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**Title:** Dose-response of myofibrillar protein synthesis to ingested whey protein during energy restriction in overweight postmenopausal women: a randomized, controlled trial

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Running head: Protein during weight loss in postmenopausal women

Abbreviations: 1-RM, one-repetition maximum; ANOVA, analysis of variance; BM, Body mass; BMI, body mass index; EBW, energy balance whey; ERW, energy restriction whey; EX, exercise; FSH, follicle-stimulating hormone; FSR, fractional synthetic rate; iAUC, incremental area under the curve; LBM, lean body mass, MPS, muscle protein synthesis; RDA, recommended dietary allowance; TG, triglycerides

Trial registry: clinicaltrials.gov (ID: NCT03326284)

Data described in the manuscript will be made available upon request pending
Abstract

Background: Diet-induced weight loss is associated with a decline in lean body mass, as mediated by an impaired response of muscle protein synthesis (MPS). The dose-response of MPS to ingested protein, with or without resistance exercise, is well characterised during energy balance but limited data exist under conditions of energy restriction in clinical populations.

Objective: To determine the dose-response of MPS to ingested whey protein following short-term diet-induced energy restriction in overweight, postmenopausal, women at rest and post-exercise.

Design: Forty middle-aged (58.6±0.4 years), overweight (BMI: 28.6±0.4), postmenopausal women were randomised to one of four groups: Three groups underwent 5 days of energy restriction (~800 kcal/d). On day 6, participants performed a unilateral leg resistance exercise bout before ingesting either a bolus of 15g (ERW15, n=10), 35g (ERW35, n=10) or 60g (ERW60, n=10) of whey protein. The fourth group (n=10) ingested a 35g whey protein bolus after 5 days of an energy balanced diet (EBW35, n=10). Myofibrillar fractional synthetic rate (FSR) was calculated under basal, fed (FED) and post-exercise (FED-EX) conditions by combining an L-[ring-13C6]phenylalanine tracer infusion with the collection of bilateral muscle biopsies.

Results: Myofibrillar-FSR was greater in ERW35 (0.043±0.003%/h, P=0.013) and ERW60 (0.042±0.003%/h, P=0.026) than ERW15 (0.032±0.003%/h), with no differences between ERW35 and ERW60 (P=1.000). Myofibrillar-FSR was greater in FED (0.044±0.003%/h, P<0.001) and FED-EX (0.048±0.003%/h, P<0.001) than BASAL (0.027±0.003%/h), but no differences were detected between FED and FED-EX (P=0.732) conditions. No differences in myofibrillar FSR were observed between EBW35 (0.042±0.003%/h) and ERW35 (0.043±0.003%/h, P=0.744).
**Conclusion:** A 35 g dose of whey protein, ingested with or without resistance exercise, is sufficient to stimulate a maximal acute response of MPS following short-term energy restriction in overweight, postmenopausal women, and thus may provide a per serving protein recommendation to mitigate muscle loss during a weight loss program. Trail registration: clinicaltrial.gov (NCT03326284).

**Key words:** Females, middle-aged, obesity, weight loss, muscle protein synthesis

1.0 **Introduction**

The worldwide prevalence of overweight and obese middle-aged (40-65 years) adults represents an increasingly important public health challenge within the discipline of human and clinical nutrition (1, 2). Accordingly, considerable attention has focused on optimising weight loss interventions that target this population demographic (3, 4). Specifically, the efficacy of complex weight loss interventions that combine non-pharmacological nutritional and exercise strategies have focussed on dietary protein manipulation with (5) or without (6-8) the inclusion of a structured resistance-based exercise training program to mitigate the counter-productive loss of lean body mass (LBM).

The efficacy of a diet-induced weight loss intervention depends, at least in part, on the retention of LBM during a period of energy deficit (9, 10). This notion is supported by clinical studies that report a clear association between muscle mass index, defined as the skeletal muscle mass:fat mass ratio, and metabolic disease risk, functional decline, and mortality (11, 12). The preponderance of evidence suggests that muscle atrophy during energy restriction is mediated by suppressed postabsorptive and postprandial rates of muscle protein synthesis (MPS) (13-16), although an upregulation in muscle protein breakdown during energy restriction also has been reported (17). Moreover, whereas similar basal rates of MPS have been observed between
obese and lean individuals (18), studies have reported a reduced postprandial response of MPS to protein ingestion in overweight/obese individuals vs. age-matched lean controls (19, 20). In addition, clinical studies have demonstrated an impaired muscle anabolic response to protein feeding and exercise training in postmenopausal women compared to older men and healthy young adults (21-25). Hence, these data provide compelling rationale for developing targeted dietary interventions aimed at mitigating muscle loss during diet-induced energy restriction specifically in postmenopausal women.

Accumulating evidence suggests that increasing the protein content of an energy-restricted diet represents an effective dietary intervention to mitigate muscle atrophy, and promote fat mass loss, during diet-induced weight loss in overweight and obese individuals (26, 27). Accordingly, a general consensus exists that the optimal daily protein intake to maintain muscle mass during weight loss is ~50% greater than the current recommended dietary allowance (RDA), ranging from 1.2-1.6 g protein/kg BM/d (26, 28). Nevertheless, acute metabolic studies that measure the response of MPS to protein feeding under conditions of energy restriction are warranted in overweight/obese individuals to refine this protein recommendation on a per-serving basis (29). Whereas the dose response of MPS to ingested protein has been characterized in young (30-33), middle-aged (34) and older (35, 36) men in energy balance, comparable studies have not been conducted in middle-aged women. Based on the apparent sexual dimorphism in response of MPS to protein feeding post menopause (23), intuitively the optimal protein dose for maximal stimulation of MPS in middle-aged and older adult men may not directly translate to age-matched postmenopausal women under conditions of energy restriction.

The specific objective of this proof-of-principle study was to examine the dose-response of MPS to ingested protein at rest (primary outcome) and during the acute (3 h) recovery period
following resistance exercise in a cohort of middle-aged, overweight, postmenopausal women following 5 days of diet-induced energy restriction. The whey protein doses (15 g, 35 g, 60 g) were selected to characterise a complete dose-response curve. In addition, to determine the influence of energy restriction on the MPS response to protein ingestion, we compared rates of MPS in postmenopausal women following ingestion of 35 g of whey protein during conditions of energy restriction and energy balance. Our primary hypothesis was that protein feeding would augment rates of MPS above basal fasting values in a dose-dependent manner (i.e., 15 g < 35 g < 60 g) following short-term energy restriction (primary outcome). Secondly, we hypothesized that rates of MPS would be augmented with resistance exercise compared to rest, regardless of protein dose. Finally, we hypothesized the MPS response to ingestion of 35 g whey protein would be attenuated following a period of energy restriction vs. energy balance in middle-aged, postmenopausal, women.

2.0 Methods

2.1 Subjects and ethical approval

Forty (n = 40) healthy, middle-aged (58.6 ± 0.4 y) women were recruited for this study (Table 1). Written informed consent was provided by all participants that were deemed healthy based on a screening interview and routine blood sample analyses. Volunteers were eligible to participate if they were aged 50-65 years, postmenopausal (defined as no menstrual bleeding for 6 months, follicle-stimulating hormone (FSH) concentration > 30 IU/L, oestrogen concentration < 50 pmol/L), non-smokers and recorded a body mass index (BMI) > 25. The study was conducted at the Department of Public Health, Aarhus University, Aarhus, Denmark between July 2017 and March 2018. The trial was registered at clinicaltrials.gov (ID:
NCT03326284) and conducted in accordance with standards of the local ethics committee of Central Denmark Region (1-10-72-56-17) and the Declaration of Helsinki.

2.2 Study design

A randomized, single blinded, parallel study design was conducted to determine the dose-response of myofibrillar fractional synthesis rate (FSR) to ingested whey protein at rest (FED) and post-exercise (FED-EX) following a 5-day period of energy restriction in middle-aged, overweight postmenopausal women. The response of myofibrillar FSR to a moderate dose (35 g) of ingested whey protein was also measured following a controlled 5-day period in energy balance to determine the influence of energy status on MPS rates. In total, 40 women were randomly assigned to one of four groups (Figure 1). Three groups underwent a 5-day energy restricted dietary intervention (ER, ~800 kcal/d; n = 30) and one group continued their habitual energy balanced diet (EB, ~1785 kcal/d, n = 10) prior to conducting an acute metabolic trial for measurement of myofibrillar FSR. Metabolic trials (Figure 2) were identical in design except for administering 15 g (ERW15; n=10), 35 g (ERW35; n=10 and EBW35; n=10) or 60 g (ERW60; n=10) of whey protein. Participants remained blinded to their assigned protein dose for the study duration. All trials included an acute bout of unilateral knee extension resistance exercise. Due to participant discomfort with the muscle biopsy procedure, we were unsuccessful in obtaining sufficient tissue from five participants and thus the measurement of plasma L-[ring-$^{13}$C$_6$]-phenylalanine enrichment and calculation of myofibrillar FSR are expressed as n = 10 (ERW15), n = 8 (ERW35), n=8 (ERW60) and n = 9 (EBW35), as displayed in Figure 1).

2.3 Screening visit

Eligible participants attended the laboratory after an overnight fast >1 wk prior to conducting the experimental trial. A blood sample was analysed for routine biomarkers of general
metabolic health and sex hormone concentrations. Women with concentrations of oestrogen <50 pmol/L, FSH < 30 IU/L, HbA1c > 7.3 mmol/mol, alanine transaminase > 45 U/L, and/or thyroid-stimulation hormone > 4.5×10⁻³ IU/L were excluded from participation. Body composition was determined using dual-energy x-ray absorptiometry (DXA; GE Lunar DXA scan, GE Healthcare, WI, USA) and a maximum strength test was conducted. At the screening visit the project coordinator performed a simple randomization procedure (participants drew lots from an opaque envelope) to allocate participants to one of the four treatments. The participants were blinded to the protein dose allocation.

2.4 Maximum strength testing

One-repetition maximum (1RM) for leg extension (Technogym-Selection line, Technogym, Italy) was estimated in accordance with the procedure described by (37). The test was conducted after a self-administered 10 min warm-up on an ergometer bike. Leg assigned to exercise was randomly selected, i.e., independent of dominance.

2.5 Diet and physical activity control

Participants commenced their assigned diets five days before the experimental visit. Energy-restricted groups (ERW15, ERW35 and ERW65) were provided with soups, shakes and meal replacement bars (Nutrilett, Orkla Health AS, Oslo, Norway) for consumption, and advised to consume 200 g of low-calorie water dense vegetables (i.e., cucumber, tomatoes, and lettuce) and > 2 L of water daily. Participants assigned to the energy balance group (EBW35) were instructed to replicate their habitual diet and register all food consumption using a diet registration mobile phone app (MADLOG mini, MADLOG Aps, Kolding, DK). Energy allowances in the energy balance group were set to provide sufficient energy to maintain energy balance as determined by using the Harris Benedict equation for estimation of basal metabolic rate, which was multiplied by a factor (1.4–1.5) corresponding to a moderate
The approximate energy requirements were as follows: 2057 ± 51 kcal/d (ERW15); 2024 ± 29 kcal/d (ERW35); 2098 ± 43 kcal/d (ERW60); 2026 ± 39 kcal/d (EBW35). Thus, the energy restricted diet would induce an estimated energy deficit of ~1200 kcal/d. Physical activity level during the experimental period was standardized by instructing participants to target a daily step count of 6,000–10,000 steps as quantified by a Yamax pedometer (Yamax PZ270 Power Walker Lite, Yamasa Tokei Keike Co., Ltd, Japan). Non-caloric drinks (e.g., black coffee and tea) were permitted ad libitum until 24-h prior to commencing the experimental day, whereas alcohol or caffeinated drinks were prohibited within 24-h of the experimental day. The participants were permitted only to drink water after 8:00 p.m. the evening before the experimental day.

2.6 Infusion protocol

Participants reported to the laboratory at 7:30 a.m. after an overnight fast. Body weight was measured and two catheters were inserted into an antecubital vein and a dorsal hand vein of the contralateral arm. A baseline blood sample was collected for determination of background phenylalanine enrichment before a primed (6.0 µmol/kg LBM), continuous (6.0 µmol/kg LBM/h) infusion of L-[ring-13C₆]-phenylalanine (Cambridge Isotopes, Andover, MA, USA) was initiated. The cannulated hand was heated for arterialized blood sampling throughout the infusion protocol. At 90 min after starting the infusion, a muscle biopsy was obtained from the leg assigned to resistance exercise (FED-EX). Next, participants rested supine before performing a single bout (5 sets × 10 repetitions) of unilateral leg extension at 80% 1RM with 2 min rest between sets. If a participant could not complete a full set, the load was lowered by 5 – 10%. A muscle biopsy was then obtained from the contralateral resting leg (FED). Immediately after the muscle biopsy, participants ingested their assigned whey protein bolus
and then rested in a supine position for 3 h before two further muscle biopsies were obtained from the exercised (FED-EX) and non-exercised (FED) leg.

2.7 Protein beverages

Whey protein beverages (Lacprodan® HYDRO.REBUILD, Arla Foods Ingredients Group P/S, Viby J, DK) were administered immediately after collection of the second muscle biopsy obtained after exercise (Table 2). Beverage flavour was chocolate or mint based on personal preference. The volume of all beverages was 300 ml. To minimize perturbations in plasma isotopic enrichment, beverages were enriched with L-[ring-\(^{13}\)C]-phenylalanine. Based on previous observations of transient elevations in plasma \(^{13}\)C\(_6\) phenylalanine enrichments following bolus ingestion of 40 g of whey protein (31), we adjusted the beverage enrichment of L-[ring-\(^{13}\)C\(_6\)]-phenylalanine as follows depending on the whey protein dose: 15 g protein dose: 10%; the 35 g dose: 8.5% and the 60 g dose: 6.25%.

2.8 Muscle biopsy and blood sampling

All blood samples were dispensed into pre-chilled coated (EDTA or lithium heparin) blood collection tubes. Serum-separator tubes were allowed to clot for 30 min before centrifugation (1,500 g for 15 min at 5°C). As described above, a total of four muscle biopsies (two from each leg; ~250 mg) were obtained from the vastus lateralis (~12–15 cm proximal to patella) under local anaesthesia (10 ml Xylocain® 10mg/ml, AstraZeneca, Sweden) using a 5 mm Bergström needle with manual suction. Muscle samples were snap frozen and stored at –80°C until further analysis.

2.9 Analytical procedures

2.9.1 Blood metabolite concentrations

Plasma amino acid concentrations and serum insulin concentrations were determined as described by Bornø and van Hall (39) and Christensen, et al. (40), respectively. Blood glucose
concentration was quantified using a HemoCue Glucose 201 RT Analyzer (HemoCue® AB, Ängelholm, Sweden) and plasma urea concentration was determined using absorption photometry (Cobas 6000, Roche, Basel, CH and Chemistry XPT System, Siemens Healthcare A/S, Ballerup, DK).

2.9.2 Stable isotope analysis

Plasma phenylalanine enrichments were determined as described previously (41). To isolate intramuscular free amino acids and myofibrillar proteins, muscle samples (25-35 mg wet weight) were homogenized by ceramic beads (lysing matrix D; FastPrep®-24 homogenizer, MP Biomedicals, Santa Ana, CA) in 1 mL of prechilled homogenization buffer (Tris 0.02 M [pH, 7.4]; NaCl 0.15 M; EDTA 2 mM, EGTA 2 mM, one protease inhibitor tablet per 10 mL buffer) and then centrifuged at 10,000 g for 15 min at 4°C. This process was repeated with the remaining pellet without the protease inhibitor tablet solubilized in the buffer. The two supernatants (~2 mL) were transferred to vials with 2 mL ice cold 100% acidic acid. The free amino acids were subsequently purified over columns with acidified cation exchange resin as described previously (42). Next, 1 mL NaOH (0.3 M) was added to the pellet from the homogenization process containing structural proteins, homogenized for 30 s and left in a heating block (50°C) for 2 × 30 min (vortexed in between) and centrifuged (10,000 g, 10 min, 4°C). Supernatants were transferred to vials suitable for hydrolysis. This process was repeated with the remaining pellet and supernatants merged. Perchloric acid (1 mL 2 M) was added to the supernatants containing myofibrillar proteins. Vials were vortexed and left on ice for 20 min. After centrifugation (3,000 g, 10 min, 4°C), supernatants were discarded and the pellets washed twice in EtOH (1 mL 70%), vortexed and centrifuged (3,000 g, 10 min, 4°C). The remaining pellets were vortexed in a mix of 2 mL HCl and 1 mL Dowex resin (Bio-Rad Laboratories, Hercules, CA), before overnight incubation (110°C). Subsequently, the
myofibrillar amino acids were purified over cation exchange resin columns using NaOH (2M) for elution. Amino acids were derivatized with N-acetyl-propyl as described previously (42). Finally, the derivatized samples were injected into a gas-chromatography combustion isotope ratio mass spectrometer (ThermoFisher Scientific, Hemel Hempstead, UK). For practical reasons, the muscle samples were analyzed at University of Birmingham and University of Nottingham. The analyses used the same protocols for sample preparation. Data was inspected visually and statistically to identify any effect of analysis-site. No effect of site was detected ($P > 0.05$).

### 2.10 Calculation of myofibrillar MPS

Myofibrillar FSR was calculated using the standard precursor equation:

$$FSR\ (\% \times h^{-1}) = \frac{\Delta E_{\text{protein}}}{E_{\text{precursor}}} \times 1/\Delta t \times 100$$

Where $\Delta E_{\text{protein}}$ is the difference in tracer enrichment in the myofibrillar protein fraction between two biopsy samples, $E_{\text{precursor}}$ is the arterialized blood precursor defined as the area under the curve (AUC) for plasma enrichments of labelled phenylalanine over the 3-h incorporation periods. $\Delta t$ is the time interval between muscle biopsies.

### 2.11 Data presentation and statistics

A sample size of 32 (8 participants/group) was calculated a priori based on previous data from comparable studies with similar participant characteristics investigating the dose-response of myofibrillar FSR to ingested protein in older men (34, 35). This calculation was based on the assumption that the minimal detectable difference in FSR between protein dosages would be 0.01 %/h when the SD of the means was set to be 0.007 %/h. The 1-$\beta$ error of probability was set at 0.8 and an $\alpha$-level of < 0.05.
Statistical analysis of myofibrillar FSR data (primary endpoint) was conducted using a repeated measures mixed effects model with *protein dose* (ERW15, ERW35, ERW60) and *condition* (BASAL, FED, FED-EX) as independent variables in the fixed part of the model. Participants were included in the random part of the model. Data were analysed for main effects and any interaction between the two independent variables. Bonferroni *post hoc* tests were applied if statistical significance of interactions or main effects were reached. *Post hoc* analyses of main effects were performed independently of the other independent variable. To determine the influence of energy status on myofibrillar FSR, a similar mixed effects model was used with *energy status* (EBW35, ERW35) and *condition* (BASAL, FED, FED-EX) as independent variables in the fixed part of the model and participants in the random part. Other endpoints (insulin, urea, glucose, amino acid concentrations and phenylalanine enrichments) were analysed using a similar mixed model with *protein dose* and *time* as fixed effects, and participants as a random effect. Main effects (*protein dose*, *time*) and interactions, as well as post hoc analyses, were performed as described above. One-way analyses of variance (ANOVA) was used for data presented as incremental area under the curve (iAUC). *iAUC* was calculated with the baseline set as timepoint 0. Normality and homogeneity of data were checked by inspecting QQ-plots and plots of residuals versus the fitted values. Serum insulin concentrations were deemed heteroskedastic from visual inspection and consequently log-transformed before statistical analyses. Data are presented as means ± SEM unless otherwise stated. All statistical analyses were performed using STATA version 14.2 (StataCorp LP, Collage Station, TX, USA) and significance was set at an *α*-level of < 0.05.
3.0 Results

3.1 Diet, exercise and body weight

Total energy and macronutrient intakes were lower in the energy-restricted diet groups than the energy balance diet group (all \( P < 0.05 \), Table 3). Average daily step count was comparable between groups (ERW15: 7502 ± 454 steps; ERW35: 8953 ± 620 steps; ERW60: 7722 ± 470 steps; EBW35: 7718 ± 573 steps; \( P > 0.05 \)). A decline in body weight was observed in all ERW groups during the 5-day energy restriction period (ERW15: \(-2.4 ± 0.2 \) kg; ERW35: \(-1.8 ± 0.2 \) kg; ERW60: \(-2.8 ± 0.3 \) kg; all \( P < 0.001 \), with no change in EBW35 (\(-0.2 ± 0.2 \) kg, \( P = 0.32 \)). Weight loss was greater in ERW60 than ERW35 (\( P = 0.03 \)). The total weight lifted throughout the exercise protocol was similar between groups (mean ± SD; ERW15: 655 ± 247 kg; ERW35: 679 ± 137 kg; ERW60: 771 ± 224 kg; EBW35: 776 ± 198 kg; ERW15 vs ERW35 vs ERW60, \( P = 0.435 \); ERW35 vs EBW35, \( P= 0.221 \)).

3.2 Amino acid concentrations

Plasma phenylalanine concentration peaked at 60 min post protein ingestion for all groups, with the magnitude of increase greater in ERW35 (105 ± 3 \( \mu \)mol/L) and ERW60 (107 ± 4 \( \mu \)mol/L) than ERW15 (83 ± 3 \( \mu \)mol/L, both \( P < 0.001 \)). Phenylalanine concentration returned to baseline at 3 h post protein ingestion in ERW15 and ERW35 but remained elevated in ERW60 (90 ± 4 \( \mu \)mol/L; \( P < 0.001 \); Figure 3a). The \( iAUC \) of phenylalanine concentration increased in a dose-dependent manner (all \( P < 0.05 \); Figure 3b), with no differences between ERW35 and EBW35 (\( P = 0.99 \)).

Plasma leucine concentration peaked at 60 min post protein ingestion in ERW15 and ERW35 and 120 min post protein ingestion in ERW60 and remained elevated for the remainder of the experimental trial (\( P < 0.001 \); Figure 4a). The \( iAUC \) of leucine concentration increased
in a dose-dependent manner (all \( P < 0.001 \)) and was greater in ERW35 than EBW35 (\( P < 0.008 \), Figure 4b).

### 3.3 Plasma glucose, serum insulin and urea concentrations

A main effect of time was observed for glucose concentration after protein ingestion (\( P = 0.03 \); Supplementary Figure 1a), but post hoc analyses showed no difference from baseline at any time (\( P > 0.05 \)). No time × dose interaction (\( P = 0.39 \)) or differences in iAUC of plasma glucose concentration was observed between groups (\( P > 0.05 \), Supplementary Figure 1b).

Serum insulin concentrations peaked 30–60 min after protein ingestion (\( P < 0.01 \)) and returned to baseline levels at 3 h post protein ingestion in ERW15 and ERW35 (Figure 5a). The iAUC of serum insulin concentration was higher in ERW35 and ERW60 than ERW15 (\( P < 0.05 \)) and higher in ERW60 than in ERW35 (\( P = 0.033 \), Figure 5b). No differences in insulin concentration were observed between ERW35 and EBW35 (\( P = 0.756 \)).

The highest plasma urea concentrations were observed at 3 h post protein ingestion in all groups (time effect: \( P < 0.001 \), Supplementary Figure 2a) and were greater in EBW35 (7.0 ± 0.3 mmol/L) and EBW60 (8.2 ± 0.3 mmol/L) compared to EBW15 (5.2 ± 0.3 mmol/L). No differences in iAUC of plasma urea concentration (all \( P > 0.05 \); Supplementary Figure 2b).

### 3.4 Plasma phenylalanine enrichments

A steady state in plasma L-(ring-\(^{13}\)C\(_6\))phenylalanine was reached 30 min after initiating the infusion (Figure 6). Despite enriching all protein beverages with tracer, a modest decline in plasma L-(ring-\(^{13}\)C\(_6\))phenylalanine enrichment was observed in EBW35, ERW35 and ERW60 post protein ingestion.

### 3.5 Myofibrillar fractional synthetic rate

A main effect of protein dose was observed across all conditions (BASAL, FED and FED-EX)
combined ($P = 0.006$) (Figure 7). Post hoc analysis revealed a greater response of myofibrillar FSR in ERW35 (32%, $+0.010 \pm 0.003\%/h$, $P = 0.013$) and ERW60 (29%, $+0.009 \pm 0.003\%/h$, $P = 0.026$) than ERW15, with no differences between ERW35 and ERW60 ($P = 1.000$). A main effect of condition was observed for all groups combined ($P < 0.001$), with myofibrillar FSR 63% greater in FED ($+0.017 \pm 0.004\%/h$, $P < 0.001$) and 79% greater in FED-EX ($+0.021 \pm 0.004\%/h$, $P < 0.001$) than BASAL, but no differences were detected between the FED and FED-EX ($P = 0.732$) conditions. In addition, no protein dose × condition interaction was detected ($P = 0.744$) (Figure 7). Moreover, no main effects of diet (energy restriction vs. energy balance, $P = 0.744$) or diet × condition interaction ($P = 0.996$) were observed for myofibrillar FSR when EBW35 and ERW35 groups only were included in the statistical model. However, a main effect of condition ($P < 0.001$) was observed for this analysis as well (Figure 7).

4.0 Discussion

This clinical randomised controlled trial investigated the dose-response relationship between ingested whey protein and in vivo postprandial rates of MPS in middle-aged, overweight postmenopausal women under conditions of diet-induced weight loss. Utilizing a unilateral leg resistance exercise model, we measured the dose-response of myofibrillar FSR to ingested protein at rest (FED) and post-exercise (FED-EX) following 5 days of energy restriction. In addition, we examined the influence of energy status (i.e., energy balance vs. energy restriction) on basal and postprandial myofibrillar FSR in response to ingestion of a moderate (35 g) dose of whey protein. By design, a modest (~2 kg) decline in body weight was observed in all energy restricted groups, with body weight stable in the energy balance group. The primary study finding was a plateau in dose response of myofibrillar FSR to ingested protein at 35 g of whey protein, with no additional stimulation of MPS with the ingestion of 60 g of whey protein (ERW15 < ERW35 = ERW60) following 5 days of energy restriction in overweight,
postmenopausal women. A secondary finding was that resistance exercise failed to potentiate the acute response of myofibrillar FSR to increasing doses of ingested whey protein following energy restriction. Finally, the acute period of energy restriction did not modulate the postprandial response of myofibrillar FSR to ingestion of a moderate dose (35 g) of whey protein. Taken together, these data indicate that ingesting a 35 g dose of high-quality protein on a per meal/serving basis, with or without resistance exercise, is sufficient to stimulate a maximal postprandial response of MPS following an acute period of energy deficit in overweight, postmenopausal women. Thus, an appropriate practical recommendation for this important clinical sub-population is to ingest 35 g of high-quality protein per meal during a weight loss programme.

Current knowledge regarding the dose-response of MPS to ingested protein is primarily based on studies in healthy young and older adults in energy balance. A general consensus exists that the dietary protein induced stimulation of MPS is finite whereby, above a certain threshold protein dose, the fate of ingested protein-derived amino acids is primarily non-anabolic (i.e., oxidation) rather than incorporation into bound new muscle protein (43). For instance, previous studies observed a plateau in the dose-response of MPS to ingested protein at a 20 g dose in healthy young men under conditions of energy balance, with the 40 g protein dose conferring no additional stimulation of MPS (30, 31, 44). The opposing argument suggests the anabolic response to ingested protein is not limited by the maximal stimulation of protein synthesis (45). This viewpoint is evidenced by studies that conducted whole-body assessments of protein synthesis, i.e., aggregate protein synthesis rates across all body tissues combined, rather than tissue-specific (i.e., muscle) measurements of MPS (46, 47). In the present study, the maximal effective protein dose for stimulation of MPS was 35 g of whey protein in middle-aged, overweight, postmenopausal, women under conditions of short-term diet-induced energy...
restriction. While the postprandial response of MPS was markedly greater in ERW35 and ERW60 than ERW15, we observed no differences in myofibrillar FSR between ERW35 and ERW60 groups. These data corroborate the findings of Robinson, et al. (34) that reported an upper limit to the stimulation of MPS with the ingestion of 36 g of beef protein in middle-aged men in energy balance. Although we did not perform a direct comparison between men and women, our results suggest that energy restricted middle-aged, overweight, postmenopausal, women respond similarly to protein feeding as their male counterparts in energy balance. Hence, taken together these data suggest that following 5 d of energy restriction, 35 g of whey protein is sufficient for the maximal stimulation of MPS in middle-aged, overweight postmenopausal woman.

The interaction of exercise training and increased dietary protein intake during a period of energy deficit represents an evidence-based strategy to mitigate the impaired response of MPS, and potential subsequent decline in muscle mass, associated with diet-induced weight loss in overweight women (48, 49). Consistent with this notion, a longitudinal study by Layman, et al. (5) demonstrated that the addition of a resistance-based exercise training program (2 d/wk resistance training + 5 d/wk walking) to a high protein diet (1.6 g/kg BM/d) promoted the loss of fat mass and retention of lean body mass in middle-aged women that undertook a 4-month weight loss trial. In addition, the impairment in basal myofibrillar FSR following 5 days of energy restriction in resistance-trained young adults was restored following a single bout of resistance exercise to levels observed at rest in energy balance (15). These authors also reported that protein ingestion increased MPS in a dose-dependent manner above rates observed at rest during energy balance (15). However, in the present study, and refuting our original hypothesis, we report no additive effect of resistance exercise on the postprandial response of MPS. Whereas myofibrillar FSR was greater in FED and FED-EX than BASAL
across dose groups, no statistical difference in MPS was observed between FED and FED-EX conditions. In contrast, previous dose-response studies, conducted under conditions of energy balance and utilizing the same unilateral exercise model as the present study, have demonstrated greater MPS rates in the exercised vs. rested leg in healthy young (31), middle-aged (34) and older (35) adults. Hence, we may deduce that 5 days in energy deficit is sufficient to inhibit the exercise-induced stimulation of MPS in middle-aged, postmenopausal woman that are less responsive to resistance exercise as an anabolic stimulus compared with their resistance-trained young adult counterparts (15, 23).

An alternative factor that may underpin the lack of exercise-induced stimulation of MPS may be the relatively short 3 h tracer incorporation period employed in the present study. Whereas protein ingestion alone elicits a rapid, but transient, stimulation of MPS, peaking 90–120 min post ingestion (50, 51), prior resistance exercise has been shown to sustain myofibrillar FSR over an extended 5 h postprandial period compared with feeding alone (30). Accordingly, previous reports of an exercise-induced increase in postprandial MPS in healthy young and older adults was measured over a 6 h incorporation period (52). Hence, it remains unclear whether the lack of exercise-induced increase in postprandial myofibrillar FSR was physiologically inherent to the studied cohort of overweight post-menopausal women under conditions of energy deficit, or merely an artefact of the tracer period for measurement of MPS.

The attenuated rate of MPS previously reported during energy restriction (14-16) has been proposed to represent an adaptive mechanism to conserve energy during weight loss. This notion is intuitive given that MPS is an energetically expensive metabolic process that requires ~4 moles of ATP to initiate the translation elongation step of MPS (53). Accordingly, studies in healthy, weight stable, young adults demonstrate an ~25% decrease in basal rates of MPS during the early (5–10 d) phase of an energy-restricted diet (13, 15, 16), with minimal changes
in muscle protein breakdown (16). Moreover, an extended period of energy restriction (21 days) was shown to elicit a suppressed postprandial response of MPS to 20 g of ingested milk protein (54) when daily protein intake was restricted to the RDA (0.8 g/kg BM/d). Hence, based on acute metabolic studies in healthy young adults, the primary metabolic driver of LBM loss during energy deficit appears to be phase dependent, with basal rates of MPS impaired during the early phase of energy restriction, and the postprandial response of MPS attenuated during later periods of energy restriction. Refuting our original hypothesis, we report no differences in basal or postprandial (FED or FED-EX conditions) myofibrillar FSR between EBW35 and ERW35 groups, despite the 2 kg decline in body mass in ERW35 following the diet period. This counter-intuitive finding was likely attributed to differences in experimental design between past (14, 15) and present studies. We utilized a parallel, between-subjects, design to determine the influence of energy status on myofibrillar FSR, whereas previous studies employed a more sensitive within-subject crossover design with participants serving as their own control (14-16). Interestingly, previous studies in physically-active young adults have demonstrated a high protein diet (1.6–2.4 g/kg/d) to be effective in preserving basal and postprandial rates of MPS, and reducing loss of LBM during short-term energy restriction (54). Hence, a follow up study that manipulates dietary protein intake during a longer-term (weeks – months) period of energy restriction is warranted in a clinical population of overweight, postmenopausal women.

A strength of the present study relates to novelty in terms of investigating the protein-dose MPS response relationship under conditions of energy deficit in a clinically relevant, homogenous sample of middle-aged, overweight, postmenopausal women. Moreover, fraction-specific measurements of myofibrillar-FSR were conducted under basal, fed and exercised-fed conditions, and thus provided comprehensive insight into postabsorptive, postprandial and
exercise-stimulated responses of MPS to energy restriction. However, we acknowledge several
limitations. First, for practical reasons, the trial was conducted as a single-blinded study. In this
regard, the investigators that performed the experimental trial and statistical analysis were not
blinded to group allocation. However, all sample analyses for the measurement of MPS (primary
endpoint) were performed by blinded investigators, and thus the single blinded nature of the
trial was unlikely to bias study findings. Second, while measurements of MPS were conducted
under multiple conditions, i.e., resting and post-exercise, energy balance and energy restriction,
the study was powered based on previous dose-response studies conducted in energy balance.
Third, the energy restriction period was severe (~800 kcal/d) and short-term (5 days) and thus
direct translation of our findings to clinically relevant (20% energy deficit for weeks to months)
periods of weight loss must be considered with caution. Fourth, due to limited available muscle
tissue, it was not possible to use intracellular \(^{13}\text{C}_6\) phenylalanine enrichments as the true
precursor in the calculation of myofibrillar FSR and instead plasma \(^{13}\text{C}_6\) phenylalanine
enrichments were used for the calculation of MPS. Finally, we did not conduct measurements
of muscle protein breakdown alongside MPS. Hence, it was not possible to calculate the
response of net muscle protein balance to protein feeding during energy deficit. Interestingly,
previous studies have reported an increased stimulation of muscle protein breakdown following
10 days of moderate (20%) energy deficit (17), suggesting a mechanistic action of muscle
proteolysis in muscle mass loss during diet-induced energy restriction, at least over prolonged
periods of weight loss. Moreover, future studies are warranted to establish the dose-response of
MPS to ingested protein during weight loss in other clinical populations that experience muscle
loss, i.e., sarcopenic obese older adults, over chronic periods of diet-induced weight loss.
Deuterium oxide tracer methodology is ideally suited to the measurement of free-living,
integrated, rates of MPS over prolonged periods of weight loss (55), and thus once fully re-
established in the field of muscle protein metabolism, may be utilised in future studies to inform protein recommendations for muscle mass retention during weight loss in clinical populations.

5.0 Conclusion

We demonstrate that ingesting a 35 g dose of high-quality protein on a per meal/serving basis, with or without resistance exercise, is sufficient to stimulate a maximal postprandial response of MPS during a short-term period of weight loss in middle-aged, overweight, postmenopausal women. These results provide a foundation for devising refined protein recommendations on a per serving/meal basis for this clinical group during a weight loss programme.

6.0 Acknowledgements

MSL, MH, OCW, KDT, LH and URM designed research; MSL, RH, MM, MBB, KML, LH, KS, and PS conducted research; URM provided the protein supplements for the study; MSL and MH analysed data; MSL, OCW and MH wrote the paper; MSL and MH had primary responsibility for final content. All authors read and approved the final manuscript. The authors are thankful for the volunteers who enthusiastically participated in this study. The authors would also like to thank Gitte K. Hartvigsen, Janni M. Jensen and Dr. Sewa Abdullah for their technical assistance and advice. All products of the energy restricted diet were kindly sponsored by Nutrilett, Orkla Health AS, Oslo, Norway. Mette Hansen reports financial support to research and supplies of products for research from Arla Foods Ingredients Group P/S and financial support from The Danish Dairy Research Foundation and Toyota Foundation, Denmark. In addition, Mette Hansen reports supplies of products from Orkla Health, Norway, to the present project. Mads S. Larsen was in the project period employed as industrial PhD at Arla Food Ingredients P/S student funded by the public fund, Innovation Fund Denmark, and Arla Food Ingredients P/S, but enrolled as PhD student at Faculty of
Health, Aarhus University. Mette Hansen from Aarhus University was the main PhD supervisor. Ulla R. Mikkelsen employed at Arla Food Ingredients Group P/S was affiliated with the project as an industrial PhD-supervisor. Lars Holm and Maike Mose reports a relationship with Arla Foods that includes funding grants. Oliver C. Witard, Kevin D. Tipton, Katrine Meyer Lauritsen, Mads Bisgaard Bengtsen, Rikke Hansen, Kenneth Smith and Paula Scaife declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Nutrilett, The Danish Dairy Research Foundation, Toyota Foundation, and Innovation Fund Denmark had no influence on study design, implementation, analysis or interpretation of the data. The data that support the findings of this study are available from the corresponding author upon reasonable request.
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## Tables

### Table 1. Participant characteristics

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<tr>
<th></th>
<th>ERW15 ($n = 10$)</th>
<th>ERW35 ($n = 10$)</th>
<th>ERW60 ($n = 10$)</th>
<th>EBW35 ($n = 10$)</th>
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<tr>
<td><strong>Mean (SD)</strong></td>
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<tr>
<td>Age (yrs)</td>
<td>58.9 (5.3)</td>
<td>57.7 (5.4)</td>
<td>57.3 (3.9)</td>
<td>57.7 (5.4)</td>
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<td>Total body mass (kg)</td>
<td>81.3 (10.0)</td>
<td>78.6 (6.7)</td>
<td>83.5 (9.0)</td>
<td>79.0 (8.8)</td>
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<td>Lean body mass (kg)</td>
<td>45.0 (5.4)</td>
<td>42.5 (2.5)</td>
<td>45.0 (4.2)</td>
<td>44.2 (3.4)</td>
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<td>Body fat (%)</td>
<td>41.4 (4.0)</td>
<td>42.6 (4.7)</td>
<td>42.7 (5.7)</td>
<td>40.8 (3.5)</td>
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<tr>
<td>BMI (kg/m$^2$)</td>
<td>28.4 (2.1)</td>
<td>28.2 (2.0)</td>
<td>29.2 (3.8)</td>
<td>28.7 (2.6)</td>
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<tr>
<td>1RM (kg)</td>
<td>17.1 (5.6)</td>
<td>18.4 (3.2)</td>
<td>19.3 (5.5)</td>
<td>19.8 (4.7)</td>
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<td>Oestrogen concentrations (pmol/L)</td>
<td>26.3 (22.3)</td>
<td>26.3 (13.1)</td>
<td>21.7 (6.3)</td>
<td>22.8 (8.7)</td>
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<td>FSH concentrations (IU/L)</td>
<td>89.2 (29.2)</td>
<td>77.7 (19.5)</td>
<td>68.5 (13.9)</td>
<td>71.2 (23.0)</td>
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<tr>
<td>Testosterone concentrations (nmol/L)</td>
<td>1.0 (0.7)</td>
<td>0.8 (0.2)</td>
<td>0.9 (0.4)</td>
<td>0.8 (0.4)</td>
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<td>Plasma cholesterol concentration (mmol/L)</td>
<td>5.6 (0.8)</td>
<td>5.6 (0.7)</td>
<td>5.6 (0.9)</td>
<td>5.7 (0.7)</td>
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<tr>
<td>Plasma TG concentration (mmol/L)</td>
<td>1.0 (0.7)</td>
<td>1.1 (0.4)</td>
<td>1.3 (0.2)</td>
<td>1.1 (0.5)</td>
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All values are means ± SD. BMI, body mass index; 1-RM, one-repetition maximum; FSH, follicle-stimulating hormone; TG, triglycerides.
Table 2. Amino acid composition of protein beverages

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<th>Amino acid</th>
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<td>Histidine</td>
<td>1.5%</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.3%</td>
</tr>
<tr>
<td>Leucine</td>
<td>10.6%</td>
</tr>
<tr>
<td>Lysine</td>
<td>9.8%</td>
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<tr>
<td>Methionine</td>
<td>2.4%</td>
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<tr>
<td>Phenylalanine</td>
<td>2.7%</td>
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<tr>
<td>Threonine</td>
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<tr>
<td>Tryptophane</td>
<td>1.3%</td>
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<tr>
<td>Valine</td>
<td>5.7%</td>
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<tr>
<td><strong>ΣEssential amino acids</strong></td>
<td><strong>47.3%</strong></td>
</tr>
<tr>
<td>Alanine</td>
<td>5.5%</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.2%</td>
</tr>
<tr>
<td>Asparagine</td>
<td>10.4%</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1.9%</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>18.0%</td>
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<tr>
<td>Glycine</td>
<td>1.5%</td>
</tr>
<tr>
<td>Proline</td>
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<tr>
<td>Serine</td>
<td>4.6%</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.4%</td>
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<td><strong>ΣNon-essetial amino acids</strong></td>
<td><strong>52.7%</strong></td>
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<td>(n = 30)</td>
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<tr>
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<tr>
<td>Absolute energy intake (kcal/d)</td>
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<td>Relative energy intake (kJ/kg/d)</td>
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<td>Absolute CHO intake (g/d)</td>
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<td>Relative CHO intake (g/kg/d)</td>
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<td>Absolute PRO intake (g/d)</td>
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<td>Relative PRO intake (g/kg/d)</td>
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<td>Absolute fat intake (g/d)</td>
<td>22 (–)</td>
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<tr>
<td>Relative fat intake (g/kg/d)</td>
<td>0.3 (0.0)</td>
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All values are means ± SD. Data were analysed using a one-factor ANOVA. *significant difference vs. energy restricted groups for corresponding measurements (P < 0.001). ER, energy restricted diet group; EB, energy balanced diet group; CHO, carbohydrate; PRO, protein.
Figures

**Figure 1.** Flowchart of enrolment process.

**Figure 2.** Overview of study design and experimental trial. Blood samples were collected prior to initiation of L-\((\text{ring}-^{13}\text{C}_6)\)phenylalanine infusion (–270 min; Baseline) and periodically thereafter during the experimental day. A single bout of unilateral leg resistance exercise was initiated 20 min prior to ingestion of whey protein beverage. Muscle biopsies were collected from the exercised leg (FED-EX) at –180, and 450 min timepoints and non-exercised leg (FED) at 0 min and 450 min timepoints. Assigned beverages containing either 15, 35 or 60 g of whey protein were ingested immediately after the muscle biopsy at 0 min.

**Figure 3.** Plasma phenylalanine concentration expressed over time (a) and as iAUC (b) in energy restricted and energy balanced groups. ERW15 (×), energy-restricted diet with ingestion of 15 g whey protein; ERW35 (▽), energy-restricted diet with ingestion of 35 g whey protein; EBW35 (▲), energy balanced diet with ingestion of 35 g whey protein; ERW60 (○), energy restricted diet with ingestion of 60 g whey protein. A repeated measures mixed effects model was used for statistical analysis of data over time. Analysis of protein dose response: Main effect of time, \(P < 0.001\); main effect of group (protein dose), \(P < 0.001\); time × group interaction, \(P < 0.001\). Analysis of energy status: Main effect of time, \(P < 0.001\); main effect of group (ERW35 vs EBW35), \(P < 0.856\); time × group interaction: \(P < 0.452\). * significant difference from ERW15 at corresponding timepoint; #significant difference from ERW35 at corresponding timepoint. A one-way ANOVA was used for statistical analysis of data expressed as iAUC. iAUC analysis of protein dose response, \(P < 0.001\). iAUC analysis of energy status (ERW35 & ERB35), \(P = 0.752\). * significant difference from
ERW15; # significant difference from ERW35. Data are expressed as means ± SEM (n = 10 for all groups). EX, exercise.

**Figure 4.** Plasma leucine concentrations expressed over time (a) and as iAUC (b) in energy restricted and energy balanced groups. ERW15 (×), energy restricted diet with ingestion of 15 g whey protein; ERW35 (▽), energy restricted diet with ingestion of 35 g whey protein; EBW35 (▲), energy balanced diet with ingestion of 35 g whey protein; ERW60 (○), energy restricted diet with ingestion of 60 g whey protein. A repeated measures mixed effects model was used for statistical analysis of data presented over time. Analysis of protein dose response, Main effect of time: P < 0.001; main effect of group (protein dose), P < 0.001; time × group interaction, P < 0.001. Analysis of energy status: Main effect of time, P < 0.001; main effect of group (ERW35 vs EBW35), P < 0.001; time × group interaction: P = 0.002. * significant difference from ERW15 at corresponding timepoint; # significant difference from ERW35 at corresponding timepoint. A one-way ANOVA was used for statistical analysis of data expressed as iAUC. iAUC analysis of protein dose response, P < 0.001. iAUC analysis of energy status (ERW35 & ERB35), P < 0.001. * significant difference from ERW15; # significant difference from ERW35. Data are expressed as means ± SEM (n=10 for all groups). EX, exercise.

**Figure 5.** Serum insulin concentrations expressed over time (a) and as iAUC (b) in energy restricted and energy balanced groups. ERW15 (×), energy restricted diet with ingestion of 15 g whey protein; ERW35 (▽), energy restricted diet with ingestion of 35 g whey protein; EBW35 (▲), energy balanced diet with ingestion of 35 g whey protein; ERW60 (○), energy restricted diet with ingestion of 60 g whey protein. A repeated measures mixed effects model was used for statistical analysis of data presented over time. Analysis of protein dose response: Main effect of time, P <
Analysis of energy status: Main effect of time, $P < 0.001$; main effect of group (ERW5 & EBR35), $P = 0.988$; time × group interaction, $P = 0.936$. *significant difference from ERW15 at corresponding timepoint; # significant difference from ERW35 at corresponding timepoint. A one-way ANOVA was used for statistical analysis of data expressed as $\text{iAUC}$. $\text{iAUC}$ analysis of protein dose response: $P < 0.001$. $\text{iAUC}$ analysis of energy status (ERW35 & ERB35), $P = 0.756$. *significant difference from ERW15; # significant difference from ERW35. Data are expressed as means ± SEM ($n = 10$ for all groups). EX, exercise.

**Figure 6.** Arterialized plasma phenylalanine enrichment expressed over time in energy restricted and energy balanced groups. ERW15 ($\times$), energy-restricted diet with ingestion of 15 g whey protein; ERW35 ($\triangledown$), energy-restricted diet with ingestion of 35 g whey protein; EBW35 ($\Delta$), energy balanced diet with ingestion of 35 g whey protein; ERW60 ($\bigcirc$), energy restricted diet with ingestion of 60 g whey protein. A repeated measures mixed effects model was used for statistical analysis of data over time. Analysis of protein dose response: Main effect off time, $P < 0.001$; main effect of group (protein dose), $P = 0.129$; time × group interaction: $P < 0.001$. Analysis of energy status: Main effect of time (ERW35 vs EBW35), $P < 0.001$; main effect of group (ERW35 vs EBW35), $P = 0.277$; time × group interaction: $P < 0.664$. *significant difference from ERW15 at corresponding timepoint; $^{a}$significant difference from time 0 for ERB35; $^{b}$significant difference from time 0 for ERW60. Data are expressed as means ± SEM (ERW15, $n = 10$; ERW35, $n = 8$; EBW35, $n = 9$; ERW60, $n = 8$). EX, exercise.

**Figure 7.** Myofibrillar fractional synthesis rate (FSR) in response to graded doses of ingested whey protein in exercised and rested muscles in energy restricted and energy balanced groups. A mixed
effect model was used for statistical analysis with protein dose (ERW15, ERW35, EBW35, ERW60) and condition (BASAL, FED, FED-EX) serving as independent variables in the fixed part of the model. Analysis of protein dose response: Main effect of group (protein dose; ERW15, ERW35, ERW60): $P = 0.006$; main effect of condition (BASAL, FED, FED-EX): $P < 0.001$; protein dose $\times$ condition interaction: $P = 0.7442$. Analysis of energy status: Main effect of group (ERW35 vs EBW35), $P < 0.744$; main effect of condition (BASAL, FED, FED-EX), $P < 0.001$; time $\times$ group interaction, $P = 0.996$. * significant difference compared to ERW15. § significant difference compared to BASAL across protein dose groups. Data are expressed as means ± SEM (ERW15, $n = 10$; ERW35, $n = 8$; EBW35, $n = 9$; ERW60, $n = 8$).
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Dose-response of myofibrillar protein synthesis to ingested whey protein during energy restriction in overweight postmenopausal women: a randomized, controlled trial

Larsen, M.S. et al

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<td>Participants</td>
<td>Settings and locations where the data were collected</td>
<td>6</td>
</tr>
<tr>
<td>Interventions</td>
<td>The experimental and control interventions for each group with sufficient details to allow replication, including how and when they were actually administered</td>
<td>8</td>
</tr>
<tr>
<td>Outcomes</td>
<td>Completely defined prespecified primary and secondary outcome measures, including how and when they were assessed</td>
<td>12</td>
</tr>
<tr>
<td>Sample size</td>
<td>How sample size was determined.</td>
<td>12</td>
</tr>
<tr>
<td>Sample size</td>
<td>When applicable, explanation of any interim analyses and stopping guidelines</td>
<td>N/A</td>
</tr>
<tr>
<td>Randomization - Sequence generation</td>
<td>Method used to generate the random allocation sequence.</td>
<td>8</td>
</tr>
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<td>Type of randomization; details of any restriction (such as blocking and block size)</td>
<td>8</td>
</tr>
<tr>
<td>Randomization - Allocation concealment mechanism</td>
<td>Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned</td>
<td>8</td>
</tr>
<tr>
<td>Randomization - Implementation</td>
<td>Who generated the allocation sequence, who enrolled participants, and who assigned participants to interventions</td>
<td>8</td>
</tr>
<tr>
<td>Blinding</td>
<td>If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how.</td>
<td>6</td>
</tr>
</tbody>
</table>
Dose-response of myofibrillar protein synthesis to ingested whey protein during energy restriction in overweight postmenopausal women: a randomized, controlled trial
Larsen, M.S. et al

Blinding #11b If relevant, description of the similarity of interventions 9

Statistical methods #12a Statistical methods used to compare groups for primary and secondary outcomes 12

Statistical methods #12b Methods for additional analyses, such as subgroup analyses and adjusted analyses 12

Outcomes #6b Any changes to trial outcomes after the trial commenced, with reasons 11

Results

Participant flow diagram (strongly recommended) #13a For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome 7

Participant flow #13b For each group, losses and exclusions after randomization, together with reason 7

Recruitment #14a Dates defining the periods of recruitment and follow-up 6

Recruitment #14b Why the trial ended or was stopped N/A

Baseline data #15 A table showing baseline demographic and clinical characteristics for each group 28

Numbers analysed #16 For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups 12

Outcomes and estimation #17a For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval) 13

Outcomes and estimation #17b For binary outcomes, presentation of both absolute and relative effect sizes is recommended N/A

Ancillary analyses #18 Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory 13

Harms #19 All important harms or unintended effects in each group (For specific guidance see CONSORT for harms) 7
Dose-response of myofibrillar protein synthesis to ingested whey protein during energy restriction in overweight postmenopausal women: a randomized, controlled trial

Larsen, M.S. et al

**Discussion**

<table>
<thead>
<tr>
<th>Section</th>
<th>Code</th>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limitations</td>
<td>#20</td>
<td>Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses</td>
<td>20</td>
</tr>
<tr>
<td>Interpretation</td>
<td>#22</td>
<td>Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence</td>
<td>15</td>
</tr>
<tr>
<td>Registration</td>
<td>#23</td>
<td>Registration number and name of trial registry</td>
<td>2</td>
</tr>
<tr>
<td>Generalisability</td>
<td>#21</td>
<td>Generalisability (external validity, applicability) of the trial findings</td>
<td>20</td>
</tr>
</tbody>
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**Other information**

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</tr>
<tr>
<td>Protocol</td>
<td>#24</td>
<td>Where the full trial protocol can be accessed, if available</td>
<td>7</td>
</tr>
<tr>
<td>Funding</td>
<td>#25</td>
<td>Sources of funding and other support (such as supply of drugs), role of funders</td>
<td>21</td>
</tr>
</tbody>
</table>

The CONSORT checklist is distributed under the terms of the Creative Commons Attribution License CC-BY. This checklist was completed on 12. April 2023 using [https://www.goodreports.org/](https://www.goodreports.org/), a tool made by the EQUATOR Network in collaboration with Penelope.ai
60 women were assessed for eligibility

Randomly assigned (n = 40)

Not randomized (n = 20)
Not meeting inclusion criteria (n = 20)

Allocated to energy restricted diet and ingestion of 15 g whey protein (n = 10); ERW15
   Analyzed (n = 10)

Allocated to energy restricted diet and ingestion of 35 g whey protein (n = 10); ERW35
   Analyzed (n = 10)
   Excluded from MPS-analysis (n = 2, were unable to obtain adequate samples from the participants)

Allocated to energy restricted diet and ingestion of 60 g whey protein (n = 10); ERW60
   Analyzed (n = 10)
   Excluded from MPS-analysis (n = 2, were unable to obtain adequate samples from the participants)

Allocated to energy balanced diet and ingestion of 35 g whey protein (n = 10); EBW35
   Analyzed (n = 10)
   Excluded from MPS-analysis (n = 1, were unable to obtain adequate samples from the participants)
5 days hypocaloric diet; n=30
5 days; energy balanced habitual diet; n=10

Experimental day
- ERW15 (n = 10) 15 g protein
- ERW35 (n = 10) 35 g protein
- ERW60 (n = 10) 60 g protein
- EBW35 (n = 10) 35 g protein

Blood sample
Muscle biopsy (exercising leg)
Muscle biopsy (rested leg)
Protein drink

90 min 3 hours 3 hours

Experimental day
- Post-absortive state
- Exercise
- Post-prandial state

Primed infusion of L-[ring-\textsuperscript{13}C\textsubscript{6}] phenylalanine

5 days; n=30
5 days; n=10

5 days;

85 g protein

85 g protein

85 g protein
Figure 3. Plasma phenylalanine concentration

(a) Plasma phenylalanine concentration (mmol/L) over time (min) in relation to drink ingestion.

(b) Plasma phenylalanine (AUC, mmol/L • 3 h) comparison between conditions.

Legend:
- ERW15
- ERW35
- ERW60
- EBW35

Statistical markers:
- * indicates significant difference compared to baseline
- # indicates significant difference compared to a specific control group

Graphical data includes error bars to represent variability.
Figure 4. Plasma leucine concentration

Plasma leucine concentration (mmol/L)

Time (min) in relation to drink ingestion

**Figure 4.** Plasma leucine concentration
Figure 5. Serum insulin

(a) Plasma insulin concentration (pmol/L) over time (min) in relation to drink ingestion.

(b) Serum insulin (AUC, pmol/L • 3 h) for different conditions.
Figure 6. Plasma phenylalanine enrichment

Plasma phenylalanine enrichment (Tracer:tracee ratio)

Time (min) in relation to drink ingestion

Drink

EX

a

b

(experimental condition, control condition, significance markers)

Baseline 240 -180 -120 -60 0 60 120 180
Figure 7. Myofibrillar protein synthesis

Myofibrillare FSR (% • h⁻¹)

- ERW15
- ERW35
- ERW60
- EBW35

Legend:
- Basal
- Fed §
- Fed-Ex §