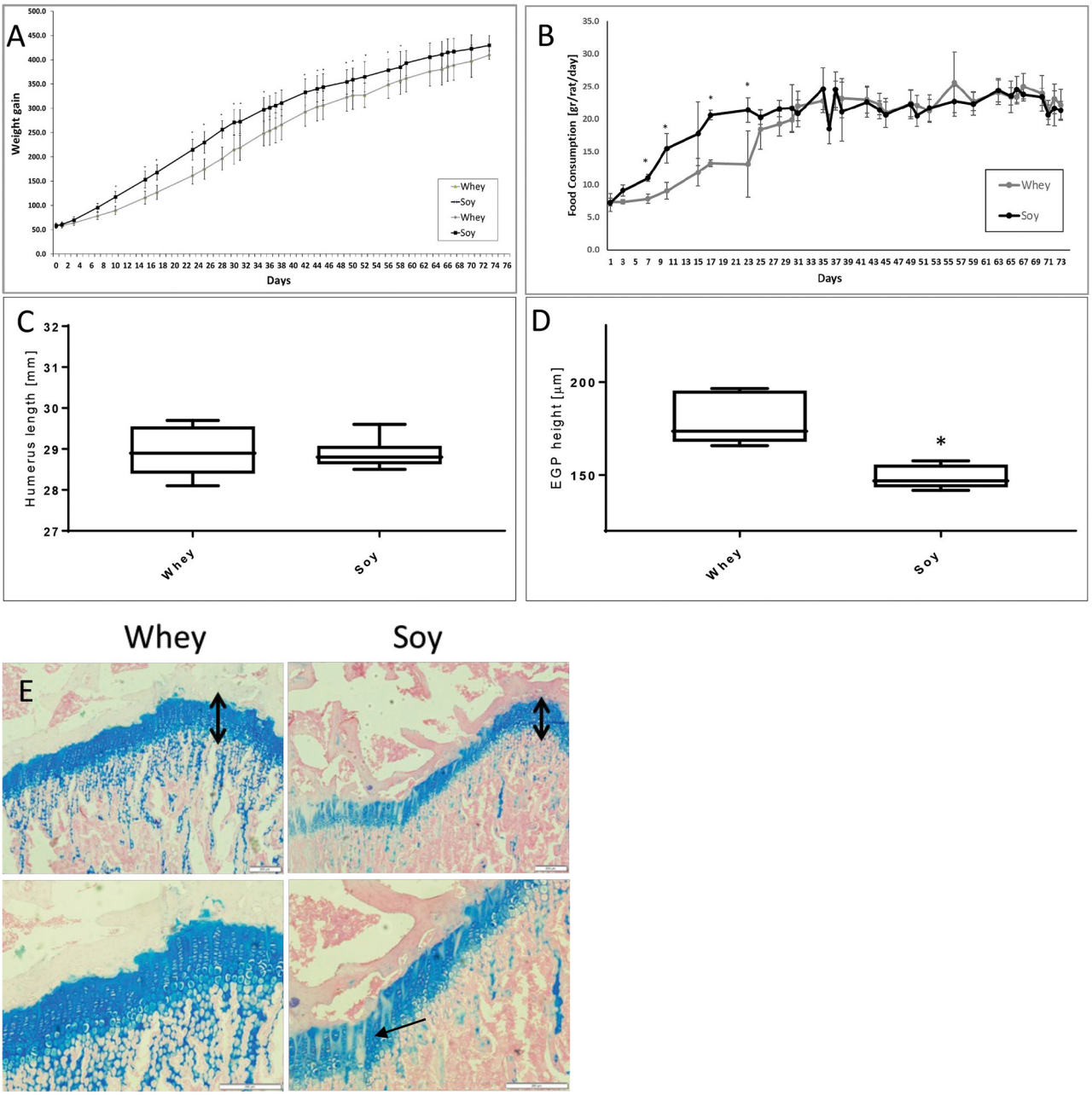

FIGURE 4 | Long-term experiment. **(A)** Animal body weight, **(B)** food consumption, **(C)** humerus length, **(D)** EGP height, **(E)** Representative stained sections of the epiphyseal growth plate (EGP) (upper panel magnification ×10; lower panel magnification X 20) (upper row magnification ×10, lower row magnification ×20). The black arrows indicate the height of the growth plates. Note the better organization of the EGP in the whey group (marked with arrows). Statistical analysis was done by Student's *T*-test. The asterisk (*) within the graphs designates significant differences at $p < 0.05$ for Soy vs. Whey. **(C,D)** The box plot shows the minimum, first quartile, median, third quartile, and maximum in humerus length **(C)** and EGP height **(D)** after 74 days free feeding. EGP, epiphyseal growth plate.

The greater effect observed in our study compared to that cited in the literature may lie in the fact that we gave the animals purified soy protein, while a more complex diet had probably been given to the humans or to the animals in those studies.

A number of epidemiological and experimental studies claimed that soy has other health benefits, including its ability to mitigate obesity, diabetes, and related complications (21). In our study, no effect on glucose levels either during fasting or in response to glucose loading was found between the groups, which may be due to the young age of the animals. The only beneficial effect that we found in soy food was a reduction in cholesterol levels, described

TABLE 1 | Serum analysis in male rats after 74 days with soy or whey diets (values are presented as average ± SD).

	Units	Whey	Soy	*p*-value	Normal range
Creatinine	mg/dl	0.45 ± 0.04	0.52 ± 0.06	0.050	0.27–0.65
Urea	mg/dl	36.31 ± 2.30	36.54 ± 2.43	0.86	29.93–59.17
SGOT	IU/L	126.57 ± 38.26	171.71 ± 2.43	0.065	57–210
SGPT	IU/L	37.14 ± 4.87	45.42 ± 9.90	0.08	30–106
Cholesterol	mg/dl	108.57 ± 17.11	92.71 ± 8.06	0.055	79.18–137.3
Trig	mg/dl	58.43 ± 15.5	59.57 ± 10.08	0.87	21–86
Total P	g/dl	6.42 ± 0.18	6.93 ± 0.26	0.001	5.92–7.46
Albumin	g/dl	4.4 ± 0.16	4.7 ± 0.20	0.01	3.96–4.73
Glob	g/dl	2.02 ± 0.10	2.23 ± 0.27	0.10	1.69–3.01
T. Bil	mg/dl	0.04 ± 0.01	0.03 ± 0.01	0.35	0.03–0.18
Alk Phos	IU/L	111.28 ± 16.59	107.29 ± 7.08	0.57	81.42–197.75
Calc	mg/dl	11.9 ± 0.053	12.01 ± 0.36	0.65	9.92–12.28
Phos	mg/dl	13.58 ± 1.08	14.1 ± 1.40	0.46	8.13–12.11
Na	mmol/L	146.4 ± 1.51	147 ± 1.73	0.52	142–147
K	mmol/L	9.01 ± 0.62	9.37 ± 1.13	0.48	5.3–7.3
Cl	mmol/L	98.71 ± 1.38	99.28 ± 1.25	0.43	94–101

Statistical analyses was done by Student's T-test. Normal range values per Sprague-Dawley rat were provided by AML Ltd. SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic-pyruvic transaminase; Trig, triglycerides; Total P, total protein; Glob, serum globulins; T. Bil, total Bilirubin; Alk Phos, alkaline phosphatase; Calc, calcium; Phos, phosphate.

TABLE 2 | Serum IGF-I levels (ng/ml).

	Whey	Soy	*p*-value
24 days AL	1102.3 ± 161.7	1230.3 ± 85.1	0.2
24 days pair-fed	1508.5 ± 93.8	1229.1 ± 92.6	0.0003
74 days AL	1712.1 ± 239.9	1412.9 ± 146.4	0.03

AL, ad libitum.

above, which indeed can be associated with reduced risk of cardiovascular diseases.

Several distinguishing features may account for the different effects of soy and whey on bone quality and linear growth in the long term:

1. The quality of a protein is primarily based upon EAA composition. EAA, defined as amino acids that cannot be synthetized by the organism and must be provided by food, are the building blocks for protein synthesis and, as such, they are required for growth. Both soy and whey contain all EAA. However, while the amino acid composition of whey is similar to that of muscle proteins and delivers the appropriate amino acid ratio upon digestion, the amino acid composition of soy has a shortage of specific amino acids, such as leucine, isoleucine, lysine, and methionine (14, 22). When matched for nitrogen content, soy reportedly stimulates protein synthesis to a lesser extent than whey (23–29). However, to the best of our knowledge, the effect of soy on linear growth and EGP has not been studied before.

The difference in protein quality between soy and whey is mostly due to soy's lower level of the BCAA leucine and the sulfuric amino acids methionine (15). BCAA are not only elementary components for building muscle and skeletal tissue, but they also stimulate protein synthesis in both animals and humans. BCAA regulate many key signaling pathways, the most classic of which is the activation of the mammalian target of the rapamycin complex 1 (mTORC1) signaling pathway. mTORC1 is an evolutionary-conserved multi-protein complex that coordinates a network of signaling cascades and functions as a key mediator of protein translation, gene transcription, and autophagy, and thus connects many diverse physiological and metabolic processes. Signal transduction through mTORC1, which is centrally involved in enhanced protein translation, is governed by an intracellular amino acid supply (30). Specifically, leucine, whose level in soy is only 58% of that in whey, was found to enhance mTORC1 signaling as well as repress proteasomal degradation (31–33), thus leading to activation of downstream signaling and subsequent stimulation of protein synthesis. As such, the leucine content of the ingested protein source forms a key characteristic that modulates activation of protein synthetic machinery after protein ingestion.

The non-proteinogenic functions of EAA should also be considered in order to better understand the physiological consequences of an insufficient intake of specific amino acids. This more notably concerns the sulfuric amino acids, methionine (Met) and cysteine (Cys) which are involved in methylation processes, participate in the control of oxidative stress, and affect metabolism and cell functions (34). The low content of Met in soy protein limits the latter's nutritive value. Met is the precursor of Cys, which is a constituent of glutathione and a precursor of taurine. The response to an insufficient Met supply has been reportedly associated with significantly reduced food intake and body weight gain, an increase in energy expenditure, and the down-regulation of genes involved in fatty acid and triglyceride synthesis in the liver, thus reducing its capacity to synthesize and export lipids to peripheral tissues (34).

TABLE 3 | Bone parameters (μCT) in male Sprague-Dawley rats after 24 or 74 days.

	Whey 24 d ($n = 6$)	Soy 24 d ($n = 6$)	*p*-value	Whey 74 d ($n = 7$)	Soy 74 d ($n = 8$)	*p*-value
			Soy vs. Whey			Soy vs. Whey
(A) Full humerus length (mm)	22.55 ± 0.38	23.55 ± 0.37	0.001	28.37 ± 0.75	28.59 ± 0.37	0.49
% BV/TV	70 ± 6	65 ± 5	0.14	71 ± 1	66 ± 1	0.002
Volumetric bone mineral density (vBMD) [mg HA/ccm]	354.57 ± 74.48	367.73 ± 56.58	0.74	651.98 ± 19.22	586.08 ± 37.53	0.011
(B) Cortical bone parameters						
Tt.Ar (mm^2)	3.88 ± 0.23	4.46 ± 0.38	0.012	6.04 ± 0.50	5.85 ± 0.58	0.49
Ct.Ar (mm^2)	2.46 ± 0.63	2.99 ± 0.47	0.13	4.7 ± 0.33	4.19 ± 0.36	0.017
Ct.Ar/Tt.Ar	0.63 ± 0.14	0.67 ± 0.09	0.56	0.78 ± 0.03	0.72 ± 0.02	0.001
Ct.Th (mm)	0.4 ± 0.16	0.47 ± 0.12	0.39	0.65 ± 0.02	0.59 ± 0.04	0.007
Dia.Dia (mm)	2.22 ± 0.07	2.38 ± 0.10	0.01	2.77 ± 0.11	2.73 ± 0.14	0.49
Med.Dia (mm)	1.33 ± 0.23	1.36 ± 0.18	0.80	1.3 ± 0.12	1.45 ± 0.11	0.03
(B1) MOI parameters						
I min (mm^4)	0.9 ± 0.21	1.26 ± 0.28	0.03	2.49 ± 0.43	2.2 ± 0.34	0.15
Polar (mm^4)	2.06 ± 0.45	2.88 ± 0.58	0.02	5.77 ± 0.86	5.26 ± 1	0.3
Areal (mm^3)	0.87 ± 0.18	1.12 ± 0.15	0.03	1.82 ± 0.20	1.73 ± 0.21	0.41
(C) Trabecular bone parameters						
Tb.BV/TV	0.23 ± 0.13	0.25 ± 0.07	0.72	0.25 ± 0.06	0.22 ± 0.03	0.27
Tb.Th (mm)	0.1 ± 0.03	0.11 ± 0.01	0.84	0.1 ± 0.01	0.11 ± 0.004	0.35
Tb.N (mm^{-1})	1.79 ± 0.49	1.93 ± 0.25	0.56	1.88 ± 0.83	1.62 ± 0.37	0.46
Tb.Sp (mm)	0.65 ± 0.12	0.60 ± 0.06	0.37	0.68 ± 0.27	0.72 ± 0.15	0.75

All values are mean ± SD. Bone length and cortical thickness and BV/TV of the distal bone were significantly different between the two diets. BV/TV, Bone volume/total volume; vBMD, bone volumetric bone mineral density; Tt.Ar, total area; Ct.Ar, cortical area; Ct.Ar/Tt.Ar, cortical area fraction; Ct.Th, cortical thickness; Dia.Dia, diaphyseal diameter; Med. Dia, medullary diameter; MOI, moment of inertia; I min, Minimum moment of inertia; Tb. Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation.

Interestingly, the animals of the soy group in the current study showed a more rapid weight gain and increased food consumption in the short-term experiments. It may be that by using a relatively large amount of protein in the diets [28%; according to the AMDR (Acceptable Macro Nutrient Distribution Rate) protein content should be 10–35% from the total protein daily intake], the lower amounts of leucine and methionine were no longer an obstacle to growth. However, we still do not have an explanation for the increased food consumption, since the animals were provided with only one type of food.

2. Our results suggest that whey leads to better calcium absorption: both the soy and whey diets contained identical amounts of calcium, but the bone mineral density was higher in the whey group. Although some studies showed that phytic acid in soy-based diets could adversely affect mineral utilization (35), our soy diet did not contain phytic acid, thus we cannot contribute to the explanation for this effect.
3. IGF-I directly stimulates the proliferation and differentiation of EGP chondrocytes (36) as well as of osteoblasts (37) and increases trabecular and cortical bone formation. Both soy and whey diets have been shown to stimulate circulating IGF-I concentrations (4, 38), however, conflicting data made it very difficult to ascertain whether soy and whey similarly affect IGF-1 (39). In the current study, we compared diets that differed only in their protein source, thus focusing specifically on the protein- IGF-I connection. Although in the short term the levels of IGF-I were similar between the groups, IGF-I increased with age in the whey group, similarly to our previous findings (4), and the levels at the long-term experiment were significantly higher. Furthermore, using the pair fed setup made it clear that when the difference in food consumption were no longer playing part, whey was more efficient in increasing IGF-I.
4. Diet composition was shown to have an effect on the gut microbiome. The gut microbiota can influence the host by regulating nutrient and energy absorption, by producing vitamins and other useful metabolic byproducts, and by stimulating the host immune system at the gut lining. The microbiome has recently been identified as a factor that can influence bone quantity and bone quality (40, 41). In one study, the colonization of germ-free mice with normal gut microbiota led to normalization of bone mass, probably by affecting the immune system (42). Subsequent studies have shown the effect of the gut microbiome on bone, either through the effect on gut-derived serotonin (40), short chain fatty acids (SCFA) (43), or through the effect on osteoclasts (42). Diets composed of animal or plant

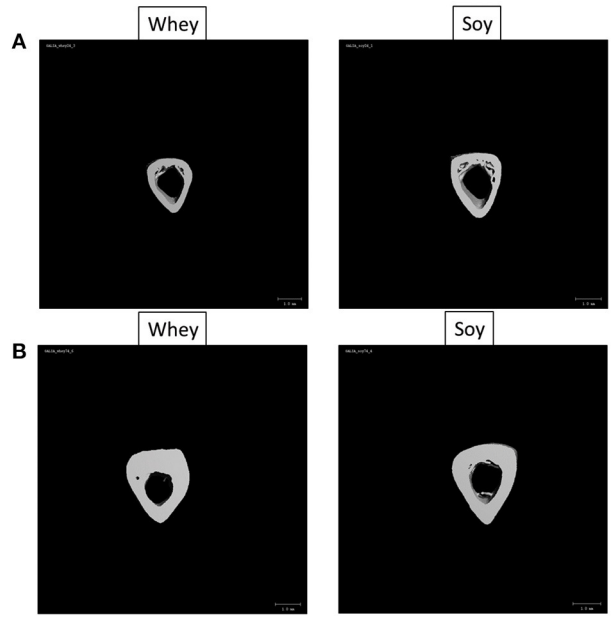

FIGURE 5 | Three-dimensional cortical bone images obtained by μCT at **(A)** 24 days or **(B)** 74 days.

TABLE 4 | Age-dependent change in bone parameters (μCT) in male Sprague-Dawley rats depending upon the diet (values are ratio of 74/24 parameters and presented as percent change).

Change	Whey	Soy	*p*-value
Total length	+26	+21	0.01
Tt. % BV/TV	+0.008	+0.02	0.65
vBMD	+83	+59	0.001
Dia.Dia	+24	+14	0.002
Med.Dia	+98	+6	0.07
Ct.Th	+62	+24	<0.001
MOI I min	+177	+173	<0.001
MOI polar	+180	+182	<0.001
MOI areal	+109	+55	<0.001
Tb.BV/TV	+8	−14	0.086
Tb.Conn.D	+19	−13	0.045
Tb.SMI	−20	+19	0.01
Tb.N	+4	−16	0.3
Tb.Th	NC	NC	0.85
Tb.Sp	+4	+20	0.4

NC, no change; BV/TV, bone volume/total volume; vBMD, bone volumetric bone mineral density; Tt.Ar, total area; Ct.Ar, cortical area; Ct.Ar/Tt.Ar, cortical area fraction; Ct.Th, cortical thickness; Dia.Dia, diaphyseal diameter; Med.Dia, medullary diameter; MOI, moment of inertia; Conn.D, connectivity density; I min, minimum moment of inertia; SMI, structure model index; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation.

constituents differentially alter the Firmicutes/Actinobacteria to Bacteroidetes ratio (21), with an animal-based diet preferentially promoting the abundance of Bacteroidetes and reducing Firmicutes compared to a plant-based diet. Soy dietary proteins were shown to alter the intestinal environment by affecting fermentation by gut microbiota and the generation of putrefactive compounds (44). However, there is currently no consensus on specific changes of gut microbiota by the soy protein, and a variety of results have been reported (21). Our previous analysis of the different effects of whey and casein on the gut microbiome showed that even proteins with high similarity could affect the gut microbiome in different ways (5).

Limitations of the study: Rats and humans are quite similar in physiology and anatomical structures, particularly the linear growth processes in both species that are composed of anatomically similar organs. Both rats and humans are omnivorous; therefore, they share strong similarities in dietary requirements. However, this study was performed on rats and not on children, and extrapolation of the findings to apply to children should be made with utmost caution. Another limitation of the study lies in the fact that only males were tested. This was due to the fact that males enter puberty later enabling a longer period of intervention (45), in the next studies, the effect on females should be completed.

CONCLUSIONS

Using more plant-based proteins in the human diet and supporting sustainability of our planet is important. However, our results clearly point to a superior effect of whey on linear growth. Efforts are being made to develop soybean lines that overexpress methionine-rich proteins in order to improve the soy protein. Alternatively, the addition of methionine to a soymilk formula was shown to increase nitrogen retention of malnourished children (16). We have no explanation why the soy diet led to comparatively increased food consumption, weight gain, and linear growth in the short term, since our animals were given only one choice of diet. However, it may suggest that soy should be used in the first steps of re-feeding rehabilitation in malnourished children, leading to a more rapid weight gain, and that whey-based diets should be used in order to keep the growth potential and limit weight gain (4, 46). Alternatively, it may be possible that a combination of soy and whey would be a more beneficial approach. Creating protein blends seems to offer benefits over increasing the dose of proteins being consumed since protein blends can provide sufficient amounts of all essential amino acids and the benefits of the better of the two worlds.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Tel Aviv University Institutional Animal Care and Use

Committee to which the Felsenstein Medical Research Center (FMRC) is affiliated. Approval was obtained before the experiments were initiated (committee protocol approval number 01-20-062).

AUTHOR CONTRIBUTIONS

GG-Y and MP: conceptualization. MB-M and CM: formal analysis. MP: financial resources. MB-M, SH-B, YG, and GG-Y: data curation. GG-Y: writing—original draft preparation, writing—review and editing, supervision, and project administration. All authors have read and agreed to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.739607/full#supplementary-material

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Whey Protein Supplementation Effects on Body Composition, Performance, and Blood Biomarkers During Army Initial Entry Training

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This study assesses if a lower dose of whey protein can provide similar benefits to those shown in previous work supplementing Army Initial Entry Training (IET) Soldiers with two servings of whey protein (WP) per day. Eighty-one soldiers consumed one WP or a calorie matched carbohydrate (CHO) serving/day during IET (WP: $n = 39$, height = 173 ± 8 cm, body mass = 76.8 ± 12.8 kg, age = 21 ± 3 years; CHO: $n = 42$, 175 ± 8 cm, 77.8 ± 15.3 kg, 23 ± 4 years). Physical performance (push-ups, sit-ups, and a two-mile run) was assessed during weeks two and eight. All other measures (dietary intake, body composition, blood biomarkers) at weeks one and nine. There was a significant group difference for fat mass ($p = 0.044$) as WP lost 2.1 ± 2.9 kg and had a moderate effect size (Cohen's d: −0.24), whereas the CHO group lost 0.9 ± 2.5 kg and had only a small effect size (d: −0.1). There was no significant group-by-time interaction on fat-free mass ($p = 0.069$). WP gained 1.2 ± 2.4 (d: 0.1) and CHO gained 0.1 ± 3 (d: 0) kg of FFM on average. There was a significant group by week 1-fat free mass interaction ($p = 0.003$) indicating individuals with higher initial fat-free mass benefitted more from WP. There were no group differences for push-up ($p = 0.514$), sit-up ($p = 0.429$) or run ($p = 0.313$) performance. For all biomarkers there was a significant effect of time as testosterone ($p < 0.01$), testosterone to cortisol ratio ($p = 0.39$), and IGF-1 ($p < 0.01$) increased across training and cortisol ($p = 0.04$) and IL-6 ($p < 0.01$) decreased. There were no differences in groups across IET for any of the biomarkers. We conclude one WP serving is beneficial for FM and for FFM in soldiers with high baseline FFM but may not significantly alter biomarker response or physical performance of IET soldiers who have high relative dietary protein intakes.

Keywords: human performance, military, supplementation, fat free mass, fat mass

INTRODUCTION

Improving physical fitness is key for the success of military personnel due to the strenuous nature of daily soldiering tasks (1, 2). Initial Entry Training (IET) is a physically and mentally rigorous training environment designed to prepare soldiers to perform their duties. Past research indicates that US Army IET soldiers participate in at least 6–7 h of daily physical activity, ranging from low to very vigorous intensity (3, 4). Recent research suggests Army IET soldiers may be inadequately fueled to respond optimally to large volumes of training (3). IET soldiers consume between 1,900-and 2,600 calories per day (3, 5). However, they are estimated to expend over 3,200 calories per day, resulting in a negative energy balance (3). This may have negative effects on performance and body composition (6). Research in US Army (5) and Marine (7) IET revealed that IET soldiers lost 1–3 kg of fat-free mass (FFM) on average across training, with only 36% of male Army IET soldiers gaining FFM during training (5). Losses in FFM may lead to decrements in physical performance for IET soldiers as has been found in non-IET in military training in the US and Australia (8, 9).

Serum biomarkers are one method of assessing responses to military training. Testosterone and Insulin-like Growth Factor 1 (IGF-1) are anabolic hormones that are positively related to body composition and performance due to their ability to stimulate anabolic mechanisms such as increased muscle protein synthesis (10, 11). While intense military training has been shown to reduce serum testosterone and IGF-1 (10), these reductions can be nutritionally modulated (12). Serum testosterone and IGF-1 are decreased during periods of negative energy balance across military training. These decreases can be restored to baseline levels when adequate nutritional provision is provided (12). Military training has also been shown to increase serum cortisol levels, a hormone that results in skeletal muscle catabolism (9, 12). The balance between anabolic and catabolic hormones is thought to be important for the promotion of muscle remodeling. Imbalances in the testosterone: cortisol (T:C) ratio, whether it is caused by decreases in testosterone or increases in cortisol, have been shown to be associated with reductions in performance (13). Studies in Army Rangers (12), Australian basic training (14), and United Kingdom section commanders' battle course (15) all report that military training results in elevated cortisol levels and a reduction in the T:C ratio. Military training has also been reported to increase serum cytokine concentrations, such as interleukin-6 (IL-6), that stimulates the inflammatory response to muscle damage and pathogens (16–19). Studies in Norway and France show that military training can lead to increases in IL-6 acutely (four days) and chronically (four weeks) (18, 19). Chronically elevated levels of IL-6 have been related to overtraining and may represent inadequate recovery (20).

Nutritional supplementation may have an important influence on physical and hormonal responses to military training. One United Kingdom study (9) reported the addition of a protein-based supplement negated the decrease in performance and FFM during 8 weeks of training. Our previous work in IET soldiers revealed supplementation with either a higher dose (two servings per day) of whey protein or a calorie-matched carbohydrate (CHO) resulted in a higher percentage of participants gaining FFM across IET in comparison to a previous investigation of non-supplemented IET soldiers (5, 21). Additionally, two servings per day of WP resulted in significantly higher push-up performance and potentiated reductions in fat mass (FM) in comparison to CHO (21). WP with small amounts of casein has been shown to increase IGF-1 and muscle mass in individuals involved in strength training (22). Another study (23) found that 6 months of protein supplementation resulted in increases in serum IGF-1 levels in individuals involved in concurrent strength and endurance training. The effects of protein supplementation on IGF-1 levels are thought to be mediated by an increased supply of amino acids that stimulate IGF-1 gene transcription in skeletal muscle (22). WP has also been reported to increase serum testosterone in comparison to soy, as well as to reduce serum cortisol levels in response to resistance training compared to soy and carbohydrate (CHO) supplementation (24). Collectively, these studies suggest that WP may be beneficial for improving the hormonal environment required to support advantageous physical performance and physiologic responses to IET.

The goal of the current study is to build upon our previous research examining the impact of WP supplementation on IET soldiers. Here we examined if one WP serving per day was more beneficial than CHO on performance, body composition, and serum-biomarker responses. If one WP serving per day provides similar benefits to those demonstrated with two WP servings per day, it would reduce preparation and distribution time, as well as supplementation costs for the military. Based on our prior data, we hypothesized WP would be beneficial for push-up performance and body composition. Additionally, we hypothesized that WP would be more beneficial than CHO for improvements in the T:C ratio and IGF-1 responses to training due to improvements in the anabolic status of the body as well as reductions in cortisol and IL-6 which may indicate improved recovery during IET.

METHODS

Study Design and Population

This was a double-blind, placebo-controlled, 2 x 2 (Group x Time) factorial-repeated measures design. The Auburn University Institutional Review Board, and the Director, Research, and Analysis Directorate Army Center approved the study procedures for Initial Military Training. Potential participants were given a description of the study. Those wishing to participate gave written consent and were enrolled in the study. Participants were cleared for military training and were apparently healthy 19–35-year-old men engaged in Army IET. All IET soldiers are required to live in barracks under the continual supervision of drill sergeants throughout the duration of IET. Daily schedules are highly regimented according to Army regulations from the time IET soldiers wake until time for bed. Daily physical fitness and occupational training events are performed in groups led by Army leadership. Daily activities consisted of morning group physical fitness (bodyweight resistance training, endurance training, general flexibility, and calisthenics) followed by soldier training tasks

(ruck marching, obstacle course, land navigation, battle tactics training, field training exercises, etc.). All soldiers in the unit completed the same tasks for the same duration each week. All soldiers consumed food from the same menu, and meals were consumed from the dining facility or from pre-packaged meals ready to eat. Participants were free from musculoskeletal injury (MSI), allergies to milk or whey protein, and had not taken supplements within the past 3 months. In total 95 participants agreed to participate in the study, 81 participants completed the study (WP: $n = 39$, height $= 173 \pm 8$ cm, body mass $= 76.8 \pm 12.8$ kg, age $= 21 \pm 3$ years old; CHO: $n = 42$, 175 ± 8 cm, 77.8 ± 15.3 kg, 23 ± 4 years old). A total of 14 participants were removed from the analysis due to prior supplementation (four participants), lack of adherence to supplementation (five participants), discontinued IET (four participants), and withdrawal of participation in the study (one participant).

Participants were supplemented with either one whey protein (Power Crunch® ProtoWhey® (BioNutritional Research Group; Irvine, CA, USA) as agglomerated, partially hydrolyzed (12.5% degree of hydrolysis) 80% whey protein concentrate (Hilmar® 8360; Hilmar Ingredients, Hilmar, CA USA) or calorie-matched CHO supplement per day. Supplement manufacturing and formulation have been described previously (21). Briefly, all supplements were manufactured at JW Nutritional, LLC (Allen, TX, USA), a United States Food and Drug Administration cGMP-compliant facility independently audited and pre-qualified by Obvium*Q, LLC (Phoenix, AZ, USA), a GMP regulatory compliance firm. Personnel at JW Nutritional, LLC and C.M.L. (Lockwood, LLC; Draper, UT, USA) formulated supplements to match for taste. These entities also maintained blinding of groups, and each supplement was assigned a randomly generated item number. The research team and participants were blinded to the contents of the packets until data collection was completed. Manufacturing batch records for production of each of the supplements were reviewed by a trained, independent expert in dietary supplement quality control, taste, and assurance (C.M.L.) before approval for use within the present study. The nutritional profile and amino acid content of both supplements were third-party tested by Covance Laboratories, Inc. (Madison, WI, USA) to verify the identity, purity, potency, and composition of the packets. The nutritional profile is described below in **Table 1**. In order to minimize interference in the IET training schedule, each week, all supplement packs were provided to the drill sergeants for their respective platoon. The drill sergeants then provided the supplements to the IET soldiers who were instructed to consume the shakes before bedtime. To assess adherence, the research team checked the boxes that were delivered to ensure distribution and asked the IET soldiers to report the number of shakes missed during the study.

TABLE 1 | Supplement nutrition information.

Macronutrient	WP Supplement	CHO Supplement
Energy (kcal)	293	291
Protein (g)	38.6	0.5
Carbohydrate (g)	19	63.4
Fat (g)	7.5	3.9
Essential AA (g)	20.1	0.1
BCAA (g)	9.5	0.0

WP, Whey Protein supplement; CHO, Carbohydrate supplement; Kcal, Kilocalories; g: grams; AA, Amino Acids; BCAA, Branched Chain Amino Acids.

Measures

The independent variables were supplementation group (WP or CHO) and time (week 1, week 9). Outcome variables were daily training volume, physical performance as measured by the Army Physical Fitness Test (APFT), body composition, dietary intake, serum biomarkers of anabolic status (testosterone, cortisol, IGF-1, T:C), and immune health/recovery (IL-6). Fasted blood and body composition were collected during weeks one and nine of training prior to breakfast and morning physical training. Urine-specific gravity (USG) testing was completed prior to all blood collections and body composition assessments to ensure hydration status. Participants with USG values above 1.03 were considered inadequately hydrated, given water to drink, and not allowed to proceed with testing until USG was below 1.03. Performance measures were performed during weeks two and eight of training. **Figure 1** summarizes the timeline of measurements for each variable during this study.

Physical Activity

The methodology employed for the evaluation of physical activity levels has been previously reported in detail (3). Briefly, physical activity was estimated using Actigraph GTX monitors (Actigraph, Pensacola, FL, USA). Each week a different set of 20 participants (10 per supplement group) were asked to wear a monitor on their right hip (Actigraph protocol) for 1 week and not remove the monitor except to shower. Monitors were initialized prior to deployment and physical activity per day was estimated using Actilife software version 13.1.1 (Actigraph, Pensacola, FL, USA). Time spent in each category of physical activity was estimated using Sasaki vector magnitude 3 (VM3) (25). The following range of counts were used for each category of physical activity: Moderate = 2,690–6,166 counts/min (3–5.99 METs), Vigorous = 6,167–9,642 counts/min (6–8.99 METs), and Very Vigorous $\geq$ 9,642 counts/min (>9 METs) (25). All VM3 counts below 200 counts/minute were classified as sedentary (26), and the difference between sedentary cut points and moderate cut points were classified as low intensity (201–2,689 counts/min). The sampling rate was 30 Hz (27) and for the physical activity data to be considered valid, wear time as estimated by Actilife software was a minimum of 600 min (26).

Dietary Intake

Diet logs were completed on three days during weeks one and nine of IET. A detailed description of this process has been previously reported (3). Briefly, members of the research team obtained the menu from the dining facility IET where soldiers were required to eat at and pre-filled the diet log with options available for that meal. Immediately after the meal the research

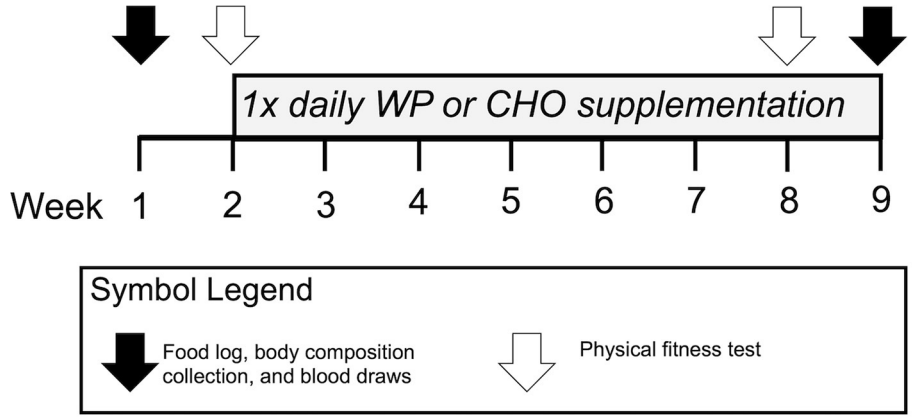

FIGURE 1 | Study timeline and measures. WP, Whey protein supplement group; CHO, Carbohydrate supplement group; 1x, Once daily.

team provided the diet logs to the participants to circle the items and amounts, they ate and were available to answer any questions. Diet log data was entered into excel spreadsheets and reviewed by two researchers for accuracy. The diet data were then imported into R statistical software (28) and dietary intake calculations were completed using R Studio (29) along with the R programming packages: dplyr (30), tidyr (31), reshape2 (32), ez (33), car (34), vars (35), and ggplot2 (36). Total calorie, protein, carbohydrate, and fat intakes were calculated for each meal and day and then averaged for training weeks one and nine. Nutritional data for the dining facility foods were retrieved from the Army Joint Culinary Center of Excellence (JCOE) website and those not available on JCOE were retrieved from the US Department of Agriculture nutrition database (21). Diet logs for all three meals were required for the day to be considered valid for dietary analysis. Days in which a participant did not complete all three logs were removed from the analysis. Participants without at least two full days of diet logs each week were removed from the summary of diet logs. A total of 60 of the 81 participants who completed, had at least two full days of diet logs and were included in the dietary analysis. Diet logs during the first week were collected before the intervention period began in order to get a baseline characterization of dietary intake in the absence of supplementation (week 1-NS). We analyzed the dietary intake data in two ways: week 1-NS compared to week 9 dietary intake with supplement nutritional information included in the overall macronutrient count (week 9-SI) and excluding the macronutrient information from the overall macronutrient count (week 9-NS). Our aim was to see if there were significant changes in food that were consumed from the dining facility.

Body Composition

Height and body mass were assessed with participants wearing Army-issued physical training shorts, socks, and shirts using a Health-O-Meter professional scale (Model 500KL, Sunbeam products INC. Boca Raton, FL. USA) and reported in centimeters and kilograms. Body composition was assessed using an ImpediMed DF50 device (ImpediMed Ltd, Brisbane, Australia). This measure is sensitive to hydration; therefore, hydration was assessed prior to measurement through urine-specific gravity (described above). Participants were asked to lay supine for ∼5 min to allow for equilibration of body fluids across intracellular and extracellular compartments prior to assessment. Measurements were taken in the supine position. Electrode placement locations on the hand and ankle were determined as per the manufacturer's recommendations. An electrode was placed on the midline of the left arm proximal to the ulnar styloid process and a distal electrode was placed on the midline 5 cm apart. Electrodes were placed on the ankle on the midline between the medial and lateral malleolus and 5 cm distal to the malleolus on the midline. All application sites were shaved to ensure optimal electrode contact. All electrode placements were performed by the same member of the research team to minimize variability. Raw output was collected from the device, and fat-free 434 mass (FFM) and fat mass (FM) were calculated using the formulas below (37):

$$\mathbf{FFM} = \frac{Height^2}{Resistance} * 0.734 + BW * 0.116 + Reactance * 0.096 + 1 * 0.878 - 4.03$$

$$\mathbf{FM} = Body\ mass - Fat\ free\ mass$$

Physical Performance

The APFT was performed during weeks two and eight of the intervention. The APFT (fitness standard of record at the time of the study) consisted of a 2-min sit-up, 2-min push-up, and two-mile run. The APFT was administered by unit drill sergeants according to the standards of the US Army field manual for physical fitness training (1). Details describing APFT administration and criteria for Army standards for the proper performance of a push-up and sit-up and two-mile run have been described previously (1, 38).

Serum Biomarkers

Blood draws were taken from the antecubital vein *via* 21-gauge, Safety-Lok needle kits (Benton, Dickinson, and Company,

Franklin Lakes, NJ, USA). Blood was collected in 10 ml serum separator vacutainer tubes (BD Vacutainer; Franklin Lakes NJ, USA) and placed on ice in a cooler (Yeti Coolers LLC, Austin TX, USA) until centrifugation the same morning of collection. Blood samples were centrifuged at 3,500 x g for 10 min at room temperature. Samples that were not fully separated were centrifuged again under the same conditions. Serum was extracted from separated blood and frozen at −80°C until analysis. Testosterone (American Laboratory Products Company, Salem, NH, USA, sensitivity: 0.022 ng/ml, CV: 2.9%), cortisol (American Laboratory Products Company, Salem, NH, USA, sensitivity: 0.4 μg/dL, CV: 4.8%), IGF-1 (American Laboratory Products Company, Salem, NH, USA, sensitivity: 0.091 ng/ml, CV: 10.5%), and IL-6 (Invitrogen, Carlsbad, CA, USA, sensitivity: 0.3 pg/ml, CV: 7.1%) were measured using ELISAs according to manufacturers' instructions. Plates were analyzed at respective wavelengths using a multispectral spectrophotometer (BioTek Eon, Winooski, VT, USA). All samples were analyzed in duplicate, and each participant's weeks 1 and 9 samples were analyzed on the same plate. All-optical densities were within the detectable range of the assays. IL-6 had four individuals whose concentrations could not be used due to being outside the normal physiologic range for the four-compartment logistic regression models and were removed from the analysis. Serum concentrations of each optical density were calculated as per manufacturer instructions using either regression or a four-parameter logistic regression.

Statistical Analysis

For statistical analysis, ANOVA was used to compare diet and serum markers between groups and across the time of the intervention. The assumption of normality of residuals testing was completed for all variables using Shapiro-Wilks (W: Wilk's Statistic), Kolmogorov Smirnov tests, and residual QQ plots were used to visually inspect the data. Data were square-root transformed and normality was recalculated for any variable for which more than 75% of the levels were non-normally distributed. An *a priori* alpha level of 0.05 was set for the determination of significant effects. Maulchy's test of sphericity was used to evaluate equality of variance and Levene's test was used to evaluate the homogeneity of variance. If sphericity was violated a Greenhouse-Geisser correction was used. Group-by-time interactions were further evaluated using paired samples *t*-test to evaluate simple main effects of time and independent samples *t*-tests were used to evaluate the simple main effect of the group.

ANCOVA was used to evaluate performance and body composition. ANCOVA has been reported to increase sensitivity to factors specified by the study design (39). Mean centered initial values for each variable were used as the covariate in the ANCOVA model.

A mixed-design ANOVA was used to detect differences in average time spent per week training across our independent variables of the training week and supplement group. Our aim with this analysis was to determine if training volume across each intensity (low, moderate, vigorous, or very vigorous) was significantly different between supplement groups and/or across each week of IET. We employed a Tukey HSD *post-hoc* test for pairwise comparisons.

Cohen's *d* effect sizes were calculated within groups across training, as well as between groups at week 9. Effect sizes are reported as effect sizes with the associated upper and lower limits of the 95% CI. Calculations are provided below:

$$\begin{aligned} Effect\ Size &= mean\,(week9) \\ &- mean\,(week1)/pooled\ standard\ deviation \end{aligned}$$

$$\begin{aligned} Pooled\ standard\ deviation &= Square\ root\ ((SD(week1)^2 \\ &+ SD\,(week9)^2)/2) \end{aligned}$$

Testosterone violated assumption of normality at all levels (WP = W: 0.78, $p < 0.01$ week 1; W: 0.7, $p < 0.01$ week 9; CHO = W: 0.8, $p < 0.01$ week 1; W: 0.7, $p < 0.01$ week 9). Testosterone was log-transformed and re-tested for normality. Only the week 9 data were non-normally distributed, but ANOVA is robust to partial violations of normality, so we chose to proceed with the analysis. IL-6 concentrations were log transformed and normality was re-tested. Following log transformation all levels of the variable were normally distributed.

RESULTS

Physical Activity

There was no statistical difference between groups for volume of training. This is indicated by a lack of significant difference between groups for light ($F[1] = 0.18$, $p = 0.67$), moderate ($F[1] < 0.01$, $p = 0.97$), vigorous ($F[1] = 0.03$, $p = 0.86$) or very vigorous ($F[1] = 0.9$, $p = 0.35$) activity. There was a significant difference in light ($F[2, 100] = 5.12$, $p < 0.01$) and moderate ($F[2, 100] = 7.02$, $p < 0.01$), but not vigorous ($F[2, 100] = 1.41$, $p = 0.25$) or very vigorous ($F[2, 100] = 2.42$, $p = 0.09$) activity levels across phase of IET. For light intensity, *post-hoc* testing revealed red phase was significantly higher than blue (adj. $p = 0.05$) and white (adj. $p = 0.01$) phases. For Moderate intensity white phase was lower than red (adj. $p = 0.02$) and blue (adj. $p < 0.01$) phases. Total training time was only found to be significantly different between white and red phase as red phase was on average 50 min higher than white (adj. $p = 0.01$). **Table 2** below summarizes the training volume during each phase.

Dietary Intake

Baseline diet was collected prior to integration of supplementation. Differences in dietary intake from the dining facility alone, between groups across IET without supplement nutritional information, and comparisons on both absolute and relative dietary intake (normalized to body weight in kg) were generated. Statistical results are listed below, and descriptive results are shown in **Table 3**.

Dietary Intake From Meals Only

Nutritional intake with no supplementation is presented in **Table 3**. There were no statistical differences between groups,

TABLE 2 | Summary of training volume per phase IET.

Phase	Group	Light	Moderate	Vigorous	Very Vig.	Total
Red	CHO	303 (37)	110 (23)*	23 (13)	5 (2)	441 (57)*
	WP	300 (41)	105 (23)*	26 (18)	6 (3)	437 (66)*
White	CHO	274 (36)$^{+}$	92 (21)	19 (16)	5 (4)	391 (61)
	WP	272 (48)$^{+}$	91 (28)	18 (11)	6 (5)	388 (78)
Blue	CHO	278 (49)$^{+}$	110 (31)*	28 (40)	3 (3)	419 (93)
	WP	271 (54)$^{+}$	122 (41)*	27 (33)	4 (4)	424 (106)

Phase, Red (weeks 1–3), White (weeks 4–6), Blue (weeks 7–9); WP, Whey protein supplement group; CHO, Carbohydrate supplement group; All values are in min/day ± SD values parenthesized. Very Vig.: very vigorous.
$^{+}$Indicates significantly different from Red Phase.
**Indicates significantly different from White Phase.*

TABLE 3 | Summary of dietary intake across IET.

Nutrient	Group	Units	Week 1-NS	Week 9-NS	Week 9-SI
Energy	CHO	kcal/day	2,759 (585)	3,472 (697)$^{+}$	3,763 (697)
	CHO	kcal/kg/day	37.4 (11.4)	46.7 (12.2)$^{+}$	50.6 (12.7)
	WP	kcal/day	2,620 (626)	3,163 (765)$^{+}$	3,456 (765)
	WP	kcal/kg/day	34.7 (10.8)	42.1 (11.9)$^{+}$	46 (12.2)
Protein	CHO	g/day	122 (26)	163 (29)$^{+}$	163 (29)$^{+*}$
	CHO	g/kg/day	1.7 (0.5)	2.2 (0.5)$^{+}$	2.2 (0.5)$^{+*}$
	WP	g/day	118 (26)	148 (30)$^{+}$	186 (30)$^{+*}$
	WP	g/kg/day	1.6 (0.4)	2 (0.5)$^{+}$	2.5 (0.5)$^{+*}$
CARB	CHO	g/day	359 (90)	456 (105)$^{+}$	519 (105)$^{+*}$
	CHO	g/kg/day	4.9 (1.6)	6.1 (1.8)$^{+}$	7 (1.9)$^{+*}$
	WP	g/day	342 (92)	423 (114)$^{+}$	442 (114)$^{+*}$
	WP	g/kg/day	4.5 (1.5)	5.6 (1.7)$^{+}$	5.9 (1.7)$^{+*}$
Fat	CHO	g/day	95 (19)	112 (27)$^{+}$	116 (27)
	CHO	g/kg/day	1.3 (0.4)	1.5 (0.4)$^{+}$	1.6 (0.4)
	WP	g/day	90 (23)	99 (27)$^{+}$	107 (27)
	WP	g/kg/day	1.2 (0.4)	1.3 (0.4)$^{+}$	1.4 (0.4)

Values are represented as mean (± SD). Week 1 of IET; Week nine of IET; Week 1-NS: Week 1-training, no supplement nutrition included in total; Week 9-NS: Week 9, no supplement nutrition information included; Week 9-SI: Week 9 with supplement nutrition information added to the total. WP, Whey protein supplement group; CHO, Carbohydrate supplement group.
**Indicates significant group difference at the respective time point.*
$^{+}$Indicates a significant effect of time (Week 1 vs. Week 9).
A priori set at $p < 0.05$.

calories, or macronutrients consumed from the dining facility across IET. This is indicated by a lack of significant group by time interactions for absolute calorie ($F[1,60] = 1.1$, $p = 0.3$), protein ($F[1,60] = 2.03$, $p = 0.16$), fat ($F[1,60] = 1.44$, $p = 0.24$), carbohydrate ($F[1,60] = 0.43$, $p = 0.51$), cholesterol ($F[1,60] = 0.54$, $p = 0.47$), and sodium ($F[1,60] = 0.58$, $p = 0.45$) intake. This was also true when intakes were normalized to body weight. There were no significant group by time interactions for calorie (kcal/kg; $F[1,60] = 0.65$, $p = 0.42$), protein (g/kg; $F[1,60] = 1.31$, $p = 0.26$), fat (g/kg; $F[1,60] = 0.89$, $p = 0.35$), carbohydrate (g/kg; $F[1,60] = 0.25$, $p = 0.62$), cholesterol (mg/kg; $F[1,60] = 0.36$, $p = 0.55$), and sodium (mg/kg; $F[1,60] = 0.15$, $p = 0.7$).

Both groups significantly increased consumption of absolute energy ($F[1,60] = 50.27$, $p < 0.01$), protein ($F[1,60] = 66.01$, $p < 0.01$), fat ($F[1,60] = 15.14$, $p < 0.01$) and carbohydrate ($F[1,60] = 44.39$, $p < 0.01$) from week 1 to week 9. This finding remained significant when these macronutrients were normalized to body weight, as there were also main effects of time for calorie ($F[1,60] = 59.58$, $p < 0.01$), protein ($F[1,60] = 78.06$, $p < 0.01$), fat ($F[1,60] = 17.29$, $p < 0.01$), and carbohydrate ($F[1,60] = 52.9$, $p < 0.01$).

Dietary Intake With Supplements Included

Nutritional information with supplementation values added to the week 9 dietary intake after week one are presented in **Table 3**. There was a significant group by time interaction for protein, absolute ($F[1,60] = 11.86$, $p < 0.01$), relative (g/kg; $F[1,60] = 10.89$, $p < 0.01$), and carbohydrate, absolute ($F[1,60] = 6.15$, $p = 0.02$) and relative (g/kg; $F[1,60] = 4.73$, $p = 0.03$). *Post-hoc t*-tests to assess differences indicated both WP and CHO groups increased absolute protein intake across IET. The WP group increased protein intake on average 68 grams ($t = 15.62$, $p < 0.001$) and CHO increased on average 41 grams ($t = 5.24$, $p < 0.001$). There was no statistical difference between protein intake at baseline between groups ($t = 0.6$, $p = 0.552$), however there was a significant difference at week 9 (-3.04, $p = 0.004$). There was a significant increase in absolute carbohydrate intake across IET in both the WP ($t = 6.2$, $p < 0.001$) and CHO ($t = 5.84$, $p < 0.001$) groups. There was no significant difference in carbohydrate intake at baseline ($t = 0.73$, $p = 0.469$), but there were significant differences at week 9 ($t = 2.77$, $p = 0.007$). Similar findings existed when protein and carbohydrate were normalized to body weight. There were significant increases in relative protein (WP: $t = 14.87$, $p < 0.01$; CHO: $t = 4.02$, $p < 0.01$) and carbohydrate ($t = 5.82$, $p < 0.01$, $t = 4.57$, $p < 0.01$) intakes across IET in both the WP and CHO groups. For relative protein and carbohydrate intake there were no significant differences in intakes at baseline (protein-baseline: $t = 0.82$, $p = 0.42$, carbohydrate baseline: $t = 0.86$, $p = 0.39$), but there were differences at week 9 (protein-week 9: $t = -2.06$, $p = 0.04$, carbohydrate-week 9: $t = 2.44$, $p = 0.02$).

Body Composition

A total of 81 participants were included in the analysis of body composition (BM, FM, FFM). Descriptive statistics and effect sizes are reported in **Table 4**. For BM, mean-centered week 1-BM was a significant predictor of week 9 BM ($F = 1{,}420.3$, $p < 0.001$). However, there were no group ($F = 0.13$, $p = 0.722$) or group by week 1-BM interactions ($F = 0.74$, $p = 0.393$).

There was a significant group by week 1-FFM interaction ($F = 9.46$, $p = 0.003$) on week 9 FFM and a significant interaction for week 1-FFM and group, thus the main effects could not be interpreted. Therefore, we conducted two follow-up analyses. First, linear models were fitted to the WP and CHO groups separately to investigate the influence of baseline FFM on the response to the treatment. Next, we conducted a standard group-by-time ANOVA to gain insight into the change across time in FFM between the groups. The interaction plot (**Figure 2**) below shows a trend in the relationship between baseline FFM and

TABLE 4 | Summary of body composition and performance.

Variable	Group	Week 1	Week 9	Mean Difference [CI]	Effect Size
BM (kg)	CHO	77.8 (15.3)	76.9 (13.1)	−0.8 [−8, 6.3]	−0.04
	WP	76.8 (12.8)	75.8 (11.6)	−0.9 [−7.5, 5.7]	−0.05
FFM (kg)	CHO	61.4 (10.5)	61.5 (8.7)	0.1 [−5.8, 5.9]	0
	WP	59.5 (8.4)	60.7 (8.5)	1.2 [−3.5, 5.8]	0.1
FM (kg)	CHO	16.3 (6.6)	15.4 (5.7)	−0.9 [−5.9, 4.1]	−0.1
	WP	17.2 (7)	15.1 (5.2)	−2.1 [−7.8, 3.6]	−0.24
		Week 2	**Week 8**		
Run (sec)	CHO	965 (146)	849 (77)	−116 [−280, 47]	−0.7
	WP	981 (144)	870 (85)	−112 [−284, 60]	−0.67
PU (reps)	CHO	44 (17)	51 (14)	8 [−17, 33]	0.35
	WP	36 (18)	48 (15)	12 [−6, 30]	0.52
SU (reps)	CHO	51 (14)	66 (11)	15 [−0.3, 31]	0.86
	WP	44 (15)	60 (13)	16 [−9, 40]	0.8

Raw values are represented as mean (± SD). Mean difference: the average difference across IET with 95% CIs at weeks 1 and 9 for BM, FFM, and FM and weeks 2 and 8 for Run, PU, and SU. Effect size, Cohen's D; BM, Body Mass; FFM, Fat-Free Mass in kg; FM, Fat Mass in kg; Run: two-mile run time in seconds; PU, Push-ups completed in 1 min; SU, Sit-ups completed in 1 min; kg, kilogram; sec, seconds; reps, number of repetitions completed; WP, Whey protein supplement group; CHO, Carbohydrate supplement group.

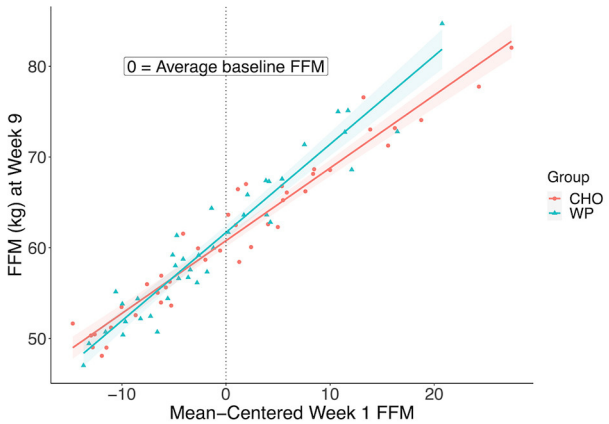

FIGURE 2 | Interaction between baseline and Week 9 FFM between groups. Plot of Week 9 FFM in kilograms and mean-centered Week 1 FFM to display the relationship between groups. FFM, Fat-Free Mass; CHO, carbohydrate supplement group; WP, Whey protein supplement group.

week 9 FFM depending on the group. For every 1 kg increase in baseline FFM, there was a related 0.97 kg increase in FFM at week 9 in the WP group compared to a 0.8 kg increase in FFM at week 9 in the CHO group. The coefficients from the multiple regression model (ANCOVA with the significant group by week 1-FFM interaction) were used to predict week 9-FFM as an illustration of the interaction of week 1-FFM and supplement group. If a soldier began IET at 5 kg above average in FFM the predicted week 9-FFM would be 1.76 kg higher if the soldier were in the WP group than if the soldier were in the CHO group. However, if the soldier were 5 kg below average, week 9 FFM is predicted to be only 0.04 kg higher if the IET soldier were in the WP vs. the CHO group. If these are extended to being 10 kg above or below average FFM beginning IET, the soldier who is 10 kg above average would be expected to have a week 9 FFM 2.62 kg higher if given WP vs. CHO, whereas if the soldier were 10 kg below average, the expected FFM at week 9 would be 0.81 lower if the soldier were in the WP vs. the CHO group. The group by time ANOVA trended toward significance (F=3.38, $p = 0.07$). The WP group increased FFM 1.2 kg on average and the CHO group increased by 0.1 kg on average, suggesting that WP may be beneficial for FFM response to IET. Both groups increased FFM across IET as there was a significant effect of time (F = 4, $p = 0.05$).

For FM, there was no group by week 1-FM interactions (F = 2.26, $p = 0.137$). Mean centered week 1-FM (F = 456, $p < 0.001$) and group (F = 4.18, $p = 0.044$) were significant factors for week 9 FM. WP lost 2.1 kg on average of FM across IET whereas the CHO group lost 0.9 kg.

Physical Performance

We were only able to obtain performance data from three out of the four platoons, creating an imbalance in sample size between groups for performance metrics. In total, there were 57 participants (WP = 37, CHO = 18) data included in the analysis for push-ups and sit-ups, and 56 participants (WP = 36, CHO = 18) for run. For sit-ups, mean centered week 1-sit-up performance was a significant predictor of week 9-sit-up performance (F = 43.85, $p < 0.001$). However, there were no group (F = 0.64, $p = 0.429$) or group by week 1-sit-up interactions (F = 0.16, $p = 0.694$). For push-ups, mean centered week 1-push-up performance was a significant predictor of week 9 push-up performance (F = 96.94, $p < 0.001$). However, there were no group (F = 0.43, $p = 0.514$) or group by week 1-push-up interactions (F = 0.97, $p = 0.33$). For run performance, mean centered week 1-run performance was a significant predictor of week 9-run performance (F = 133.52, $p < 0.001$). However, there were no group (F = 1.04, $p = 0.313$) or group by week 1-run interactions (F = 0.02, $p = 0.899$).

Serum Biomarkers

A total of 48 participants (WP = 23, CHO = 25) were included in the analysis of serum testosterone. The ANOVA was conducted on the log transformed testosterone data due to violation of the assumption of normality of residuals. There was a significant main effect of time (F = 13.74, $p < 0.01$), however, there was no main effect of group (F = 0.89, $p = 0.35$) or group by time interactions (F = 0, $p = 0.95$). A total of 47 participants (WP = 23, CHO = 25) were included in the analysis of serum cortisol. There was a significant main effect of time (F = 4.38, p = 0.04), however, there was no main effect of group (F = 0.34, $p = 0.56$) or group by time interactions (F = 1.88, $p = 0.18$). A total of 48 participants (WP = 23, CHO = 25) were included in the analysis of serum T:C. There was a significant main effect of time (F = 20.15, $p < 0.01$), however, there was no main effect of group (F = 0.75, $p = 0.39$) or group by time interactions (F = 0.8, $p = 0.38$).

A total of 48 participants (WP = 23, CHO = 25) were included in the analysis of serum IGF-1. There was a significant main effect of time (F = 8.07, $p < 0.01$), however, there was no main effect of

group (F = 2.81, $p = 0.1$) or group by time interactions (F = 1.30, $p = 0.26$). Lastly, we investigated the effects of supplementation on IL-6, a marker of inflammation. A total of 36 participants (WP = 17, CHO = 19) were included in the analysis of serum IL-6. Due to violation of the assumption of normality of residuals, the ANOVA was conducted on the log transformed IL-6 data. There was a significant main effect of time (F = 17.92, $p < 0.01$), however, there was no main effect of group (F = 0.02, $p = 0.9$) or group by time interactions (F = 0.15, $p = 0.71$). The biomarker responses are summarized in **Figure 3**.

DISCUSSION

This project examined if 8 weeks of a single daily serving of WP compared to CHO supplementation influenced physical performance, blood biomarkers, and body composition across IET. Our primary findings were: (1) WP was related to a significant reduction in FM during IET; (2) WP had differential effects on FFM depending on the soldiers' FFM upon entry into IET; (3) there was no statistically significant benefit between supplements for physical performance or the anabolic or inflammatory biomarker response to IET. Two important secondary findings were that soldiers increased dietary intake from meals across IET and that training volume was higher in the initial phases of IET in comparison to the later phases. Below we discuss these findings and how these findings (with once-daily WP supplementation) relate to our findings using twice daily supplementation with WP daily in the same population.

Soldiers consuming WP daily had a significant reduction in FM during IET. The WP group lost an additional 1.2 kg of FM with a larger effect size than CHO (WP = −0.24, CHO = −0.1). There were no significant differences in overall caloric intake or training volume completed between groups. Thus, the losses in FM seen here were likely not influenced by those variables. This is similar to our previous work in IET soldiers that found a significant reduction in FM in the WP group that consumed 2 servings (80 g total) of WP daily. IET soldiers consuming 2 servings per day lost an additional 1.8 kg of FM in comparison to the group consuming two CHO servings per day. The potential impact of WP on FM agrees with studies in non-military populations as well (40, 41). WP has been shown to promote FM loss in conjunction with exercise in healthy (41, 42)

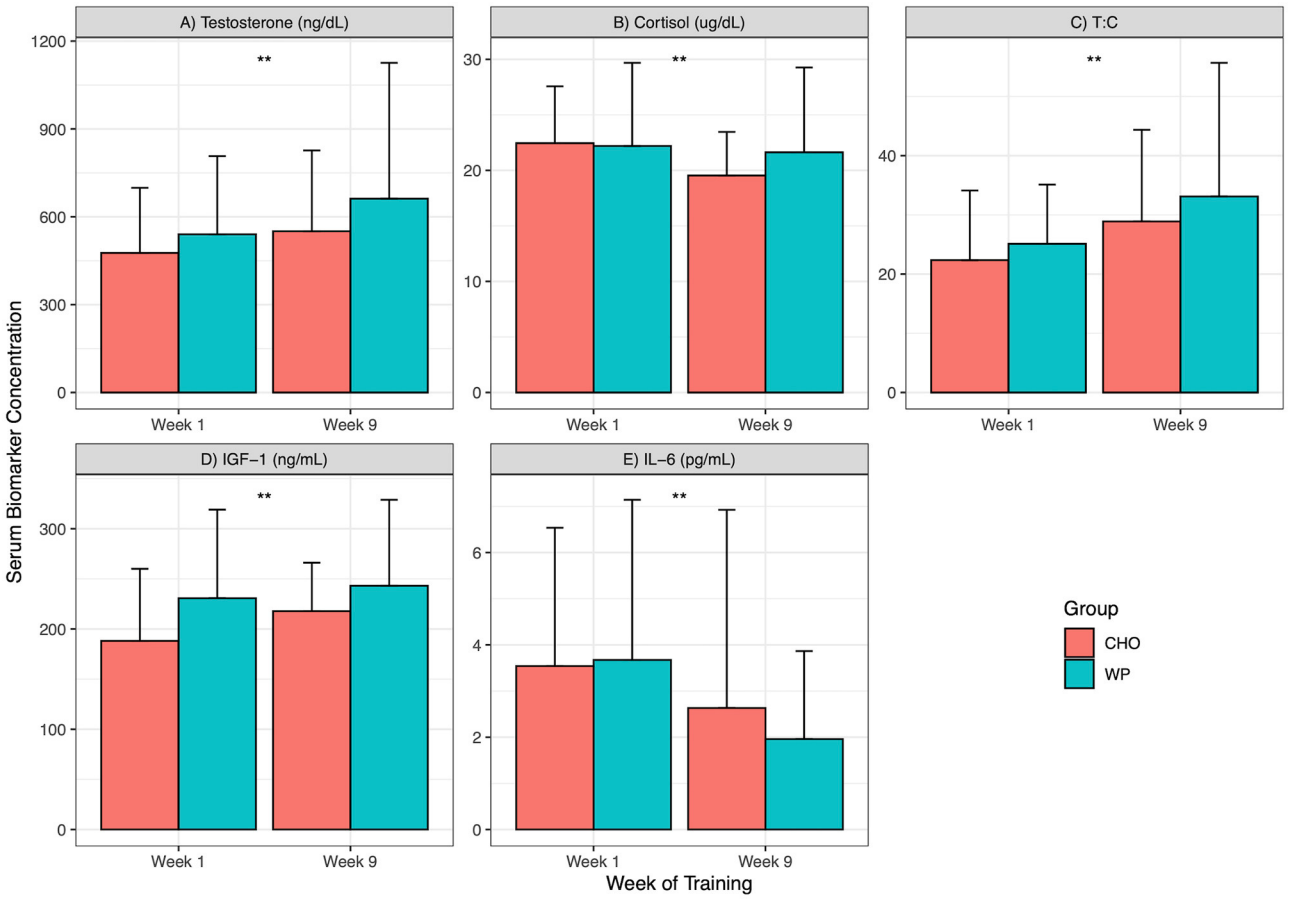

FIGURE 3 | Biomarker response across IET. Biomarkers at weeks 1 and 9. ng/dL, nanogram per deciliter; ug/dL, microgram per deciliter; T:C, Testosterone to Cortisol ratio; ng/mL, nanogram per milliliter; pg/mL, pictograms per milliliter; CHO, Carbohydrate supplement group; WP, Whey protein supplement group; Data presented as mean ± standard deviation. **indicates significant main effect of time (Week 1 vs. Week 9) for both groups; ++indicates a significant group by time interaction. Panels: **(A)** Testosterone; **(B)** Cortisol; **(C)** Testosterone to Cortisol Ratio; **(D)** Insulin-Like Growth Factor 1; **(E)** Interleukin 6.

and obese individuals (40, 41). Animal and cell culture models suggest WP may promote fat metabolism by influencing both adipose and muscle (43, 44). WP has been shown to impact adipose tissue by upregulating signaling pathways associated with the breakdown of triglycerides into FFA (43, 44), fat oxidation (45), thermogenesis (43), and antagonists to fatty acid synthesis (44). Conversely, in myotubes, WP has been shown to upregulate signaling pathways related to free fatty acid transport to mitochondria (43) and mitochondrial ability to oxidize free fatty acids (44). Additionally, myotubes cultured in serum from humans who consumed WP in the fasted state had improved GLUT4 translocation which would promote uptake of glucose to the muscle (46). Thus, WP may contribute to FM reductions by promoting the breakdown of adipose, suppressing the synthesis of FFA, and improving transport and oxidation of free fatty acids in both adipose and muscle tissue. Another potential way WP can impact FM is the thermic effect of food. Previous work has shown that protein has a higher thermic effect than both carbohydrate and fat intake (47). WP has been shown to increase the thermic effect of food (48) and to a greater extent than other protein sources such as soy and casein (49). Therefore, WP supplementation may be a viable option for IET soldiers and individuals who are engaging in exercise training while trying to reduce FM.

WP was also found to be more beneficial for FFM in individuals who had higher FFM at week 1 relative to soldiers with lower FFM and those in the CHO group. This is evidenced by the significant group by baseline FFM interaction. We then used the multivariate model to predict what a soldier's week 9 FFM would be if the individual were in the WP vs. the CHO group at different baseline FFM (5, 10 kg above or below average). In summary, an individual who has higher FFM at week 1 would have a higher predicted week 9 FFM if he were in the WP vs. the CHO group. Interestingly, we followed this up with correlational analysis and found that body weight was significantly, inversely correlated with relative protein intakes at weeks one ($R^2 = -0.66$, $p < 0.001$) and nine ($R^2 = -0.56$, $p < 0.001$). Further exploration showed that when IET soldiers were binned into groups based on baseline BM, only those where week one BM five kg or more below average consumed 2 or more g/kg/day of protein. Interestingly individuals 10 kg above average week one BM consumed only 1.7 g/kg/day of protein. Organizational recommendations and systematic reviews of the literature suggest that daily protein intakes should be between 1.6 and 2 (50) and 1.7 and 2.2 g/kg/day (51). Therefore, individuals with lower BM in our cohort were able to consume protein intakes on the higher end of the recommended ranges whereas individuals with higher BM were closer to the lower end of this range. These daily requirements may increase to 2–3 g/kg/day in individuals who train in energy-restricted conditions (52), such as may occur in IET (3). Collectively, this suggests that individuals with higher BM or FFM entering into IET may benefit from additional supplementation to help elevate protein intake to optimal levels to optimize the FFM response to training.

The overall group-by-time interaction for FFM was not significant ($p = 0.07$). However, the low p-value considered in light of that the WP group gained on average 1.2 kg of FFM vs. only 0.1 kg in CHO and had a larger effect size (WP = 0.1, CHO = 0), suggests that WP may have a clinically relevant effect on FFM in IET soldiers. The lack of statistical significance in the current work may be due to the large response heterogeneity in the cohort. The change in FFM was 0.1 on average with a standard deviation of 3 kg in CHO and 1.2 with a standard deviation of 2.4 kg in the WP group. One potential driver of the variability in response in the CHO group is that protein intake from diet alone was adequate to maximize the FFM response to IET. Previous work in British IET, suggests that nitrogen balance can be attained, at least in the initial weeks, by consumption of 1.5 g/kg/day of protein intake (53). Additionally, a meta-analysis summarizing the literature on supplementation in strenuous military environments suggested protein intakes between 1.7 and 2.2 g/kg/day are recommended (51). Here we report that the CHO group consumed on average 2.2 g/kg/day. It is also possible that additional caloric intake from supplementation, in general, may be beneficial for FFM response to IET. Previously we reported that 90% of IET soldiers gained FFM when consuming two supplement servings per day (21). Overall, in this study, approximately 69% of all IET soldiers gained FFM when consuming one supplement serving per day. Other work reported only 36% of male soldiers gained FFM when no supplementation is given during IET (5). Thus, it is possible there is a dose-response benefit of additional energy intake during IET to combat the negative energy balance that has been previously reported during IET training (3). However, this distinction cannot be made in the current study due to the lack of direct comparison of one vs. two servings with a non-supplemented control group. Future research needs to expand our work by comparing WP and CHO at various doses with a non-supplemented control group.

The body composition presented here should be interpreted with caution, due to the method used. Single-frequency bioelectrical impedance analysis of body composition, as used in the current investigation, has been reported to be a valid and reliable method for assessing body composition (54–56), but may under/over-predict FM and FFM (55, 57). Thus, caution should be used when drawing conclusions about the precise characterization of body composition of IET soldiers from the current investigation. However, the body composition responses to IET and supplementation presented in the current investigation should be considered reliable as controls were in place to optimize the reliability of the results. Estimation equation (54) and conditions prior to assessment (58) can impact the accuracy of SF BIA results. Here we used the Lukaski equation, which has been shown to be a valid estimator of FFM in comparison to hydrostatic underwater weighing and Dual X-ray Absorptiometry (DXA) (56, 57). To address the influence of conditions prior to assessment, we performed body composition measures at the same time of day (early morning), prior to exercise, in the fasted, hydrated state, all of which may impact body composition assessments in SF-BIA (58) and non-SF-BIA methods such as DXA (59, 60). Additionally, we aimed to minimize the influence of electrode placement by having the same team member perform electrode placement on

all soldiers. Specifically, the body composition device used in this investigation has reliability in relation to FFM and FM as measured by DXA in obese and athletic populations but may underestimate FM and overestimate FFM (55, 57).

Both the WP and CHO groups improved in overall performance during IET training. This was expected as the physical fitness program is designed to take untrained civilians and make them into trained tactical athletes. The lack of difference in endurance between the groups may be explained in the similar levels of carbohydrate consumption. Current recommendations for athletes involved in moderate, high, and very high volumes of exercise are 5–7 g/kg/day, 6–10 g/kg/day, and 8–12g/kg/day respectively to restore muscle glycogen stores that fuel endurance exercise (61, 62). IET soldiers experience training volumes in the high to very high range but are consuming carbohydrate intakes that are below or at best, on the lower range of the recommendations for their physical activity levels regardless of supplement groups (3, 4, 61, 62). Additionally, there was only a 1.1 g/kg/day difference in carbohydrate intake between groups. Previous work has shown that a difference of 2 g/kg/day of carbohydrate showed no difference in pro or macro-glycogen (subfractions of muscle glycogen that are responsive to diet), only the combined total muscle glycogen levels (63). Therefore, the 1.1 g/kg/day difference between groups may not have been large enough to elevate muscle glycogen stores to a level that would lead to substantial differences in endurance performance. Dietary intakes may also have contributed to the lack of statistical difference in push-up performance. Although there was a significant group difference at the end of IET, both groups increased relative protein intakes to at least 2.2 (WP: 2.5, CHO: 2.2) g/kg/day. Protein intakes at this level are at or above the upper amounts of the current recommended protein intakes for military populations and may have been adequate to support the strength adaptations in IET soldiers (51). Another possible contributor to this is the large variability in individual response. On the group level, there was a large effect size of WP (Cohen's D: 0.52) and a medium effect size of CHO (Cohens D: 0.35) and the WP group gained on average four more push-ups relative to the CHO group. This is similar to what we have previously reported (21). However, the standard deviation of the mean difference was thirteen for CHO with an average improvement of eight push-ups and nine for WP with an average improvement of twelve push-ups. Large variability in the response to IET has been shown elsewhere revealing very large improvements (over 100% improvement) to even losses in push-up performance across training (64). The large variability along with the knowledge that we were only able to obtain physical performance data from three out of the four platoons, may have contributed to the lack of statistical significance in physical performance. Regarding the large variability in response across IET, future work would be highly impactful that is designed to explore the factors that contribute to the response variability so that the IET soldier's response to IET can be optimized.

Serum IGF-1, testosterone, and the T:C ratio significantly increased, whereas IL-6 decreased regardless of the supplementation group across the 8 weeks of IET. Physiologically, IGF-1 and testosterone play important roles in stimulating muscle protein synthesis (65, 66) and enhancing satellite cell activity to increase the myonuclear number and enhance hypertrophy (67, 68). Conversely, cortisol has catabolic effects on skeletal muscle (69) and its increase relative to the concentration of testosterone (T:C) has been related to decreases in performance in athletic environments (13). Studies in similar IET environments outside of the United States consistently show decreases in IGF-1 and increases in Cortisol. The testosterone response is more heterogeneous. One study showed an increase, another shows no change, whereas another shows an increase in initial weeks (1–4) followed by a decrease in the final weeks (5–7). Studies in US Army Ranger training have reported that IGF-1 and testosterone decrease in response to large volumes of training and inadequate energy intake (8, 70). Here, we report that regardless of supplement group, IGF-1 increased, and cortisol decreased, both of which the opposite typically occurs in military training environments. The biomarker decrease in previous studies was thought to reflect an imbalance between training volume and nutritional intake. This imbalance can be restored by increased nutritional intake (12). Therefore, it is possible that additional nutritional intake by supplementation, in general, is beneficial for the biomarker response to IET. However, this statement is limited in that the base, typical hormonal response to IET is not adequately characterized. More work is needed to establish the typical hormonal response to IET in United States IET environments.

We also report that IL-6 decreased across IET. IL-6 is released post-exercise and plays a variety of roles, one of which is stimulating the inflammatory response to muscle damage (71). Chronic elevations in IL-6 have been linked to overtraining (20). Previous work in Israeli IET revealed there was no statistically significant change in IL-6 in male (72) and female (72, 73) recruits across 4 months of training. Another study in Australian IET reported no change in IL-6 across 8 weeks of IET (74). We did not replicate these findings in the current investigation. One potential factor was that IET soldiers in our cohort consumed high levels of protein from their diet. One study in marathon runners showed that while supplementation with soy protein did not have an effect, individuals who consumed higher dietary protein (over 20% of daily caloric intake from protein) had a reduced IL-6 and overall inflammatory response to a marathon (75). Studies on the acute effect of protein supplementation vary in protein dose, type, and results as some report a reduction in IL-6 post-exercise (76) while others report no effect and suggest that meeting energy intake needs may be more important (77). These are all important considerations for the current results as they lend potential explanations for the IL-6 response observed here. Overall participants: consumed on average 19% (±2%) of daily calories from protein at week 9 and received additional caloric intake via calorie-matched supplements. Furthermore, participants for both groups had access to dietary protein in the post-exercise period as physical fitness training for Army IET soldiers occurs early in the morning and is followed by breakfast. It is also important to note that IL-6 also plays a key role in stimulating the immune response to pathogens (16) and is elevated by psychological stress (78). Therefore, it is possible that week 1 levels of IL-6 could have been elevated at pre-intervention

due to immunizations, close exposure to a new group of people coming from diverse locations, or stress. This would create an artificial elevation in IL-6 at week 1 and appear to be a reduction in IL-6 across IET. Overall, similar to the hormonal response to IET, the inflammatory response to Army IET is not well characterized and more work needs to be done to characterize the typical inflammatory response of IET soldiers. Considering the collective catabolic (cortisol), inflammatory (IL-6), and anabolic (testosterone, IGF-1) hormonal response observed here, the physiologic environment seems to be one that is beneficial for optimal response to IET.

Although secondary, there were two interesting findings regarding diet and physical activity. Of concern was that supplementation would not be additional nutrition to the soldier's diet but would instead lead to decreased caloric consumption during meals. To address this, we collected diet logs before implementation of supplementation during the baseline week, allowing us to compare dietary intake from the dining facility alone at baseline to see if there was an increase in food consumption across IET. Here, as in our past investigation (21), we report that IET soldiers increased dietary intake from meals consumed from the dining facility and that both groups in the current investigation increased absolute and relative (relative to body weight) macronutrient intake across IET. It is important to note that this was not a primary aim of this investigation and, therefore, future research needs to be conducted to determine if supplementation does negatively impact food consumption. Another secondary important finding from this study is that physical activity (i.e., training volume) was significantly different across the IET phase. The red phase (first 3 weeks of IET) was significantly higher in time spent in light activity than all other phases and on average had the highest total training volume. This is in agreement with our previous work that found training volume was higher during the initial weeks of training (3, 4). Reports from the Center for Disease Control (CDC) suggest that <30% of individuals in the U.S. aged 18–35 participate in 300 min per week of moderate or 150 min of vigorous-intensity exercise (79). Here, we report that IET soldiers participate in over 400 min per day of at least light intensity exercise. Thus, IET soldiers may experience rapid increases in training volume as they perform more physical activity in 1 day than much of the US population performs in 1 week. US Army training command has been working to resolve these issues.

What should finally be noted is how the current dataset relates to our previous study where IET Soldiers were provided two servings of WP vs. a calorie-matched CHO supplement (21). The current investigation was conducted in a separate cohort, with different IET cadre, military occupation specialty, and training, with only one serving of WP or CHO once per day. The mean differences (WP minus CHO) for FFM were 1.1 (single serving) and 0.6 (two servings) kg higher in the WP vs. CHO groups. WP decreased FM 1.2 (single serving) and 1.8 (two servings) kg more on average and improved push-up performance on average about 4.3 (single servings) and 4.2 (two servings), relative to the change in the CHO group. Though not all of these were determined to be statistically significant, the consistency of these results, even though IET cohort and leadership were different, suggest that WP may benefit body composition changes and strength endurance during IET. More information on comparisons between our prior and current studies can be found in **Table 5**.

TABLE 5 | Consistency of between group mean differences across cohorts.

Variable	Group	Single WP/CHO serving Mean Difference [CI]	Two WP/CHO servings Mean Difference [CI]
FFM (kg)	CHO	0.1 [−5.8, 5.9]	3.6 [2.3, 4.9]
	WP	1.2 [−3.5, 5.8]	4.2 [3.1, 5.4]
	Diff	1.1	0.6
FM (kg)	CHO	−0.9 [−5.9, 4.1]	−2.7 [−4.0, −1.3]
	WP	−2.1 [−7.8, 3.6]	−4.5 [−5.8, −3.2]
	Diff	−1.2	−1.8
PU (reps)	CHO	7.8 [−17.3, 32.9]	2.6 [−0.7, 6.0]
	WP	12.1 [−5.8, 30.1]	6.8 [2.9, 10.7]
	Diff	4.3	4.2

Mean difference: the average difference across IET with 95% CIs at weeks 1 and 9 for FFM and FM and weeks 2 and 8 for PU. Diff, Mean Difference in WP minus Mean Difference in CHO. FFM, Fat-Free Mass; FM, Fat Mass; PU, Push-ups completed in 1 min; WP, Whey protein supplement group; CHO, Carbohydrate supplement group.

There are limitations to this study. One limitation in this study was that performance data was obtained from 75% of the participants. This resulted in more data being collected for the WP group in comparison to the CHO. We were still able to obtain 55 (WP = 37, CHO = 17) data points. Another limitation is that performance data was collected by multiple testers. While inter-rater reliability could influence the findings of this study, it is notable that drill sergeants administered all tests and are highly trained in conducting the APFT. They administer the test often and IET soldier graduation is dependent upon the APFT. Caution should be taken regarding the body composition results (critiqued in detail above). While the reliability of a single frequency has been established previously (54–56), the characterization of true FFM and FM may not be as precise as other methods such as underwater weighing and DXA (55, 57). Regarding the analysis of biomarkers, it is notable that blood draws were collected only at the week 1 and week 9-time points due to limited access to the soldiers during the IET period. Ideally, more sampling time points would be completed to better describe the typical hormonal and inflammatory response of soldiers to Army IET environments, which is not well characterized. Another limitation is the timing of supplement consumption. We were able to record adherence to consuming supplementation but were not able to gather adherence as to the time of consumption/dispersion of supplements being before bed. Drill sergeants were asked to disperse and IET soldiers were instructed to consume supplements before bed, but the research team was not present due to trying to be minimally invasive into the IET training schedule. Finally, our discussion of the potential influence of supplement dose (one vs. two servings) must be considered in the context that we did not perform a direct comparison in this investigation. Our work investigating two servings per day was completed previously in a different IET cohort (21).

CONCLUSION

Once-daily supplementation with WP significantly decreased FM and enhanced gains in FFM in individuals who entered IET with higher FFM compared to those with lower FFM. The consistency of mean changes and effect sizes in FM and FFM and previous cohorts of Army IET suggest that WP may be beneficial for soldiers' body composition response during IET. However, there was no significant influence of WP on physical performance or biomarkers of the physiologic response to IET. The lack of response may be due to high relative dietary protein intakes in IET soldiers in the current cohort or may suggest that more than one serving is needed to optimize performance.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because of Army data sharing restrictions. They may be available upon special arrangement. Requests to access the datasets should be directed to jms0018@auburn.edu.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Auburn University Institutional Review Board, and the Director, Research and Analysis Directorate Army Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JM, JS, KY, KRL, and MDR: conceptualization. JM, JS, DB, PM, KY, KRL, MDR, and KDL: methodology. JM, CH, PM, and KRL: software/analysis. JM, JS, CH, PM, PR, KY, KRL, and MDR: validation. JM, PM, KY, and KRL: formal analysis. JS, JM, KDL, DB, CH, MAR, PM, PR, KY, KRL, and MDR: investigation. JS and MDR: resources. JS, JM, KDL, DB, CH, MAR, PM, PR, KY, and MDR: data collection and quality assurance. JM, JS, and MDR: writing-original draft preparation and funding acquisition. JS, KDL, DB, CH, MAR, PM, PR, KY, KRL, and MDR: writing-review and editing. JM and MDR: visualization. JS and JM: supervision. JS, JM, KDL, and MDR: project coordination/administration. All authors contributed to the article and approved the submitted version.

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High Protein Diets Improve Liver Fat and Insulin Sensitivity by Prandial but Not Fasting Glucagon Secretion in Type 2 Diabetes

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Glucagon (GCGN) plays a key role in glucose and amino acid (AA) metabolism by increasing hepatic glucose output. AA strongly stimulate GCGN secretion which regulates hepatic AA degradation by ureagenesis. Although increased fasting GCGN levels cause hyperglycemia GCGN has beneficial actions by stimulating hepatic lipolysis and improving insulin sensitivity through alanine induced activation of AMPK. Indeed, stimulating prandial GCGN secretion by isocaloric high protein diets (HPDs) strongly reduces intrahepatic lipids (IHLs) and improves glucose metabolism in type 2 diabetes mellitus (T2DM). Therefore, the role of GCGN and circulating AAs in metabolic improvements in 31 patients with T2DM consuming HPD was investigated. Six weeks HPD strongly coordinated GCGN and AA levels with IHL and insulin sensitivity as shown by significant correlations compared to baseline. Reduction of IHL during the intervention by 42% significantly improved insulin sensitivity [homeostatic model assessment for insulin resistance (HOMA-IR) or hyperinsulinemic euglycemic clamps] but not fasting GCGN or AA levels. By contrast, GCGN secretion in mixed meal tolerance tests (MMTTs) decreased depending on IHL reduction together with a selective reduction of GCGN-regulated alanine levels indicating greater GCGN sensitivity. HPD aligned glucose metabolism with GCGN actions. Meal stimulated, but not fasting GCGN, was related to reduced liver fat and improved insulin sensitivity. This supports the concept of GCGN-induced hepatic lipolysis and alanine- and ureagenesis-induced activation of AMPK by HPD.

Keywords: glucagon, insulin sensitivity, liver fat content, alanine, type 2 diabetes, non-alcoholic fatty liver disease (NAFLD), high protein diet

INTRODUCTION

Glucagon (GCGN) increases glucose production in the liver, stimulates insulin release from beta cells and contributes to maintaining normal levels of glucose in a close interplay with insulin in healthy subjects (1, 2). Hyperglucagonemia was proposed as an early driver of hyperglycemia and as an initial step in the pathogenesis of type 2 diabetes mellitus (T2DM) (3, 4) although the causes of hyperglucagonemia remain controversial (5). Insulin resistance of the alpha-cell was proposed to impair the inhibition of glucagon secretion by insulin and may thereby increase GCGN levels (3). Glucagon release is directly and acutely stimulated by amino acids (AA) (6) and drives their hepatic degradation in the urea cycle (7, 8), which generates a liver-alpha-cell feedback loop. Non-alcoholic fatty liver disease (NAFLD) is a frequent consequence of obesity and associated with increased levels of AA (9) which was proposed to result from fatty liver-induced hepatic resistance to the GCGN-induced degradation of AA. The ensuing hyper-aminoacidemia may in turn stimulate GCGN-release and induce fasting and postprandial hyperglucagonemia in obesity and diabetes mellitus. The increase of fasting GCGN is thought to increase glucose production and to induce hyperinsulinemia which will further aggravate NAFLD and insulin resistance (10). The product of GCGN and alanine was recently proposed as an indicator of hepatic GCGN resistance and was associated with hepatic fat content (11). Fatty liver is closely linked to insulin resistance and increased levels of AAs, such that the overlap and interdependence of both phenomena make it difficult to separate the causes.

Although GCGN antagonists reduced blood glucose levels in T2DM patients they increased hepatic transaminases, induced fatty liver and dyslipidemia (5, 12–14). This raised awareness of the positive actions of GCGN such as the induction of lipolysis and lipid oxidation, inhibition of appetite and increase in energy expenditure (5, 15, 16). Moreover, recent work unraveled an important role of intra-islet GCGN release from alpha cells in maintaining beta cell responses (5, 17, 18). This work was backed by the development of GCGN agonists in peptide polyagonists combining GCGN, GLP-1, and/or GIP to treat T2DM (5). As AAs are potent inducers of GCGN secretion, high protein diets (HPDs) might be used to increase GCGN release and thereby profit from its benefits (16). Indeed, we recently tested HPDs without restriction of calorie intake in patients with T2DM and observed improvements of insulin sensitivity, hepatic fat content, circulating fatty acids, uric acid, and markers of inflammation and redox metabolism (19–23).

This raises the question, whether (a) fatty liver is quantitatively linked to fasting glucagon secretion and hepatic GCGN resistance in T2DM as reflected by elevated fasting AA and the GCGN–alanine index and (b), whether a reduction of liver fat would improve the hepatic GCGN resistance in people with T2DM as might be expected if NAFLD is a primary cause of hyperglucagonemia. As NAFLD is also closely linked to insulin resistance, the reduction of liver fat should improve alpha-cell insulin sensitivity and may thereby reduce fasting and postprandial GCGN release. Because alpha-cell-GCGN-stimulated insulin secretion is largely mediated by GLP-1 receptors, GCGN-resistance might not alter the response to protein- and AA intake-induced insulin secretion in mixed meal tolerance tests (MMTTs).

A second aspect arises from potential beneficial effects of GCGN in obesity and T2DM: GCGN specifically drives intrahepatic lipolysis and lipid oxidation through a recently discovered inositol trisphosphate-receptor-1 (INSP3-R1) dependent signal pathway and thereby is a powerful stimulus to reduce liver fat (24). Preclinical studies moreover suggest a centrally mediated inhibition of hepatic lipogenesis by GCGN (16). Indeed, isocaloric HPDs which strongly stimulate GCGN release, have been used to reduce liver fat in patients with T2DM by over 40% which most likely was mediated by the increase in GCGN-induced hepatic lipolysis (19, 20). This raises the question whether GCGN resistance of the liver would impair the action of GCGN and thereby serve as a marker of the prospective effectiveness of HPD for the reduction of liver fat in people with NASH/NAFLD.

This analysis was performed to assess the interplay of intrahepatic lipids (IHLs) with plasma levels of GCGN and hepatic GCGN-resistance in study participants with T2DM before and after extensive loss of liver fat achieved by the intake of HPDs (30%E of protein) for 6 weeks. We assessed whether there is (a) a correlation of IHL with insulin sensitivity and GCGN resistance determined by the GCGN–alanine index at baseline and after the intervention, (b) whether an extensive reduction of IHL by isocaloric HPD affects insulin or GCGN sensitivity, (c) whether GCGN sensitivity at baseline determines the effect of the HPD on loss of IHL, and whether (d) GCGN sensitivity affects the secretion of insulin induced by a mixed meal, i.e., whether the ultra-short loop feedback between alpha- and beta-cells changes.

MATERIALS AND METHODS

The analysis is based on the "LeguAN" intervention trial in subjects (18–80 years) with T2DM, which was registered at ClinicalTrials.gov (NCT02402985). Participants with orally treated T2DM, matched for age, sex, body mass index (BMI), glycated hemoglobin A1c (HbA1c), and anti-diabetic medications, were randomized using computer algorithm to 6 weeks of isocaloric diets which contained 30% of energy intake (%E) as protein, 40%E as carbohydrates, and 30%E as fat (20). All participants received individually adapted dietary instructions and meal plans by an experienced dietician and Master in Nutrition (SS) and were partially supplied with foods during the 6 weeks. The overall composition of SAFA (10%E), MUFA (10%E), and PUFA (10%E) was kept similar as much as possible and dietary intake was calculated with the computer program PRODI as described in detail in the supplements of refs (19, 20). The study participants completed MMTTs before and at the end of the study which consisted of breakfast (MMTT1) and lunch (MMTT2) with detailed profiles of insulin, GCGN, glucose, and AA over 360 min. The original study compared plant vs. animal protein rich diets which showed similar improvements of IHL, insulin sensitivity, fasting glucose,

HbA1c, visceral adipose tissue (VAT), inflammatory, liver, and redox markers ref (19–23). The groups were therefore combined in the current analysis. The separation into two groups with changes of liver fat above vs. below the median comprised animal/plant protein of 7/8 in the higher and 9/7 in the lower liver fat change groups. Changes of protein intakes, blood urea nitrogen (BUN) and urinary nitrogen excretion relative to changes in IHL, GCGN, and homeostatic model assessment for insulin resistance (HOMA-IR) are shown in **Supplementary Figures 2–4**. The free fatty acid (FFA) in serum showed a decrease of all saturated fatty acids (C14–C22), no change of linoleic acid and a small increase of alpha-linoleic acid as reported previously (20). All subjects signed informed consent prior to participation. A total of 31 subjects were included who performed proton magnetic resonance spectroscopy (^{1}H-MRS) of the liver and MRI for VAT on a 1.5 T whole body imager (Magnetom Avanto, Siemens Healthcare, Erlangen, Germany) at baseline and after 6-weeks of high-protein dietary intervention (19–21). Body composition (fat mass and lean mass) was determined by Air Displacement Plethysmography (BOD POD, COSMED, Italy). Routine parameters were measured in serum using ABX Pentra 400 (Horiba, Japan). Insulin and glucagon in serum samples were measures by ELISA (Mercodia, Sweden). Plasma AA levels were determined by liquid chromatography tandem mass spectrometry analysis.

Calculations

Index of whole-body insulin resistance (HOMA-IR) was calculated as: fasting insulin (mU/L) × fasting glucose in (mmol/L)/22.5 (25). Matsuda index was calculated according to Matsuda and DeFronzo (26).

The GCGN–alanine index and the GCGN–AA-index were calculated as fasting glucagon × fasting alanine or other AA, respectively, according to the previous publication (12). The glucose disposal rate (*M*-value) was calculated from the infusion rate of exogenous glucose during steady state of the hyperinsulinemic euglycemic clamp (HEC) as previously described.

Statistical Analysis

For statistical analysis, all variables are described as mean ± SD. Normal distribution was evaluated by Shapiro–Wilk-test. According to the normal or non-normal distribution, statistical comparison of variables at baseline and after 6-weeks high protein intervention between two groups was performed by independent *t*-test or Mann–Whitney *U*-test; Paired *t*-test or Wilcoxon signed rank test was used within groups. The repeated measures ANOVA was used to analyze differences at different time-points.

For correlation analysis, non-normally distributed data (GCGN–AA index, IHL, and HOMA-IR) were logarithmically transformed to approximate a linear distribution. Spearman's non-parametric rank or Pearson correlations were conducted depending on the normality of data distribution. Areas under the curve (AUC) and incremental areas under the curve (iAUC) were calculated by GraphPad prism 8 (CA, United States) using the trapezoid rule.

A p-value < 0.05 was considered statistically significant. All statistical calculations were performed using SPSS 26.0 (IBM, United States). All graphs were generated by GraphPad prism 8 (CA, United States).

RESULTS

We studied 31 study participants with orally treated T2DM whose characteristics are shown in **Table 1**. The intrahepatic lipid content (IHL) was 15.4 ± 9.8% determined by ^{1}H-MRS and correlated highly with insulin sensitivity measured as HOMA-IR ($\rho = 0.554$, $p = 0.001$) (**Figure 1A**) and with fasting GCGN levels ($\rho = 0.454$, $p = 0.012$) (**Figure 1B**). VAT, determined by MRI,

TABLE 1 | Parameters at baseline (Week 0) and after the HPD intervention of all study participants (Week 6).

Parameter (n = 31)	Week 0	Week 6	p-Value
Age (years)	64.6 ± 6.0		
Gender (male/female)	19 m/12 f		
Liver fat content (MR-S; %)	15.4 ± 9.8	8.8 ± 8.1	<0.001***
Body weight (kg)	89.4 ± 14.2	87.4 ± 14.0	<0.001***
BMI (kg/m^2)	30.6 ± 3.7	29.9 ± 3.5	<0.001***
Waist circumference (cm)	102.9 ± 10.9	100.6 ± 10.7	<0.01**
Fasting glucose (mmol/L)	9.6 ± 1.5	8.8 ± 1.5	<0.001***
Fasting insulin (mU/L)	8.4 ± 4.7	7.9 ± 5.4	0.16
Fasting glucagon (pmol/L)	8.2 ± 3.5	8.4 ± 3.7	0.63
Fasting C-P (μg/L)	1.9 ± 0.8	1.9 ± 0.9	0.40
Insulin/glucagon ratio	1.1 ± 0.72	0.89 ± 0.42	0.056
C-P/glucagon ratio	0.27 ± 0.17	0.23 ± 0.08	0.23
iAUC glucagon (pmol/L)	992.1 ± 577.4	829.3 ± 502.3	0.313
HbA1c	6.8 ± 0.70	6.4 ± 0.69	<0.001***
HOMA-IR	3.5 ± 1.9	3.1 ± 2.0	<0.05*
Matsuda index	4.5 ± 3.1	5.0 ± 2.9	<0.05*
M-value	4.9 ± 2.1	5.5 ± 1.9	<0.01**
AST (U/L)	25.2 ± 8.7	21.8 ± 6.1	<0.01**
ALT (U/L)	28.2 ± 9.9	26.5 ± 8.4	0.13
AST/ALT ratio	0.87 ± 0.21	0.84 ± 0.19	0.54
GGT (U/L)	44.1 ± 26.2	30.8 ± 15.9	<0.001***
TG (mmol/L)	1.7 ± 0.59	1.6 ± 0.66	0.22
TC (mmol/L)	5.3 ± 0.97	4.62 ± 0.95	<0.01**
LDL-C (mmol/L)	3.4 ± 0.89	2.9 ± 0.85	<0.01**
HDL-C (mmol/L)	1.1 ± 0.26	0.96 ± 0.17	<0.01**
CREA (μmol/L)	81.3 ± 16.2	77.5 ± 16.7	<0.05*
BUN (mmol/L)	6.0 ± 0.95	7.8 ± 1.8	<0.001***
eGFR (mL/min/1.73 m^2)	78.6 ± 15.2	82.6 ± 15.2	<0.05*
Urine urea (mmol/24 h)	403.0 ± 134.2	564.0 ± 200.2	<0.001***
VAT (L)	6.0 ± 2.1	5.8 ± 1.9	<0.01**
Fat mass (%)	35.8 ± 7.3	33.9 ± 7.0	<0.05*
Lean mass (%)	64.0 ± 7.3	66.2 ± 7.0	<0.05*

*BMI, body mass index; C-P, C-peptide; iAUC, incremental area under curve; HbA1c, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment for insulin resistance; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; TG, triglycerides; TC, total cholesterol; CREA, creatinine; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; VAT, visceral adipose tissue. *p < 0.05; **p < 0.01; ***p < 0.001.*

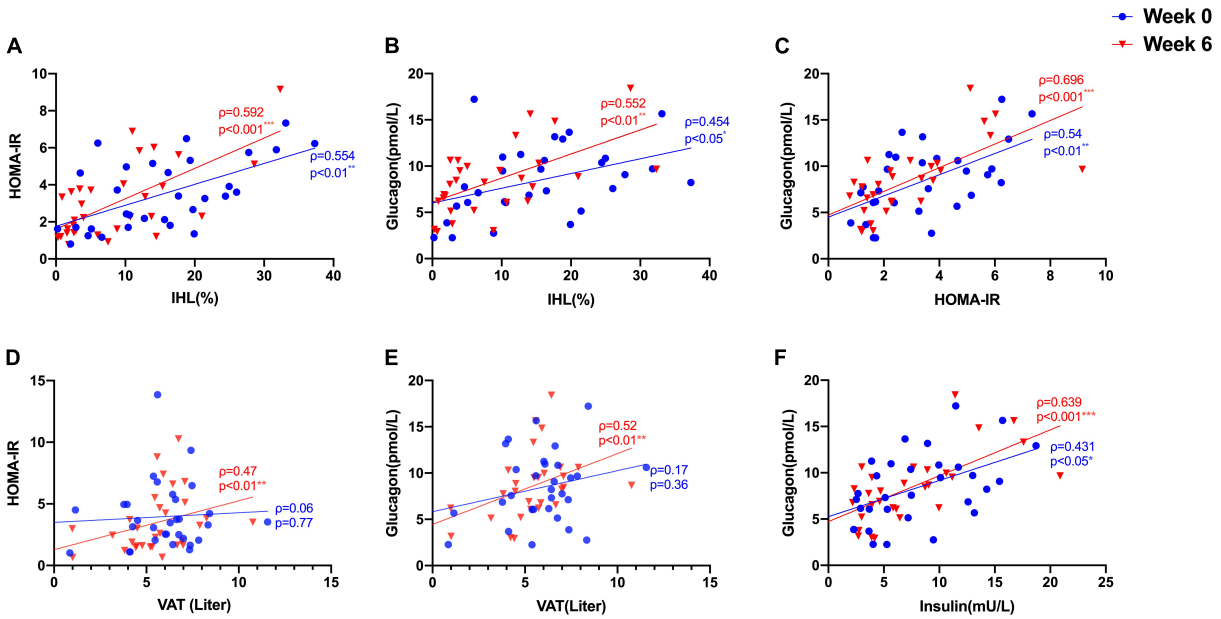

FIGURE 1 | Correlations between **(A)** IHL (%) and insulin sensitivity (HOMA-IR); **(B)** IHL (%) and GCGN; **(C)** HOMA-IR and GCGN; **(D)** visceral adipose tissue (VAT) and HOMA-IR; **(E)** VAT and GCGN; **(F)** correlations between fasting insulin and GCGN before (Week 0, blue) and after high protein intake intervention in the entire study group (Week 6, red). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

did not correlate with GCGN ($\rho = 0.17$, $p = 0.36$) (**Figure 1E**). The intervention resulted in markedly reduced liver fat content by 6.6%, slightly but significantly reduced VAT and significant improvements of HbA1c, fasting glucose, and insulin sensitivity (HOMA-IR, Matsuda index, and *M*-value) (**Table 1**) (19, 20). The levels of fasting GCGN did not change significantly (**Table 1**).

Correlation of Glucagon, Glucagon–Alanine Index, and Insulin Sensitivity With Intrahepatic Lipid and Visceral Adipose Tissue

Glucagon levels correlated with IHL and insulin sensitivity before and after the intervention (**Figures 1B,C**) and with VAT after the intervention ($\rho = 0.52$, $p = 0.004$) (**Figure 1D**). In order to assess hepatic GCGN sensitivity, we calculated the GCGN–alanine index as proposed (12) which correlated modestly with IHL at baseline ($\rho = 0.369$, $p < 0.05$). Insulin sensitivity calculated by HOMA-IR correlated trendwise and non-significantly with the GCGN–alanine index at baseline ($\rho = 0.352$, $p = 0.057$) (**Figure 2A**). Remarkably, the correlations of the GCGN–alanine index became highly significant upon the high protein intake for 6 weeks for IHL ($\rho = 0.652$, $p < 0.001$) (**Figure 2B**) and for insulin sensitivity ($\rho = 0.644$, $p < 0.001$) (**Figure 2A**). Similarly, increased correlations were observed between GCGN–alanine index and BCAA, glutamine, or histidine as well as between total AAs and with IHL or HOMA-IR (**Supplementary Table 3**). The intake of the high-protein diet thus greatly increased the alignment of GCGN and AA as reflected by their increasing correlation with liver fat and insulin sensitivity.

Improvements of Insulin Sensitivity Upon Reduction of Liver Fat Are Dissociated From Changes of the Glucagon–Alanine Index

Glucagon is likely a key player in the protein-induced reduction of liver fat by high protein intake (24). The reductions of liver fat in our study showed large differences between individuals. We therefore hypothesized that these differences might be related to hepatic GCGN resistance resulting in impaired GCGN-induced hepatic lipolysis and induction of ureagenesis.

We therefore analyzed the participants according to changes above or below the median of liver fat change. This resulted in a significant difference of liver fat reduction between the groups although baseline levels of IHL did not differ significantly (**Table 2**). The lesser liver fat reduction group shifted from 17.4 to 12.7% IHL and thus maintained a high liver fat content even after the relative reduction by 27%. The greater liver fat reduction group decreased IHL by 65% from 13.3 to 4.6 ± 3.8% and thus – in average – below the defined threshold of fatty liver of 5.56% IHL. The modest reduction of weight and waist circumference was around 2 kg and 2 cm, respectively, identical in both groups as were modest reductions of visceral and total adipose tissue and modest increases in muscle mass (**Table 2**).

Fasting glucose decreased significantly in both groups while fasting insulin decreased significantly in the greater liver fat reduction group only. Fasting GCGN did not change significantly in either group. Insulin sensitivity expressed by HOMA-IR, Matsuda index, or *M*-value improved significantly in the group with greater IHL reduction but not in the lesser IHL-reduction group resulting in a significant difference between the groups.

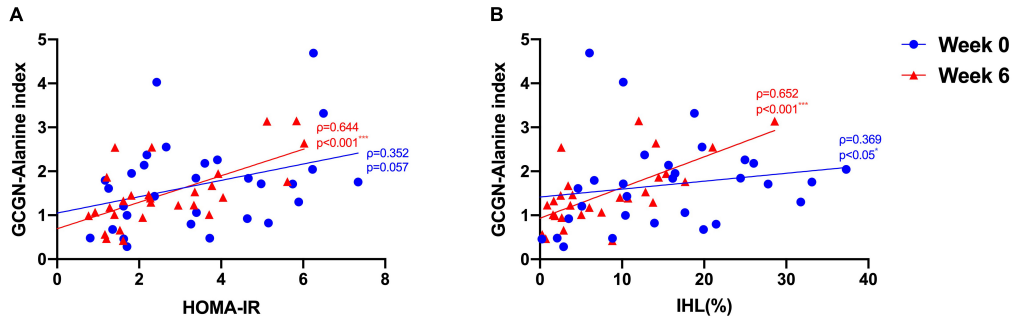

FIGURE 2 | Correlations between **(A)** GCGN–alanine index and HOMA-IR; **(B)** GCGN–alanine index and IHL (%) before (Week 0, blue) and after the intervention in the entire study group (Week 6, red). *$p < 0.05$; ***$p < 0.001$.

TABLE 2 | Parameters at baseline (Week 0) and after HPD intervention (Week 6) of study participants with lower (below median) and higher (above median) reduction of intrahepatic lipid content (IHL).

Parameter	Lower liver fat reduction (n = 16) below median			Higher liver fat reduction (n = 15) above median			$p$week 6 vs week 0
	Week 0	Week 6	p	Week 0	Week 6	p	
Age (years)	63.0 ± 5.7			66.3 ± 6.0			
Gender (male/female)	8 m/8 f			11 m/4 f			
Liver fat content (MR-S; %)	17.4 ± 10.7	12.7 ± 9.2	<0.001***	13.3 ± 8.6	4.6 ± 3.8	<0.001***	<0.05*
Body weight (kg)	89.0 ± 14.0	86.7 ± 13.6	<0.001***	89.6 ± 15.6	86.8 ± 15.4	<0.001***	0.96
BMI (kg/m^2)	31.0 ± 4.1	30.2 ± 4.0	<0.001***	30.2 ± 3.3	29.5 ± 3.1	<0.001***	0.96
Waist circumference (cm)	102.5 ± 10.4	100.7 ± 10.3	0.07	103.2 ± 11.8	100.6 ± 11.5	<0.01**	0.54
Fasting glucose (mmol/L)	9.3 ± 1.0	8.8 ± 1.1	<0.05*	10.0 ± 1.8	8.9 ± 1.8	<0.01**	0.12
Fasting insulin (mU/L)	8.4 ± 4.9	8.9 ± 6.4	0.28	8.3 ± 4.6	6.9 ± 4.1	<0.05*	<0.05*
Fasting glucagon (pmol/L)	8.2 ± 3.2	9.2 ± 4.0	0.24	8.7 ± 4.5	7.6 ± 3.4	0.51	0.18
Fasting C-P (ug/L)	1.9 ± 0.9	1.9 ± 1.0	0.59	1.8 ± 0.8	1.7 ± 0.8	0.07	0.11
AUC insulin (MMT1)	8915.3 ± 6880.0	9039.2 ± 7201.4	0.75	10163.1 ± 6425.7	8503.3 ± 4852.5	<0.05*	<0.05*
AUC insulin (MMT2)	6322.0 ± 4262.6	5923.3 ± 3508.7	0.14	6062.9 ± 4109.5	4926.7 ± 2844.5	0.06	0.35
AUC glucagon (MMT1)	2917.4 ± 869.5	3051.3 ± 1018.9	0.35	2925.1 ± 1004.8	2672.3 ± 1046.9	<0.05*	0.08
AUC glucagon (MMT2)	2988.1 ± 829.9	2755.9 ± 831.8	0.08	2651.7 ± 1089.3	2439.4 ± 1181.6	0.08	0.98
HbA1c	6.7 ± 0.54	6.3 ± 0.47	<0.01**	7.0 ± 0.81	6.6 ± 0.84	<0.05*	0.80
HOMA-IR	3.4 ± 1.9	3.4 ± 2.4	0.77	3.6 ± 2.0	2.6 ± 1.5	<0.01**	<0.05*
Matsuda index	4.8 ± 3.7	4.7 ± 2.9	0.72	4.2 ± 2.5	5.4 ± 3.0	<0.01**	<0.05*
M-value	5.0 ± 2.4	5.3 ± 2.0	0.28	4.8 ± 1.8	5.8 ± 1.7	<0.01**	0.11
AST (U/L)	26.4 ± 9.7	21.8 ± 5.8	<0.05*	24.0 ± 7.7	21.7 ± 6.5	0.16	0.34
ALT (U/L)	29.9 ± 12.7	27.8 ± 9.4	0.15	26.4 ± 5.5	25.1 ± 7.2	0.48	0.42
AST/ALT ratio	0.88 ± 0.24	0.82 ± 0.16	0.61	0.87 ± 0.18	0.86 ± 0.22	0.81	0.67
GGT (U/L)	48.4 ± 23.6	36.0 ± 17.9	<0.001***	39.5 ± 28.7	25.2 ± 11.5	<0.05*	0.81
TG (mmol/L)	1.7 ± 0.54	1.8 ± 0.74	0.33	1.7 ± 0.66	1.4 ± 0.52	<0.05*	<0.05*
TC (mmol/L)	5.2 ± 0.88	4.8 ± 1.0	<0.01**	5.4 ± 1.1	4.5 ± 0.88	<0.001***	<0.05*
LDL-c (mmol/L)	3.3 ± 0.86	3.0 ± 0.91	<0.05*	3.5 ± 0.94	2.9 ± 0.82	<0.01**	0.58
HDL-c (mmol/L)	1.1 ± 0.27	0.95 ± 0.14	<0.01**	1.2 ± 0.27	0.96 ± 0.21	<0.001***	0.18
Creatinine (μmol/L)	82.6 ± 17.5	79.9 ± 18.5	0.41	79.9 ± 15.2	74.9 ± 14.7	<0.05*	0.49
BUN (mmol/L)	6.0 ± 1.0	7.8 ± 1.7	<0.01**	5.9 ± 0.94	7.8 ± 1.9	<0.01**	0.38
eGFR (mL/min/1.73 m^2)	77.1 ± 16.1	80.2 ± 15.9	0.38	80.3 ± 14.6	85.1 ± 14.6	<0.05*	0.61
Urine urea (mmol/day)	377.6 ± 79.3	507.6 ± 158.5	<0.01**	430.4 ± 175.0	624.7 ± 227.9	<0.01**	<0.05*
VAT (L)	6.0 ± 2.2	5.6 ± 2.1	<0.01**	5.9 ± 2.1	5.5 ± 1.9	0.12	0.92
Fat mass (%)	36.4 ± 9.0	34.8 ± 8.9	<0.01**	35.2 ± 4.9	32.6 ± 3.8	0.11	0.52
Lean mass (%)	63.6 ± 9.0	65.2 ± 8.9	<0.01**	64.8 ± 4.9	67.4 ± 3.8	0.11	0.52

*BMI, body mass index; C-P, C-peptide; HbA1c, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment for insulin resistance; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; TG, triglycerides; TC, total cholesterol; CREA, creatinine; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.*

By contrast, the GCGN resistance indices calculated for alanine or AA did neither change significantly within, nor differ between the groups (**Supplementary Tables 1, 2**, **Figure 3**). However, the reduction of liver fat showed a borderline correlation with the change of GCGN ($\rho = 0.344$, $p = 0.077$) but not with the change of the GCGN–alanine index.

Notably, the correlations of the GCGN–AA indices with IHL and insulin sensitivity became highly significant for virtually all AA from baseline to follow-up, indicating a close alignment of GCGN-regulated AA-metabolism with IHL and insulin sensitivity (**Supplementary Table 2**). Thus, the reduction of liver fat is linked to a reduction of insulin resistance but not of GCGN resistance estimated by the GCGN–alanine index even upon extensive reductions of liver fat. However, the role of the GCGN–AA-hepatic axis appears to become enhanced which we interpret to reflect beneficial actions of GCGN.

Does Glucagon Resistance Impair the High Protein Diet-Induced Loss of Liver Fat?

We then asked whether hepatic GCGN resistance may relate to impaired degradation of IHL by GCGN in response to HPD and therefore compared participants above with those below the median of the GCGN–alanine index regarding responses of IHL to high-protein diet. Indeed, the GCGN–alanine index in the upper half was associated with higher liver fat compared to the lower half both at baseline (20.9 ± 9.2 vs. $11.9 \pm 9.4\%$; $p < 0.05$) and after 6 weeks (11.4 ± 7.3 vs. $4.1 \pm 3.9\%$; $p < 0.01$). However, the absolute magnitude of liver fat reduction did not differ between the groups (6.8 ± 5.3 vs. $6.6 \pm 5.1\%$; $p > 0.05$) and we did not find an indication that a higher GCGN–alanine index impairs the HPD-induced reduction of liver fat (**Table 3** and **Supplementary Figure 1**).

Does Glucagon Play a Role for Circulating Free Fatty Acids?

We previously reported that HPDs reduced circulating saturated FFAs which associated with the changes in IHL (19). In view of the regulation of hepatic lipid metabolism by GCGN we assessed associations between circulating GCGN and FFA.

TABLE 4 | Correlations between GCGN and FFA and DNL-index before (Week 0) and after (Week 6) HPD intervention.

Parameters ($n = 31$)	Week 0	Week 6
C14:0	$\rho = 0.253$ $p = 0.186$	$\rho = 0.417$ $p < 0.05^*$
C15.0	$\rho = -0.071$ $p = 0.713$	$\rho = 0.094$ $p = 0.626$
C17.0	$\rho = 0.315$ $p = 0.096$	$\rho = 0.272$ $p = 0.153$
C16:0	$\rho = 0.388$ $p < 0.05^*$	$\rho = 0.524$ $p < 0.01^{**}$
C18.0	$\rho = 0.522$ $p < 0.01^{**}$	$\rho = 0.489$ $p < 0.01^{**}$
DNLindex = 16:0/18:2n6	$\rho = 0.531$ $p < 0.01^{**}$	$\rho = 0.456$ $p < 0.05^*$

*C14:0: myristic acid; C16:0: palmitic acid; C18:0: stearic acid; C15:0: pentadecanoic acid; C17:0: heptadecanoic acid; DNLindex: de novo lipogenesis index. *p < 0.05; **p < 0.01.*

Indeed, GCGN correlated significantly with palmitic and stearic acid and the *de novo* lipogenesis index, both before and after the intervention supporting a role of GCGN in the regulation of lipogenesis. This would be expected due to the AMPK induced inhibition of ACC (**Table 4** and **Supplementary Table 7**). In agreement, there was no correlation with odd numbered FFA or unsaturated FFA or indices of desaturase or elongase activities (**Supplementary Table 7**).

Assessment of Beta-Cell Stimulation by High Protein Diet – Does the Glucagon Response Play a Role?

Capozzi and coworkers recently proposed that GCGN-induced insulin secretion contributes to lowering of blood glucose concentrations particularly in mixed meals (17, 18). We wondered whether changes of AAs and GCGN responses to protein challenges occurred in response to the reductions of liver fat by HPD. Fasting levels of AA did not change in response to the intervention. Fasting levels of GCGN and insulin were highly correlated ($\rho = 0.431$, $p < 0.05$), and the

TABLE 3 | Parameters at baseline (Week 0) and after HPD intervention (Week 6) between lower (below median) and higher (above median) basal GCGN–alanine index groups.

Parameters	Week 0 Glucagon–alanine index			Week 6 Glucagon–alanine index		
	Lower ($n = 16$) below median	Higher ($n = 15$) above median	p	Lower ($n = 16$) below median	Higher ($n = 15$) above median	p
Liver fat content (MRS; %)	11.9 ± 9.4	20.9 ± 9.2	$<0.05^*$	4.1 ± 3.9	11.4 ± 7.3	$<0.01^{**}$
Insulin/glucagon ratio (fasting)	1.4 ± 0.84	0.85 ± 0.48	0.052	0.89 ± 0.42	0.83 ± 0.32	0.88
Insulin/glucagon ratio (60 min)	5.2 ± 3.7	2.9 ± 1.6	0.07	3.7 ± 2.0	4.2 ± 2.8	0.84
Insulin/glucagon ratio (120 min)	4.6 ± 2.8	3.5 ± 2.2	0.18	3.8 ± 2.0	4.3 ± 2.7	0.77
Insulin/glucagon ratio (180 min)	2.6 ± 1.6	2.4 ± 1.6	0.33	2.3 ± 1.1	2.4 ± 1.3	0.95

$^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

TABLE 5 | Area under the curve-insulin and AUC-GCGN levels at baseline (Week 0) and after HPD intervention (Week 6) in MMTT 1 and MMTT 2.

Parameters	MMTT 1			MMTT2		
	Week 0	Week 6	*p*	Week 0	Week 6	*p*
AUC insulin	9482.6 ± 6410.0	8722.8 ± 5890.0	0.17	6233.7 ± 4015.9	5394.6 ± 3102.0	<0.05*
AUC glucagon	2917.4 ± 899.8	2856.6 ± 1007.4	0.51	2907.4 ± 964.2	2656.0 ± 979.8	<0.05*

*AUC, area under the curve. *p < 0.05; **p < 0.01.*

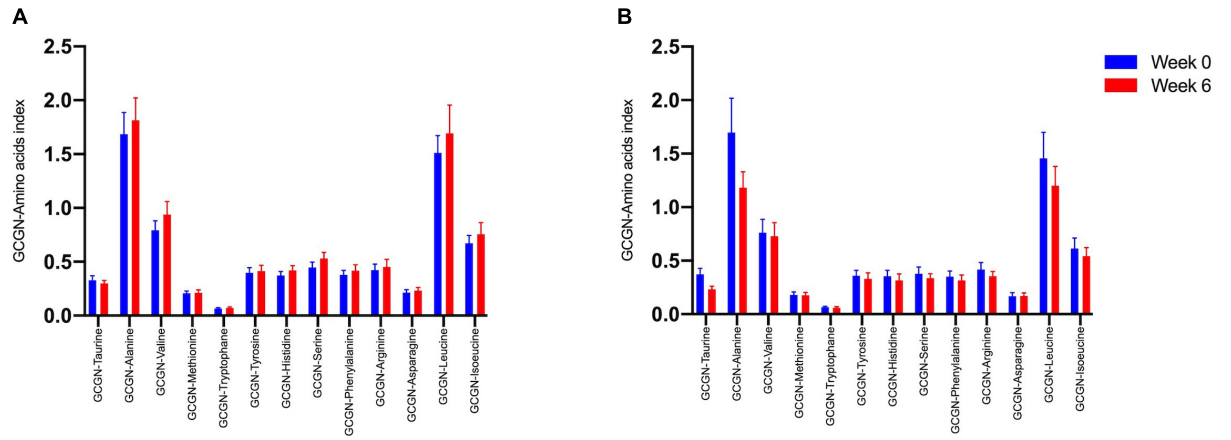

FIGURE 3 | Glucagon–alanine index between **(A)** lower IHL reduction groups; **(B)** higher IHL reduction group before (Week 0, blue) and after the intervention (Week 6, red).

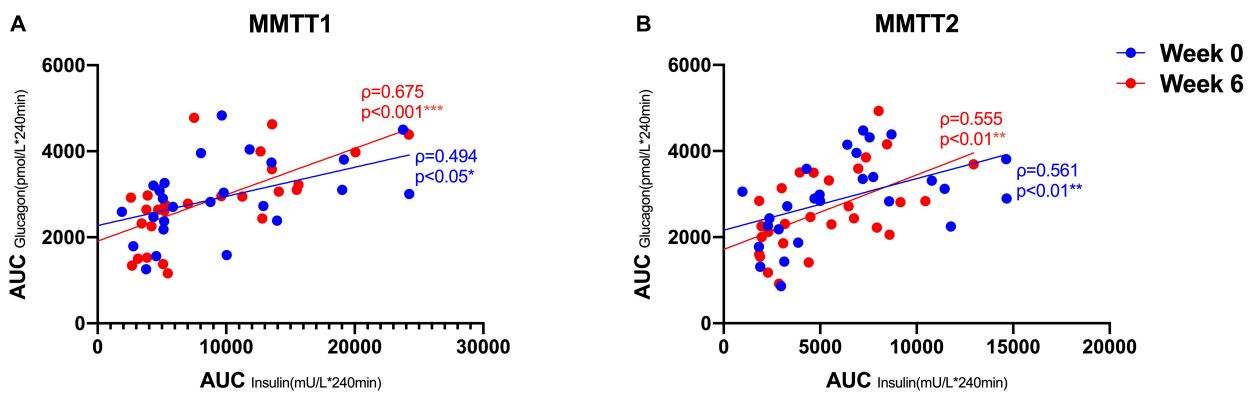

FIGURE 4 | Correlations between AUC (*240 min) insulin and AUC (*240 min) GCGN in MMTT 1 **(A)** and MMTT 2 **(B)** before (Week 0, blue) and after the intervention (Week 6, red). *p < 0.05; **p < 0.01; ***p < 0.001.

correlation increased markedly after the intervention ($\rho = 0.639$, $p < 0.001$) (**Figure 1F**).

We therefore tested whether the AA and GCGN responses to intake of 30 g protein/mixed meal were related to the liver fat content by analyzing identical successive breakfast (MMTT1) and lunch (MMTT2) before and after the intervention. The reduction of IHL resulted in reduced overall responses of insulin and GCGN in the MMTTs (**Table 5** and **Figure 4**). This was accompanied by significantly and selectively reduced increases of alanine but not of other AAs (**Figure 5**). We then performed the same calculations for the groups above and below the median with greater and lesser liver fat reduction. Indeed, the reductions of insulin-, GCGN-AUC in the meal tests were only observed in the greater liver fat reduction group while alanine-AUC was reduced in both groups (**Table 2** and **Figures 6A,B**).

The insulin/GCGN ratios were, moreover, significantly higher in participants with a fasting and postprandial GCGN–alanine index below compared to above the median at baseline indicating relatively less GCGN release (**Table 3**). Remarkably, the insulin/GCGN ratio decreased markedly from 1.42 ± 0.84 to 0.89 ± 0.42 at 0 min and also over the meal test in the lower

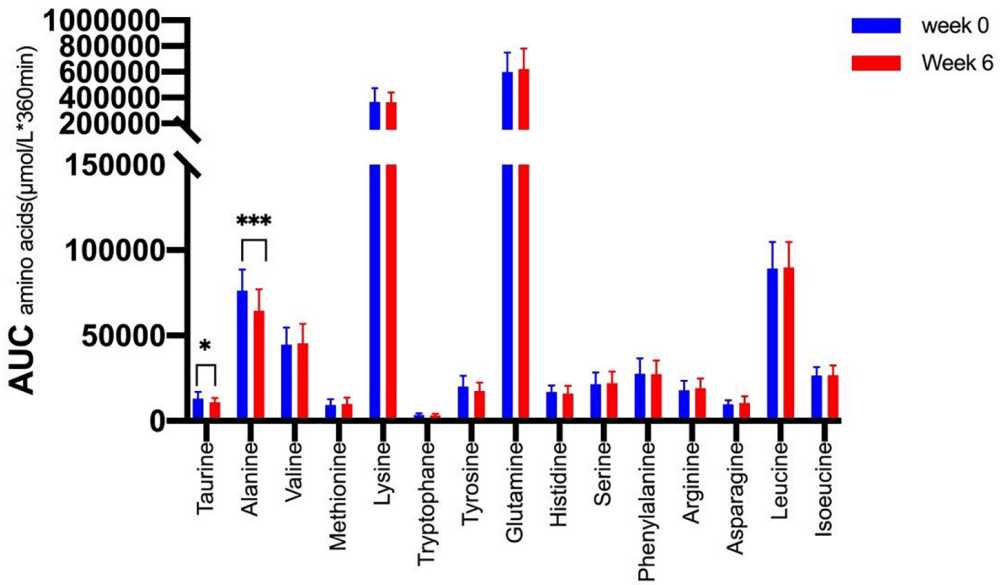

FIGURE 5 | Area under the curve (*360 min) of amino acids in the MMTTs before (Week 0, blue) and after the intervention (Week 6, red). *$p < 0.05$; ***$p < 0.001$.

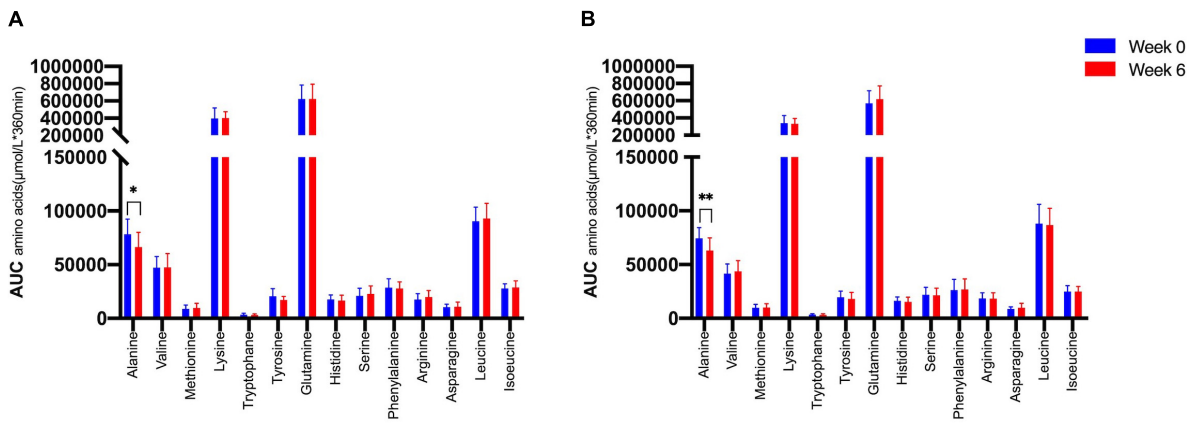

FIGURE 6 | Area under the curve (*360 min) of amino acids in the MMTTs between **(A)** lower IHL reduction group; **(B)** higher IHL reduction group before (Week 0, blue) and after the intervention (Week 6, red). *$p < 0.05$; **$p < 0.01$.

GCGN–alanine index group (with lower liver fat) indicating a greater relative secretion of GCGN. By contrast, the higher GCGN–alanine index group showed no significant change.

The ratios did not differ significantly between the higher and lesser IHL-reduction group before or after the intervention although the insulin/GCGN-ratio also decreased numerically in the greater liver fat reduction group (**Supplementary Table 4**).

DISCUSSION

The role of GCGN in the dysregulation of glucose and lipid metabolism is debated at present (5, 10, 15). GCGN is consistently elevated in people with fatty liver and T2DM and contributes to hyperglycemia as shown with GCGN antagonists (27, 28). However, GCGN antagonists increased dyslipidemia, IHL and liver enzymes (13). Glucagon selectively induces hepatic lipolysis and enhances insulin secretion within pancreatic islets (17, 24).

Our findings confirm (A) a positive correlation of fasting GCGN with hepatic fat content and insulin sensitivity in subjects with T2DM, obesity, and fatty liver. We report (B) that extensive, but not moderate, reductions of IHL after 6 weeks HPD induce the expected improvement in insulin sensitivity but do not alter fasting levels of GCGN. However, (C) postprandial stimulation of GCGN is reduced in parallel to reductions of insulin due to the better insulin sensitivity. However, the insulin/GCGN ratio in MMTTs decreased in participants with a greater reduction of liver fat and extensive metabolic improvements. Thus, the fasting and postprandial levels of GCGN relative to insulin increased indicating that higher GCGN responses were associated with

metabolic improvements. Moreover, (D) a selective reduction of the postprandial response of alanine was observed which may indicate enhanced hepatic GCGN sensitivity which strongly regulates alanine metabolism (see below). Therefore, increasing GCGN by HPD indeed allows metabolic improvements which suggests that the beneficial actions of GCGN outweigh the negative role in glucose production and parallel the positive results of GCGN co-agonists.

Correlation of Glucagon or the Glucagon–Alanine Index With Intrahepatic Lipid and Insulin Sensitivity

Glucagon is potently stimulated by increases of AA and then regulates not only the hepatic degradation of AA in the urea cycle but also increases hepatic glucose production and stimulates insulin secretion (5). This increase of glucose production is physiologically compensated for by increased insulin release and glucose disposal in healthy subjects (29) which remains functional in people with T2DM despite of an impaired insulin response to glucose (21).

The correlation of IHL with the GCGN–alanine index was borderline significant at baseline and GCGN alone showed a higher correlation with IHL than the GCGN–alanine index which thus reflects variable alanine levels. The correlation of the GCGN–alanine index with insulin sensitivity (HOMA-IR or *M*-value) was non-significant at baseline while a significant correlation with GCGN was observed. The GCGN–alanine index did not improve the association of GCGN with insulin sensitivity or fatty liver as might be expected if the dysregulation of fasting alanine plays a primary role. This was also true for all other GCGN–AA indices. As the GCGN–alanine index multiplies Ala (or other AA) with GCGN one would expect a higher correlation of the index than of GCGN alone if alanine contributes to the increased GCGN levels.

Remarkably, after the HPD intervention, the correlation of GCGN–alanine index with IHL and HOMA-IR became highly significant. This also applied to other GCGN–AA indices. We interpret this to reflect a greater impact of AA in the regulation of IHL and insulin sensitivity in combination with GCGN due to the increased protein intake. High protein intake results in the oxidation of AAs in muscle which employs the alanine–glucose (or Cahill) cycle to shuttle the amino groups to the liver for detoxification in the urea cycle (30). GCGN was shown to directly regulate both ALT enzymes (GPT and GPT-2) at the transcriptional level (31) as the first step of AA detoxification in the hepatic urea cycle. This may reflect the primary role of GCGN in the reduction of IHL due to GCGN induced hepatic lipolysis through the INSP3R1 mediated pathway which was recently described and provides an important explanation for the effect of HPD (24). The urea cycle was moreover shown to activate AMPK due to the consumption of ATP by arginino-succinate synthase which results in AMPK-induced inhibition of hepatic acetyl-CoA carboxylase and thus of lipogenesis (32). The high correlations of GCGN with saturated FFA and the *de novo* lipogenesis index support the role of GCGN in the regulation of lipid metabolism which became apparent in human studies with GCGN antagonist-induced dyslipidemia and fatty liver. Obviously, this also applies to HPD-induced increases of GCGN. The reduction of the metabolically toxic saturated FFA, in particular palmitic acid (C16:0) by HPD likely involves a regulation of *de novo* lipogenesis by GCGN, possibly due to inhibition of ACC1 by increased AMPK activity in the liver.

Dissociation of Improvements of Fasting Insulin- and Glucagon-Sensitivity in Response to Reduced Intrahepatic Lipid

The associations of GCGN with increased fasting AA, insulin resistance and fatty liver appear to support its negative role in the obesity and diabetes-associated metabolic dysregulation. A stimulation of GCGN by high protein intake should therefore further deteriorate metabolism (10). The alternative view interprets the increase of GCGN as a defensive response in an attempt to reset metabolism (5). Indeed, the HPD induced marked improvements of metabolism (19, 20, 22). However, an extensive reduction of IHL by 42% upon consumption of HPD for 6 weeks did not alter fasting GCGN, AA-levels, or the GCGN–alanine index, indicating that IHL is not directly related to fasting GCGN or AA levels in people with T2DM. By contrast, the reduction of liver fat resulted in a significant improvement of insulin sensitivity as shown by either HOMA-IR, Matsuda index, or *M*-value. Moreover, other markers of metabolism improved such as uric acid, CRP, and blood lipids (19, 20). Therefore, the HPD induced *meal related* increase most likely explains the metabolic improvements while fasting GCGN may be of minor importance.

The decrease in liver fat with HPDs was remarkably variable which might be related to hepatic GCGN resistance, because GCGN most likely drives the liver fat reduction by specifically enhancing hepatic lipolysis and inhibiting lipogenesis (24, 32). We therefore compared subjects above the median and below the median of liver fat reduction. The upper 50th percentile lost 27% of IHL which resulted in 12.7% IHL after the intervention while the lower 50th percentile lost 65% of liver fat which led to 4.6% IHL on average which is below the threshold definition of fatty liver. Although all indices of insulin resistance improved significantly only in the greater IHL-reduction group, there was no significant difference in fasting GCGN, GCGN–alanine index, or other fasting GCGN–AA indices. There were also no significant changes in the fasting levels of AA. This shows that changes of insulin sensitivity and GCGN sensitivity as calculated by the GCGN–alanine index in response to metabolic improvements can be dissociated in T2DM mellitus. Therefore, the alpha-cell response in the fasting state appears to be less responsive to reductions of liver fat than other metabolic parameters.

Improvements of Meal-Related Insulin and Glucagon Responses

Meal related responses of GCGN are thought to be exaggerated in T2DM although this has received little attention with regards to responses to protein intake previously. We assessed whether the reduction of IHL would alter the GCGN response to protein

intake. The same protein rich MMTTs were performed before and after the intervention such that each individual could serve as its own control. This showed a reduction of insulin and GCGN responses in the MMTTs in the presence of greater reduction of liver fat. Moreover, the levels of GCGN relative to insulin increased supporting a contribution of GCGN to the metabolic improvements.

Altered Alanine Responses May Reflect Changes of the Glucose-Alanine Cycle

Remarkably, the plasma levels of alanine in the meal challenge tests were selectively reduced after 6 weeks of HPD, accompanied by a pronounced reduction of IHL, while the other AA and total AA did not change. The glucose-alanine cycle is well known to play a key role in glucose and AA metabolism (33). Alanine is generated by transamination from other AA used as energy substrates in muscle and transports amino groups to the liver which detoxifies the ammonium groups by delivery to the urea cycle. GCGN was shown to preferentially increase hepatic alanine uptake several-fold as compared to other AA (33). Alanine was recently shown to directly regulate mitochondrial oxidative metabolism in fasted humans (34). Mouse studies identified alanine as an intracellular activator of AMPK in hepatocytes which was dependent on ALT1 and the extraction of intermediate metabolites of the TCA-cycle (35). Alanine supplementation resulted in improved glucose metabolism of lean or obese mice. The alanine metabolic pathway was shown to be reversibly dysregulated in obese mice and humans and associated with impairments of ureagenesis (36, 37). We interpret the selective reduction of alanine in the MMTTs therefore as an indication of more effective use of alanine and improved mitochondrial oxidative function which may partially explain the improvements of glucose metabolism. As GCGN primarily regulates hepatic alanine uptake and metabolism, the reduced levels may indicate an improved prandial hepatic GCGN sensitivity. Notably, insulin resistance of protein metabolism was shown to be more pronounced in the fasting state while postprandial responses were close to normal (38). In analogy, postprandial GCGN actions may adapt preferentially to metabolic improvements.

A remarkable observation was that levels of urea were higher in subjects with greater liver fat reduction and showed a greater increase during the HPD intervention. This may indicate that there was a higher efficiency of GCGN to induce AA degradation and ureagenesis which may support the loss of IHL (32) as discussed above. In addition, GCGN was shown to specifically induce hepatic secretion of cAMP into the bloodstream to regulate kidney function which is a further energy-expensive signaling pathway (39). The improvements of IHL and insulin sensitivity in the entire cohort indicate a sufficiently preserved capacity of the liver to handle AA metabolism and to profit from its consequences in response to HPD. However, there appear to be subgroup-specific differences in the capacity to respond to HPD which are not well understood at present.

An important concern regarding high protein intake is a potential impairment of renal function due to the increased delivery of urea (40). GCGN was shown to participate in the adaptation of the kidney to increased protein intake (41). However, there is no conclusive evidence that limitation of protein intake prevents the progression of renal failure in T2D in randomized prospective studies (42, 43). Nevertheless, high protein intake should be avoided in patients with renal impairment until better evidence is available.

Limitations of the study apply to the relatively small number of patients who displayed a well-controlled non-insulin requiring diabetes and were characterized in considerable detail. The study used plant or animal protein supplements which differed in AA composition and there was a gender dysbalance in the groups above or below the median of liver fat reduction. The patients were Caucasian and of moderately advanced age. We did not study direct responses to exogenous administration of GCGN which may allow more sensitive assessment of GCGN responses. However, the high protein MMTTs reflect the real-life situation.

CONCLUSION

Although fasting levels of GCGN are positively correlated with insulin resistance and IHL, increasing prandial GCGN secretion by HPD improves IHL, insulin sensitivity, fasting glucose, and circulating free saturated fatty acids. This associates with a selective reduction of alanine in meal challenge tests which is known to be primarily regulated by GCGN. Alanine links GCGN-stimulated glucose and AA-metabolism and might play a key role in augmenting insulin sensitivity and in inhibition of lipogenesis through AMPK-dependent pathways. Moreover, the metabolic improvements are associated with a reduction of meal stimulated insulin and GCGN secretion but a greater GCGN relative to insulin secretion. Together these findings suggest a primary role of prandial GCGN in the HPD-induced metabolic improvements which appears to be associated with an increased GCGN sensitivity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the University of Potsdam. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MM, OP-R, and SH conducted the experiments by dietary consultation and collected the data. SK, MM, OP-R, and SH analyzed and interpreted the experimental data. SR analyzed

the samples, designed the study, and interpreted the data. JZ performed the statistical analysis, designed the figures and tables, and wrote the manuscript. AP designed the study and wrote the manuscript. All authors read and revised the manuscript, contributed to discussion, and approved the final version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.808346/full#supplementary-material

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