Effects of resistance exercise and whey protein supplementation on skeletal muscle strength, mass, physical function, and hormonal and inflammatory biomarkers in healthy active older men: a randomised, double-blind, placebo-controlled trial

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Abbreviations

1RM, one repetition maximum

6MWT, 6-min walk test

CRP, C-reactive protein

CONSORT, Consolidated Standards of Reporting Trials

eGFR, estimated glomerular filtration rate

ELISA, enzyme-linked immunosorbent assay

EWGSOP, European Working Group of Sarcopenia in Older People

FFM, fat-free mass

IGF-1, insulin-like growth factor 1

IL-6, interleukin-6

IL-10, interleukin-10

MPS, muscle protein synthesis

RCT, randomised controlled trial

RDA, recommended dietary allowance

RE, resistance exercise

SPPB, short physical performance battery

SMM, skeletal muscle mass

TNF-α, tumor necrosis factor-alpha

Abstract

Purpose: To determine the individual and combined effects of 12 weeks of resistance exercise (RE) and whey protein supplementation on skeletal muscle strength (primary outcome), mass and physical function, and hormonal and inflammatory biomarkers in older adults.

Methods: Thirty-six healthy older men [(mean ± SE) age: 67 ± 1 y; BMI: 25.5 ± 0.4 kg/m^2] were randomised to either control (CON; *n* = 9), whey protein (PRO; *n* = 9), RE + control (EX+CON; *n* = 9), or RE + whey protein (EX+PRO; *n* = 9) in a double-blinded fashion. Whole-body RE (2 sets of 8 repetitions and 1 set to volitional failure at 80% 1RM) was performed twice weekly. Supplements (PRO, 25 g whey protein isolate; CON, 23.75 g maltodextrin) were consumed twice daily.

Results: EX+CON and EX+PRO increased leg extension (+19 ± 3 kg and +20 ± 3 kg, respectively) and leg press 1RM (+27 ± 3 kg and +39 ± 2 kg, respectively) greater than the CON and PRO groups ($P < 0.001$, Cohen's $d = 1.50$-1.90). RE (EX+CON and EX+PRO groups pooled) also increased fat-free mass (FFM) (+0.9 ± 0.3 kg) and 6-min walk test distance (+21 ± 5 m) and decreased fat mass (-0.4 ± 0.4 kg), and interleukin-6 (-1.0 ± 0.4 pg/mL) and tumor necrosis factor-alpha concentration (-0.7 ± 0.3 pg/mL) greater than non-exercise (CON and PRO groups pooled; $P < 0.05$, Cohen's $f = 0.37$-0.45). Whey protein supplementation (PRO and EX+PRO groups pooled) increased 4-m gait speed greater than control (CON and EX+CON groups pooled) (+0.08 ± 0.03 m/s; $P = 0.007$, $f = 0.51$).

Conclusion: RE increased muscle strength, FFM and physical function, and decreased markers of systemic inflammation in healthy active older men. Whey protein supplementation alone increased gait speed. No synergistic effects were observed.

Key words: ageing, resistance exercise, whey protein, sarcopenia, systemic inflammation

1. Introduction

Age-related declines in skeletal muscle mass (SMM), strength, and physical function, termed sarcopenia (Cruz-Jentoft et al., 2019), progress at rates of ~0.5-1%, ~1-3%, and ~0.5% per annum, respectively, manifesting around the fifth decade of life (Clark and Manini, 2008; Daly et al., 2013; Janssen, 2010). Sarcopenia is associated with various adverse health outcomes, including an increased risk of falls and fractures, reduced physical function (Beaudart et al., 2017), and greater cardiovascular, metabolic disease and mortality risk (Bahat and Ilhan, 2016; de Buyser et al., 2016; Hunter et al., 2019). In economic terms, in the United Kingdom, the annual cost associated with muscle weakness is estimated at £2.5 billion (Pinedo-Villanueva et al., 2019). Hence, interventions that attenuate sarcopenia are imperative.

Resistance exercise (RE) is an effective stimulus to increase muscle strength (Peterson et al., 2010), fat-free mass (FFM) (Peterson et al., 2011), and physical function (Yoshimura et al., 2017). Meta-analyses also suggest that increased dietary protein intake may augment the adaptive response of skeletal muscle to RE (Cermak et al., 2012; Finger et al., 2015; Kirwan et al., 2021; Liao et al., 2017; Morton et al., 2018). However, whilst several individual studies in older adults have demonstrated greater increases in muscle strength, skeletal muscle and/or FFM, and physical function following combined RE and increased dietary protein intake compared to RE alone (Bell et al., 2017; Daly et al., 2014; Huschtscha et al., 2021; Junior et al., 2018; Kang et al., 2019; Rondanelli et al., 2020, 2016; Tieland et al., 2012b; Verreijen et al., 2015; Yamada et al., 2019; Zdzieblik et al., 2015), the majority of studies have not observed such effects (Arnarson et al., 2013; Candow et al., 2006; Chalé et al., 2013; de Carvalho Bastone et al., 2020; Dulac et al., 2020; Englund et al., 2018; Fielding et al., 2017; Gryson et al., 2014; Hofmann et al., 2016; Holm et al., 2008; Holwerda et al., 2018; Kim et al., 2012; Kirk et al., 2020, 2019; Krause et al., 2019; Kukuljan et al., 2009; Leenders et al., 2013; Maesta et al., 2007; Maltais et al., 2016; Oesen et al., 2015; Ottestad et al., 2017; Shahar et al., 2013; Thomson et al., 2016; Verdijk et al., 2009; Verreijen et al., 2017).

Inconsistent findings may be explained by the population studied, habitual protein intake of participants, and characteristics of the protein intervention. To explain the latter, several studies that observed synergistic effects evaluated a multi-ingredient supplement, which contained nutrients such as vitamin D, creatine and fatty acids in addition to protein that may have contributed to the augmented effect (Bell et al., 2017; Rondanelli et al., 2020, 2016; Verreijen et al., 2015; Yamada et al., 2019). Furthermore, studies in healthy older adults that observed synergistic effects increased dietary protein intake by 0.5-0.6 g/kg/d (Bell et al., 2017; Huschtscha et al., 2021; Junior et al., 2018), which exceeds the proposed increase required to elicit gains in SMM (≥0.4 g/kg/d) (Moore et al., 2015; Park et al., 2018). In contrast, studies that failed to observe amplified effects increased dietary protein intake by ≤0.3 g/kg/d (Arnarson et al., 2013; Dulac et al., 2020; Gryson et al., 2014; Hofmann et al., 2016; Holwerda et al., 2018; Kirk et al., 2020, 2019; Kukuljan et al., 2009; Leenders et al., 2013; Maesta et al., 2007; Maltais et al., 2016; Verdijk et al., 2009).

Moreover, a meta-regression conducted by Morton et al. (2018) showed, whilst driven by data in young adults, that ~1.6 g protein/kg/d might be required to maximally augment RE-induced gains in FFM in healthy adults. In support, protein intakes up to 1.6 g/kg/d [twice the recommended dietary allowance (RDA)] have also been recommended and established to mitigate sarcopenia (Mitchell et al., 2017; Morley et al., 2010). However, this level of dietary protein intake was only achieved by two of the aforementioned studies (Bell et al., 2017; Huschtscha et al., 2021). Additionally, the protein intervention employed by Huschtscha et al. (2021) elicited an evenly distributed dietary protein intake of >0.4 g/kg/meal (at breakfast, lunch, and dinner), the reported dose required to maximally stimulate rates of muscle protein synthesis (MPS) (Moore et al., 2015) and has been associated with increased muscle strength (Loenneke et al., 2016) and physical function (ten Haaf et al., 2018) in older adults. Taken together, current evidence suggests that an increase of ≥0.4 g protein/kg/d and a total protein intake of ~1.6 g/kg/d, which is evenly distributed across all three main meals (>0.4 g/kg/meal),

may be required to amplify RE-induced effects on sarcopenia outcomes in healthy older adults.

A limitation of most studies investigating the synergistic effects of RE and increased dietary protein intake on sarcopenia outcomes was the failure to include a protein only group. Of the studies [excluding Huschtscha et al. (2021)] that examined the synergistic effects compared to both RE and increased dietary protein alone (de Carvalho Bastone et al., 2020; Gryson et al., 2014; Huschtscha et al., 2021; Kim et al., 2012; Kirk et al., 2020; Krause et al., 2019; Kukuljan et al., 2009; Maesta et al., 2007; Shahar et al., 2013; Verreijen et al., 2017), the increase and daily dose of dietary protein intake (≤0.3 g/kg/d and <1.6 g/kg/d, respectively) was suboptimal according to previously mentioned data (Moore et al., 2015; Morton et al., 2018; Park et al., 2018). However, whilst Huschtscha et al. (2021) increased dietary protein intake by >0.4 g/kg/d to ≥1.6 g/kg/d, this study did not employ a double-blind, placebo-controlled design, which has been recommended for trials investigating the effectiveness of interventions to treat or prevent sarcopenia by an expert working group (Reginster et al., 2016). Lack of such experimental control significantly increased the risk of bias in this study. Consequently, to our knowledge, data on the synergistic effects compared to each intervention alone employing a double-blind, placebo-controlled design and utilising the optimal dietary protein regimen is currently unavailable, highlighting the need for further robustly designed studies.

Several physiological factors are involved in the pathogenesis of sarcopenia. These include, but are not limited to, chronic systemic inflammation [e.g., elevated interleukin (IL)-6, C-reactive protein (CRP) and tumor necrosis factor-alpha (TNF-α), and reduced IL-10] and changes in the hormonal milieu [e.g., reduced insulin-like growth factor 1 (IGF-1), flattened diurnal cortisol secretion, and increased myostatin] (Beyer et al., 2012; McKee and Morley, 2019; White and Lebrasseur, 2014). Previous work has shown that RE and increased dietary proten intake independently decrease markers of systemic inflammation (Liberman et al.,

2019; Sardeli et al., 2018) and increase IGF-1 (Bauer et al., 2015; Bo et al., 2019; Jiang et al., 2020) in older adults. Others have also demonstrated decreases in fasting concentrations of cortisol (Häkkinen et al., 2002; Izquierdo et al., 2003) and myostatin (Bagheri et al., 2020, 2019) following RE. However, there is currently limited evidence of the combined effects of these interventions on these biomarkers compared to each intervention alone in older adults. Such data may identify mechanisms which explain the synergistic sarcopenic-mitigating effects.

The primary aim of the present study was to investigate the individual and combined effects of RE and whey protein supplementation [aimed to increase dietary protein intake by ≥0.4 g/kg/d to ~1.6 g/kg/d (>0.4 g/kg/meal)] using a double-blind, placebo-controlled design, on muscle strength in healthy active older men. Secondary aims were to examine the effects on other sarcopenia outcomes (i.e., skeletal muscle/FFM and physical function) and multiple hormonal and inflammatory biomarkers associated with sarcopenia, and to determine whether changes correlate with changes in sarcopenia outcomes. We hypothesised that RE combined with whey protein supplementation would augment the effects on SMM, strength and physical function and elicit a superior systemic hormonal and inflammatory profile compared to each intervention alone. We also postulated that changes in sarcopenia outcomes would correlate with changes in hormonal and inflammatory biomarkers.

2. Materials and methods

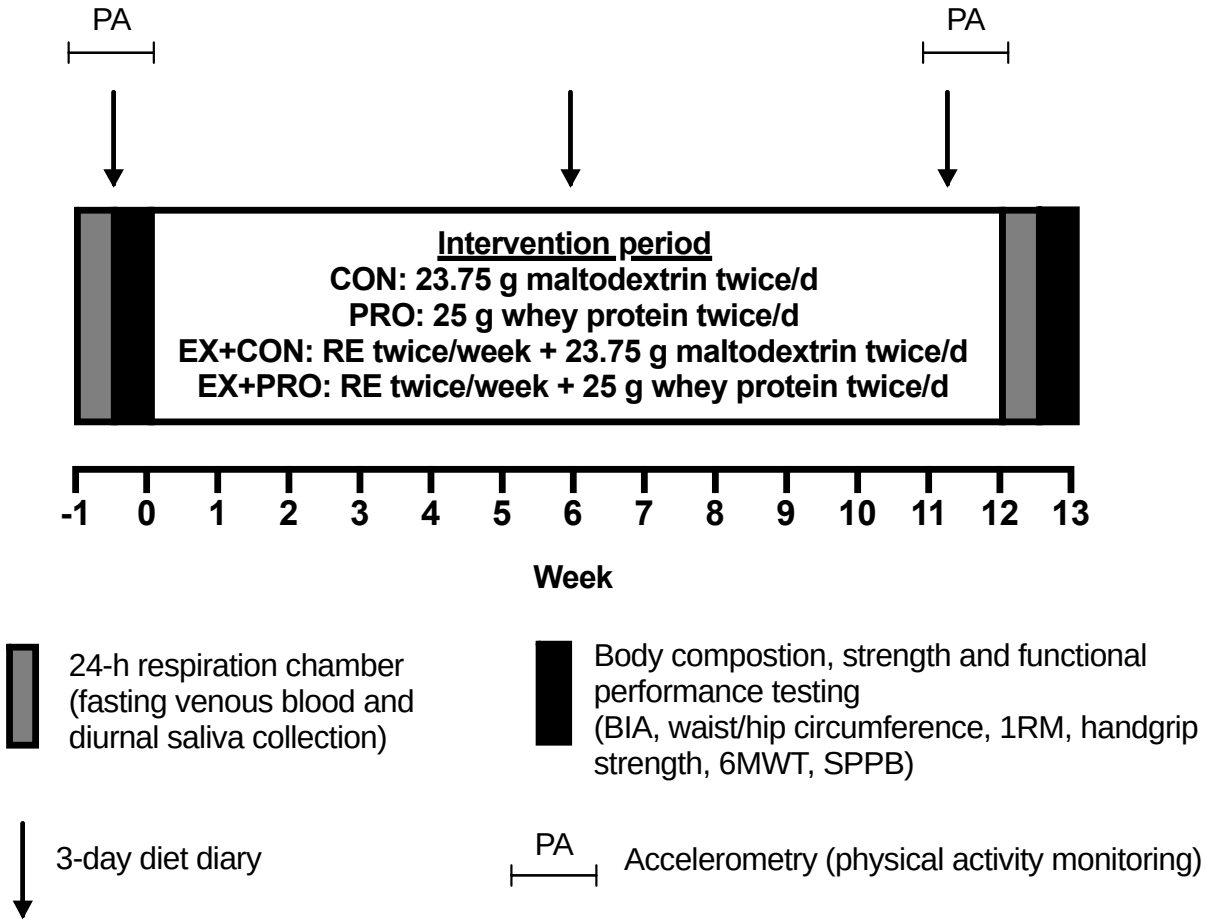
2.1 Participants

Thirty-six healthy, community-dwelling older men (mean ± SE age: 67 ± 1 y) participated in this study. The following eligibility criteria applied: i) aged 60-80 y; ii) BMI between 18.5 and 30 kg/m^2; iii) non-smoker; iv) weight stable (±<3 kg change in the previous 6 months); v) no participation in RE in the previous 6 months; vi) no past or existing history of cancer, diabetes mellitus, or cardiovascular, thyroid, or renal disease; and vii) not taking statins, or non-steroidal

anti-inflammatory or metabolism-affecting drugs. Participants were recruited from Coventry, UK, and surrounding areas by newspaper advertisements, contact with local groups and organisations, and via word of mouth. The study was approved by Coventry University Ethics Committee (project code: P59723), registered at clinicaltrials.gov as NCT03299972, and is reported in accordance with Consolidated Standards of Reporting Trials (CONSORT) guidelines (Schulz et al., 2010). All participants provided written informed consent in accordance with the Declaration of Helsinki.

2.2 Experimental design

This was a 12-week randomised, controlled, double-blind, 4-arm parallel group trial, which was conducted between October 2017 and May 2019. Participants were randomised to either control (CON; *n* = 9), whey protein (PRO; *n* = 9), RE + control (EX+CON; *n* = 9), or RE + whey protein (EX+PRO; *n* = 9). A coded (A, B, C or D) randomisation scheme was used. Randomisation was performed using the minimization allocation method, with stratification for age and body mass index (BMI) using free online software (QMinim; http://rct.mui.ac.ir/q/). A key to the randomisation code was held by an investigator who was not directly involved with participant recruitment, exercise training, or testing. All measurements were taken at baseline and following the 12-week intervention. A schematic of the trial design can be seen in Fig. 1. To minimise diurnal variation, muscle strength, body composition, and physical function measures were performed at the same time of day (± 1 h) at both testing sessions. In addition to the main analysis, exploratory analyses were also conducted between pooled exercise (EX+CON and EX+PRO groups; *n* = 18) and non-exercise groups (CON and PRO groups; *n* = 18), and between pooled whey protein (PRO and EX+PRO groups; *n* = 18) and control supplement groups (CON and EX+CON groups; *n* = 18).



228

229 **Figure 1** Schematic of the experimental design. 1RM, one repetition maximum; 6MWT, 6-min
230 walk test; BIA, bioelectrical impedance analysis; SPPB, short-physical performance battery;
231 PA, physical activity.

232

233 *2.3 Exercise training*

234 Supervised whole-body RE was performed twice weekly at Coventry University. Sessions
235 occurred at least 48 h apart. A frequency of 2 sessions per week was chosen to maximise
236 adherence and adaptation of sarcopenia outcomes whilst performing the minimalist amount
237 of RE in this population. This was based on prior work that has reported that older adults prefer
238 to perform RE twice as opposed to three thrice weekly (Foley et al., 2011), and that thrice
239 weekly RE does not provide an additive benefit on sarcopenia outcomes over twice weekly
240 RE (Grgic et al., 2019; Kneffel et al., 2020; Silva et al., 2017; Stec et al., 2017). Each session

consisted of a 5-min warm up on a cycle ergometer at a self-selected cadence, followed by 3 sets of leg press, lateral row, hamstring curl, chest press, leg extension and shoulder press (in that order) on fixed RE machines (Life Fitness, Rosemont, Illinois, USA). These exercises were chosen to target major muscle groups using multi-joint movements to stimulate whole-body increases in SMM and strength and to improve physical function (Fragala et al., 2019). During the first 4 weeks of training, RE load began at 60% one repetition maximum (1RM) (10-12 repetitions per set) and was gradually increased by ~5-7% per week to 80% 1RM (8 repetitions per set), where it remained until the end of the intervention. Training volume was selected based on meta-analyses which suggest that 2-3 sets per exercise and ~8 repetitions per set elicits superior increases in muscle hypertrophy and strength in older adults (Borde et al., 2015; Peterson et al., 2010). Exercise load was chosen based on meta-analyses which suggest for optimisation of muscle strength, which is considered the primary index of sarcopenia by the European Working Group of Sarcopenia in Older People (EWGSOP) (Cruz-Jentoft et al., 2019), high-load RE (~70-80% 1RM) elicits the largest effects (Borde et al., 2015; Steib et al., 2010). The final set of each exercise was performed to volitional failure, which was defined as the inability to perform an additional repetition with the correct form. Completion of repetitions was monitored during each session. Participants were allocated 60 s and 3 min recovery between sets and exercises, respectively. Exercise load was adjusted according to 1RM tests performed every 4 weeks to mimic typical changes in muscle fibre type and strength (Kraemer and Ratamess, 2004) and when participants were able to complete >12 repetitions on the final set of each exercise. Sessions concluded with a 5-min cool-down on a cycle ergometer. Compliance was monitored using a training log.

2.4 Nutritional supplements

Participants ingested either 25 g whey protein isolate (including ~3 g leucine) (Instantized BiPRO; Agropur, Quebec, Canada) or an energy-matched control (23.75 g maltodextrin; Myprotein, Northwich, UK) twice daily, consumed directly after breakfast and lunch. On RE training days, participants in the EX+CON and EX+PRO groups consumed their second

supplement immediately following the session. The nutritional composition of the experimental supplements can be seen in Table 1. Supplements were unflavoured, similar in powder weight, and were provided in opaque sachets in a double-blinded manner (Flexible Packaging Services Ltd, Wirral, UK). Participants were instructed to dissolve the contents of their supplements into ~200 mL of water combined with a no-added sugar cordial of choice immediately prior to consumption using a handheld shaker (Myprotein, Northwich, UK). Flexibility in cordial use was provided to mitigate flavour fatigue. Compliance was assessed by the number of empty sachets returned by participants at the end of the study and through the use of a supplementation log. To test the success of supplement blinding, participants completed an exit questionnaire on completion of the study.

297 **Table 1** Nutritional composition of the experimental supplements (per serving)[1]

Component	Whey protein isolate (PRO)[1]	Control (CON)
	25 g	23.75 g
Energy (kcal)	95	95
Carbohydrate (g)	0	23.75
Protein (g)	22.8	0
EAA (g)	11.1	0
Histidine (g)	0.5	0
Lysine (g)	2.2	0
Methionine (g)	0.4	0
Phenylalanine (g)	0.8	0
Threonine (g)	1.1	0
Tryptophan (g)	0.7	0
BCAA (g)	5.4	0
Leucine (g)	2.9	0
Isoleucine (g)	1.3	0
Valine (g)	1.2	0
NEAA (g)	11.7	0
Alanine (g)	1.0	0
Arginine (g)	0.5	0
Aspartic Acid/Asparagine (g)	2.5	0
Cysteine (g)	0.6	0
Glutamic Acid/Glutamine (g)	3.6	0
Glycine (g)	0.4	0
Proline (g)	1.1	0
Serine (g)	0.8	0
Tyrosine (g)	0.8	0
Fat (g)	0.4	0

298

299 [1]Whey protein isolate also contained per serving: vitamin A (<25 IU), vitamin C (<0.5 mg),
300 vitamin D (0.2 µg), iron (0.25 mg), calcium (21.3 mg), phosphorus (85 mg), magnesium (2.5
301 mg) chloride (20 mg), sodium (172.5 mg), potassium (17.5 mg). BCAA, branched-chain amino
302 acids; EAA, essential amino acids; NEAA, non-essential amino acids.

303

304 The whey protein dosing regimen employed was chosen based on previous studies that have
305 demonstrated that older adults typically consume insufficient amounts of dietary protein at

breakfast and lunch to maximally stimulate rates of MPS (~0.2 and ~0.3 g/kg, respectively (Farsijani et al., 2017; Smeuninx et al., 2020; Tieland et al., 2012a). Based on a hypothesised habitual protein intake of ~1.0 g/kg/d and a mean body mass of ~80 kg of the cohort in this study to that of others (Bell et al., 2017; Kirk et al., 2019; Smeuninx et al., 2020), 25 g of whey protein (~0.25 g protein/kg) at breakfast and lunch was postulated to yield a daily protein distribution of ~0.45 g/kg, ~0.55 g/kg, and ~0.5 g/kg at breakfast, lunch and dinner, respectively. Thus, meeting the per meal protein (≥0.4 g/kg) and leucine (≥2.5 g) thresholds required to maximally stimulate rates of MPS in older adults (Moore et al., 2015). In addition, it was hypothesised that the whey protein dosing regimen would increase daily dietary protein intake from ~1.0 to 1.6 g/kg/d, the intake recommended to curb sarcopenia (Phillips et al., 2016) and maximise SMM accretion during RE training (Morton et al., 2018), whilst also surpassing the suggested required increase of ≥0.4 g/kg/d to stimulate gains in SMM in healthy older adults (Park et al., 2018).

2.5 Dietary intake and habitual physical activity

Participants completed a 3-day diet diary (2 weekdays and 1 weekend day) at baseline (prior to commencing the intervention) and during weeks 6 and 12. Dietary records were analysed using dietary analysis software (Nutritics Version 5.097; Nutritics, Dublin, Ireland). To control for changes in habitual physical activity levels/intensity during waking hours [i.e., step count and time spent sedentary, and in light and moderate-vigorous physical activity (MVPA)], participants wore a tri-axial accelerometer on the dominant wrist for 7 days at baseline and week 12 (Freedson et al., 1998). The accelerometer was sampled at 80 Hz and analysed in 60-s EPOCHs. Participants were instructed to not alter their habitual diet or physical activity levels for the duration of the study.

2.6 Muscle strength

Muscle strength (primary outcome) was assessed by 1RM tests on the leg press and leg extension machines (in that order) (Life Fitness, Rosemont, Illinois, USA) using the guidelines

of Kraemer et al. (2006). Prior to baseline testing, proper lifting technique was demonstrated and practiced by participants to minimise a potential learning effect (Levinger et al., 2009; Phillips et al., 2004). During 1RM testing, participants first completed 5-10 repetitions at 40-60% of perceived 1RM followed by 3-5 repetitions at 60-80% of perceived 1RM. The load was then gradually increased by 5-10%, and participants performed one repetition at each increased load until they were unable to achieve a complete repetition. One repetition maximum was determined as the last successful lift prior to failure. Three min rest was allocated between each maximal lift. Handgrip strength was measured using a JAMAR hydraulic handgrip dynamometer (Jamar 5030J1; Sammons Preston, Bolingbrook, Illinois, USA) using standardised procedures (Roberts et al., 2011).

2.7 Body composition

Body composition (SMM, FFM and fat mass) was measured in the morning by bioelectrical impedance analysis (BIA) (BC-418 MA; Tanita Corporation, Tokyo, Japan). Skeletal muscle mass was estimated using the formula of Janssen et al. (2000). This method has been cross-validated against magnetic resonance imaging for measurement of SMM in older adults (Janssen et al., 2000). Skeletal muscle and fat mass index (kg/m^2) were calculated by dividing SMM and FM by height squared, respectively. Waist circumference was measured at the midpoint between the lowest rib margin and the iliac crest. Hip circumference was measured at the widest portion of the hips. Both outcomes were measured to the nearest 0.1 cm using a measuring tape (Seca 201; Seca GmbH, Hamburg, Germany).

2.8 Physical function

Physical function was assessed by the short physical performance battery (SPPB) and the 6-min walk test (6MWT). The SPPB followed standard procedures, which consisted of three timed tests: 4-m gait speed, time to perform five chair raises, and standing balance (feet together, semi-tandem and tandem) (Guralnik et al., 2000). Each test was scored equally between 0 and 4. The total score between 0 and 12 was used for analysis. The 6MWT was

362 performed adhering to guidelines set by the American Thoracic Society (Crapo et al., 2002).
363 A 30 m indoor track was marked out with cones at either end. Participants were informed that
364 the aim of the test was to cover as much distance as possible in six minutes.
365
366 *2.9 Biochemical analysis*
367 Venous blood was collected at 0815 h following a >10 h overnight (observed) fast and >72 h
368 following the final RE session to allow for biomarkers to return to basal levels (Schoenfeld,
369 2012). Whole blood was collected into ethylenediaminetetraacetic acid (EDTA), heparin and
370 serum separator tube (SST) vacutainers (BD 3 mL vacutainers; BD, New Jersey, USA) then
371 immediately centrifuged at 1900 x g for 10 min at 4°C (Eppendorf 5702R; Eppendorf UK Ltd,
372 Stevenage, UK). Serum samples were rested for 30 min prior to centrifugation to allow for
373 sufficient clotting. Aliquots containing plasma and serum were stored at -80°C until analysis.
374 Due to difficulty in blood collection, blood was unable to be drawn from two participants (n = 1
375 participant in the CON and EX+CON groups). Therefore, n = 34 participants had full blood
376 data. Commercially available enzyme-linked immunosorbent assay (ELISA)'s were used to
377 detect and quantify concentrations of plasma IL-6 (Item # D6050 and HS600C), IL-10 (Item #
378 HS100C), TNF-α (Item # HSTA00E) and CRP (Item # DCRP00), serum IGF-1 (Item #
379 DB100B) and myostatin (Item # DGDF80) (R&D Systems Inc., Abbington, UK), and plasma
380 annexin A1 (Item # ab222868; Abcam, Cambridge, UK) and insulin (Item # EIA-2935; DRG
381 Instruments GmbH, Marburg, Germany). Serum creatinine was determined using an
382 enzymatic method on an automated clinical chemistry analyser (Cobas c720 analyser, Roche,
383 Mannheim, Germany). Estimated glomerular filtration rate (eGFR) was calculated using the
384 Modification of Diet in Renal Disease (MDRD) equation (Levey et al., 2006).
385
386 Saliva samples were collected whilst participants (n = 33) resided in respiration chambers for
387 24 h under highly controlled conditions, as described in Supplementary Materials. Samples
388 were collected immediately upon waking at 0650 h, and at 0805 h, 1225 h, 1700 h, and 2000
389 h using a synthetic swab (Salivette; Sarstedt, Nümbrecht, Germany). Samples were

centrifuged at 1900 x g for 2 min and stored at -80°C until analysis. Saliva samples were analysed for cortisol by ELISA (Item # 1-3002; Salimetrics, Pennsylvania, USA). Salivary cortisol data was used to calculate multiple indices. Firstly, all five samples were used to calculate salivary cortisol area under the curve (AUC) (nmol/L x 790 min) using the trapezoidal method. Secondly, salivary cortisol concentration upon waking (0650 h) and in the evening (2000 h) are reported as separate indices. Lastly, salivary cortisol slope (peak-to-evening) was calculated as the rate of salivary cortisol change from peak morning (0650 or 0805 h, depending on the highest concentration) to 2000 h (Adam et al., 2017). Cortisol was measured as it plays a key role in influencing metabolic functions, including gluconeogenesis, glycogenolysis and proteolysis (Coderre et al., 1991; Simmons et al., 1984). Diurnal salivary cortisol and elevated evening (2000 h) cortisol concentration have also been associated with sarcopenia (Gonzalez et al., 2018; Rodriguez et al., 2021), and a limitation of previous studies assessing cortisol following RE and increased dietary protein intake in older adults was the sole measurement of fasting cortisol (Häkkinen et al., 2002; Huschtscha et al., 2021; Izquierdo et al., 2003; Park et al., 2019). The intra-assay CVs were 9.5%, 9.8%, 11.8%, 2.7%, 9.9%, 9.9%, 9.1%, 4.2%, 7.7% and 9.0% for plasma insulin, IL-6, high sensitivity IL-6, IL-10, TNF-α, CRP and annexin A1, serum IGF-1 and myostatin, and salivary cortisol, respectively.

2.10 Statistical analysis

Based on change in muscle strength from previously published data in older adults following 12 weeks of RE and oral protein supplementation (Esmarck et al., 2001), an *a priori* power calculation using G*Power (Version 3.1.9.2; Dusseldorf, Germany) for a repeated measures ANCOVA with one covariate indicated a minimum of 36 participants (n = 9 per group) were required to observe a significant group-by-time interaction on 1RM strength measures [α = 0.05; β = 0.8; effect size (Cohen's f) = 0.6].

Statistical analysis was performed using SPSS version 25 (IBM Corporation, New York, USA). Data are presented as means ± SE (data on mean difference ± SD between groups is also

reported in supplementary materials). All data were checked for normality using the Shapiro-Wilk test. Non-parametric data were transformed using appropriate transformation (i.e., log, square root, or reciprocal). When transformation was unsuccessful, non-parametric tests were utilised. Participant baseline characteristics were analysed by one-way ANOVA. A mixed-model ANCOVA with time as the within-subjects factor, group as the between-subjects factor, and respective baseline values as covariates was conducted to determine group-by-time interactions. Following significant group-by-time interactions, significant between-group differences were identified using post-hoc tests with a Bonferroni correction for multiple comparisons. For exploratory analyses comparing pooled groups (i.e., exercise and non-exercise groups, and whey protein and control groups), supplement consumed and RE participation were also controlled for in the ANCOVA model, respectively. Non-parametric data were analysed using the Scheirer-Ray-Hare two-way ANOVA of ranks test (including baseline rank as a covariate) with post-hoc analysis conducted using the Mann-Whitney U test. Longitudinal changes within groups were analysed using 2-tailed paired samples *t*-tests. Correlations between changes in SMM, strength and physical function and changes in hormonal and inflammatory biomarkers were analysed by partial correlation controlled for intervention group (Pearson's for parametric data and Spearman's rank order coefficients for non-parametric data). Significance was set at $P < 0.05$. Effect sizes were calculated for *t*-(Cohen's *d*) and F tests (Cohen's *f*) to quantify the magnitude of change (within and between groups) using previously published formulae (Cohen, 1988). The standard definitions of Cohen's *d* are: very small, 0.01-0.19; small, 0.20-0.49; medium, 0.50-0.79; large, 0.80-1.29; and very large, ≥1.20 (Cohen, 1988; Sawilowsky, 2009). The standard definitions of Cohen's *f* are: small, 0.10; medium, 0.25; and large, 0.40 (Cohen, 1988).

3. Results

3.1 Participants and safety

Thirty-nine older men were randomised between October 2017 and February 2019: 36 completed the study and 3 withdrew (see Fig. 2 for participant flow). Baseline characteristics of the 36 participants who completed the study are shown in Table 2. Resistance exercise was well tolerated, with only adverse event reported (muscle soreness), which resulted in one participant missing a single session. Following whey protein supplementation, renal function was not adversely affected, confirmed by an eGFR of >60 mL/min/1.73m^2 in all participants following the intervention.

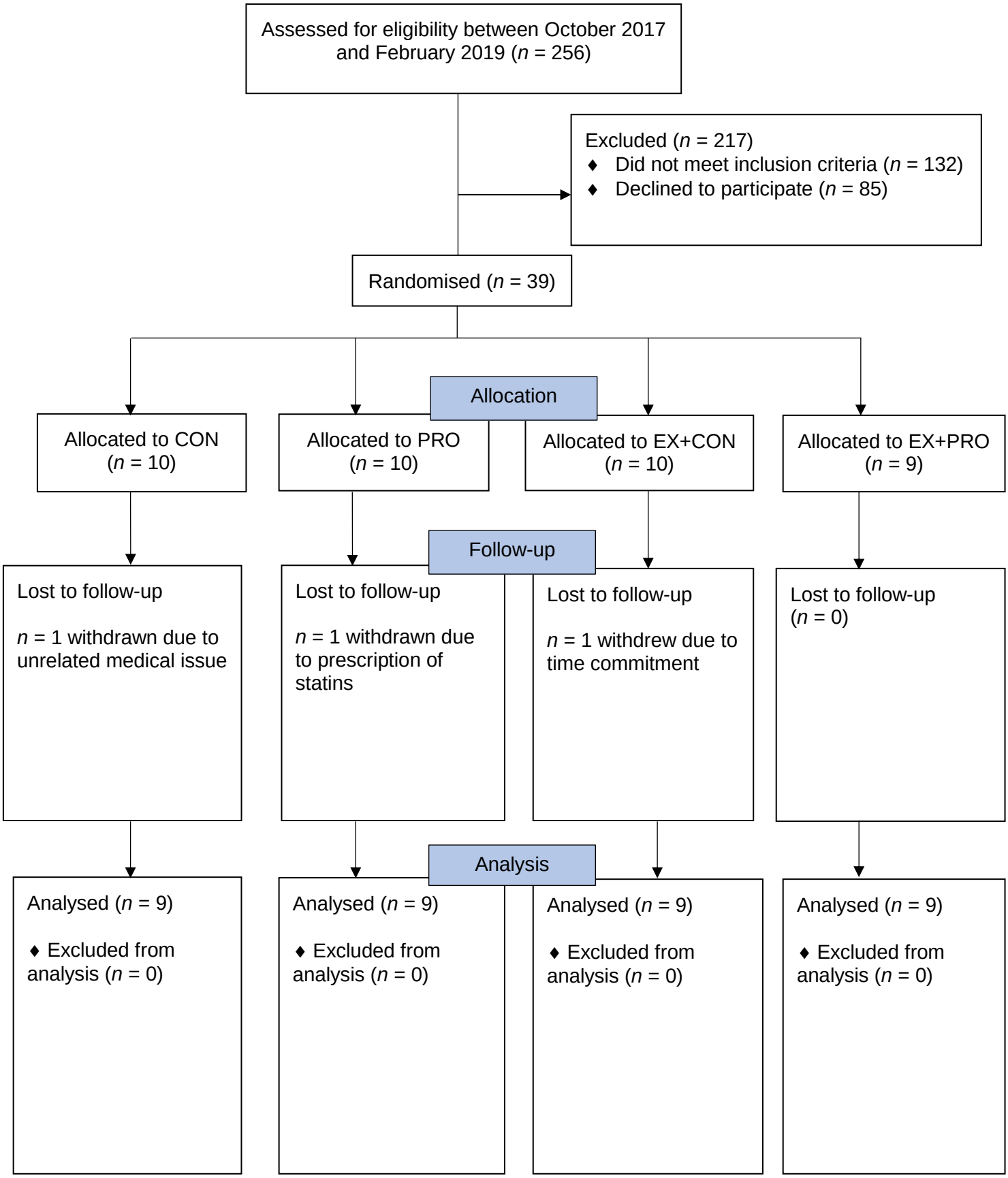


Figure 2 Flow of participants throughout the study.

Table 2 Baseline characteristics of participants[1]

	CON	PRO	EX+CON	EX+PRO	*P* value[3]	Overall
n	9	9	9	9	-	36
Age (y)	67 ± 2	66 ± 2	67 ± 1	68 ± 1	0.75	67 ± 1
Height (m)	1.77 ± 0.01	1.76 ± 0.03	1.77 ± 0.02	1.74 ± 0.03	0.71	1.76 ± 0.01
Body mass (kg)	79.0 ± 3.4	78.0 ± 3.1	78.2 ± 3.9	80.9 ± 4.0	0.94	79.0 ± 1.8
BMI (kg/m^2)	25.1 ± 1.0	25.0 ± 0.6	25.1 ± 0.9	26.6 ± 0.8	0.50	25.5 ± 0.4
FFM (kg)	59.8 ± 1.5	60.0 ± 1.7	58.5 ± 2.6	60.5 ± 2.9	0.94	59.7 ± 1.1
SMM (kg)	26.7 ± 0.6	27.2 ± 0.7	25.9 ± 1.1	26.9 ± 1.3	0.79	26.7 ± 0.5
SMI (kg/m^2)	8.5 ± 0.2	8.8 ± 0.2	8.3 ± 0.2	8.9 ± 0.3	0.19	8.6 ± 0.1
Fat mass (kg)	19.2 ± 2.4	18.0 ± 1.7	19.6 ± 2.0	20.4 ± 1.5	0.85	19.3 ± 0.3
Fat mass (%)	23.8 ± 2.0	22.7 ± 1.5	24.8 ± 1.7	25.1 ± 1.2	0.74	24.1 ± 0.8
Handgrip strength (kg)	41.8 ± 1.8	36.5 ± 2.5	39.9 ± 4.1	41.8 ± 2.1	0.37	40.1 ± 1.3
Leg extension 1RM (kg)	63 ± 6	58 ± 3	52 ± 5	59 ± 4	0.52	58 ± 2
Leg press 1RM (kg)	116 ± 9	107 ± 7	107 ± 9	118 ± 7	0.66	112 ± 4
SPPB (points)	11.7 ± 0.2	11.4 ± 0.2	11.2 ± 0.3	11.8 ± 0.1	0.38	11.5 ± 0.1
6MWT (m)	639 ± 21	616 ± 18	627 ± 30	591 ± 26	0.54	618 ± 12
4-m gait speed (m/s)	1.09 ± 0.08	1.13 ± 0.04	1.14 ± 0.04	1.22 ± 0.05	0.51	1.15 ± 0.03
Step count (steps/d)	10,766 ± 594	12,670 ± 1263	12,061 ± 1023	11,346 ± 907	0.55	11,710 ± 483
MVPA (min/d)	110 ± 8	127 ± 15	137 ± 18	135 ± 10	0.50	127 ± 7

[1]Values are means ± SE. [3]*P* value refers to differences between groups analysed by one-way ANOVA. No significant differences in baseline characteristics occurred between pooled exercise and non-exercise groups, or between pooled whey protein and control groups (data not shown). 1RM, one repetition maximum; 6MWT, 6-min walk test; BMI, body mass index; FFM, fat-free mass; MVPA, moderate-vigorous physical activity; SMI, skeletal muscle index; SMM, skeletal muscle mass; SPPB, short physical performance battery.

456 *3.2 Exercise and supplement adherence*

457 Participants in the EX+CON and EX+PRO groups attended 98.2 ± 1.0% and 98.2 ± 1.2% of
458 their prescribed RE sessions, respectively (P = 0.63, d = 0.00). All participants completed their
459 prescribed repetitions for sets 1 and 2 of each exercise. During the final set (to volitional
460 failure), the mean number of completed repetitions was 9.1 ± 0.3 in the EX+CON group and
461 9.1 ± 0.2 in the EX+PRO group (P = 0.97, d = 0.00). Compliance with the dietary supplements
462 was 94.1 ± 1.2%, 96.8 ± 1.0%, 96.1 ± 1.3%, and 96.1 ± 1.3% in the CON, PRO, EX+CON and
463 EX+PRO groups, respectively (P = 0.50, f = 0.08). Eighty percent of participants were unable
464 to judge treatment allocation based on the supplement exit questionnaire.

465

466 *3.3 Dietary intake*

467 Significant group-by-time interactions were observed for total dietary protein intake (expressed
468 as g/d, g/kg/d, and % energy; P < 0.001, f = 1.45-1.70), meal-specific relative protein intake
469 (g/kg) at breakfast and lunch (P < 0.001, f = 1.25-1.49), and carbohydrate intake (expressed
470 as g/d and % energy; P < 0.05, f = 0.54-0.65; Table 3). Total dietary protein intake increased
471 over time in the PRO and EX+PRO groups greater than the CON and EX+CON groups at
472 weeks 6 (P < 0.001, d = 1.94-2.20) and 12 (P < 0.001, d = 2.19-2.39). These increases were
473 driven by increased intakes at breakfast and lunch (P < 0.001, f = 2.18-2.84). Carbohydrate
474 intake increased over time in the EX+CON group greater than the PRO and EX+PRO groups
475 at weeks 6 (P < 0.05, d = 1.07-1.28) and 12 (P < 0.05, d = 1.02-1.09). Total energy intake
476 increased over time in the EX+PRO group at week 6 (P = 0.03, d = 0.48) and in the CON
477 group at weeks 6 and 12 (P < 0.05, d = 0.36-0.57).

Table 3 Dietary intake during the intervention period (including experimental supplements)[1]

	Pooled	CON		PRO		EX+CON		EX+PRO		
	Baseline	6 weeks	12 weeks	6 weeks	12 weeks	6 weeks	12 weeks	6 weeks	12 weeks	*P* value[2]
Energy (kcal/d)[3]	1964 ± 59	1944 ± 111[#]	2013 ± 107[#]	2055 ± 130	1937 ± 140	2177 ± 83	2176 ± 118	2238 ± 97[#]	2159 ± 141	0.30
Protein (total)										
(g/d)[3]	81 ± 2	77 ± 5	74 ± 4	129 ± 4[*¥#]	125 ± 5[*¥#]	86 ± 4	82 ± 5	131 ± 6[*¥#]	125 ± 3[*¥#]	< 0.001
(g/kg/d)[3]	1.03 ± 0.02	0.97 ± 0.05	0.93 ± 0.03	1.64 ± 0.07[*¥#]	1.60 ± 0.05[*¥#]	1.10 ± 0.05	1.04 ± 0.07	1.63 ± 0.07[*¥#]	1.58 ± 0.07[*¥#]	< 0.001
(%)	16.8 ± 0.4	15.9 ± 0.7	15.1 ± 0.9	25.6 ± 1.3[*¥#]	26.4 ± 1.4[*¥#]	15.8 ± 0.7	15.2 ± 0.8	23.5 ± 0.6[*¥#]	23.8 ± 1.4[*¥#]	< 0.001
Protein (meal specific)										
Breakfast (g/kg)[3]	0.22 ± 0.02	0.23 ± 0.06	0.16 ± 0.03	0.54 ± 0.04[*¥#]	0.51 ± 0.05[*¥#]	0.22 ± 0.02	0.20 ± 0.02	0.52 ± 0.06[*¥#]	0.50 ± 0.04[*¥#]	< 0.001
Lunch (g/kg)[3]	0.28 ± 0.02	0.26 ± 0.05	0.22 ± 0.03	0.59 ± 0.03[*¥#]	0.58 ± 0.05[*¥#]	0.27 ± 0.04	0.28 ± 0.03	0.62 ± 0.03[*¥#]	0.55 ± 0.04[*¥#]	< 0.001
Dinner (g/kg)[3]	0.46 ± 0.02	0.45 ± 0.05	0.45 ± 0.05	0.57 ± 0.04	0.49 ± 0.04	0.48 ± 0.04	0.46 ± 0.07	0.43 ± 0.04	0.47 ± 0.02	0.41
Carbohydrate										
(g/d)[3]	232 ± 8	235 ± 11	250 ± 15	200 ± 21	209 ± 16	279 ± 16[‡$#]	280 ± 11[‡$#]	221 ± 11	238 ± 16	0.007
(%)[3]	48.5 ± 1.4	48.8 ± 3.6	49.9 ± 2.5	39.0 ± 3.6	43.1 ± 1.5	51.2 ± 2.0[‡$]	52.1 ± 2.2[‡$#]	39.7 ± 1.8	44.4 ± 2.1	0.03
Fat										
(g/d)[3]	68 ± 3	68 ± 5	71 ± 6	71 ± 10	55 ± 8	70 ± 5	68 ± 8	78 ± 5	62 ± 8	0.06
(%)[3]	31.0 ± 1.0	29.4 ± 1.3	31.4 ± 1.4[$]	32.5 ± 1.7	25.3 ± 1.9	31.5 ± 2.5	27.6 ± 2.1	30.7 ± 2.2	25.3 ± 1.7	0.08

[1]Values are means ± SE. Baseline values for individual groups are not shown but no significant differences occurred between groups for any dietary marker. [2]*P* value refers to respective group-by-time interaction.[3]Significant main effect of time ($P < 0.05$). *Significantly ($P < 0.05$) greater than CON group at respective time point. [‡]Significantly greater than PRO group at respective time point. [¥]Significantly greater than EX+CON group at respective time point. [$]Significantly greater than EX+PRO group at respective time point. [#]$P < 0.05$ from baseline value.

3.4 Habitual physical activity

No differences in daily step count (P = 0.61, f = 0.24), or time spent sedentary (P = 0.45, f = 0.30), or in light (P = 0.67, f = 0.22) or MVPA (P = 0.80, f = 0.21) occurred between groups over time. No significant within-group differences occurred.

3.5 Muscle strength

Significant group-by-time interactions were observed for both leg extension (P < 0.001, f = 1.13; Fig. 3A) and leg press 1RM (P < 0.001, f = 1.76; Fig. 3B). Both variables significantly increased over time in the EX+CON (+38%, P < 0.001, d = 1.20; +28%, P < 0.001, d = 1.18, respectively) and EX+PRO groups (+36%, P < 0.001, d = 1.74; +33%, P < 0.001, d = 1.81, respectively) greater than the CON and PRO groups (P < 0.001, d = 1.50-1.90). No differences were observed between either the CON and PRO groups (P > 0.98; d = 0.11-0.31), or the EX+CON and EX+PRO groups (P > 0.17; d = 0.00-0.53). When whey protein supplement groups were pooled, leg press 1RM did, however, tend to increase with a medium effect greater than control supplement groups pooled (P = 0.058, f = 0.35; Fig. 3C). No significant within- or between-group differences in handgrip strength occurred.

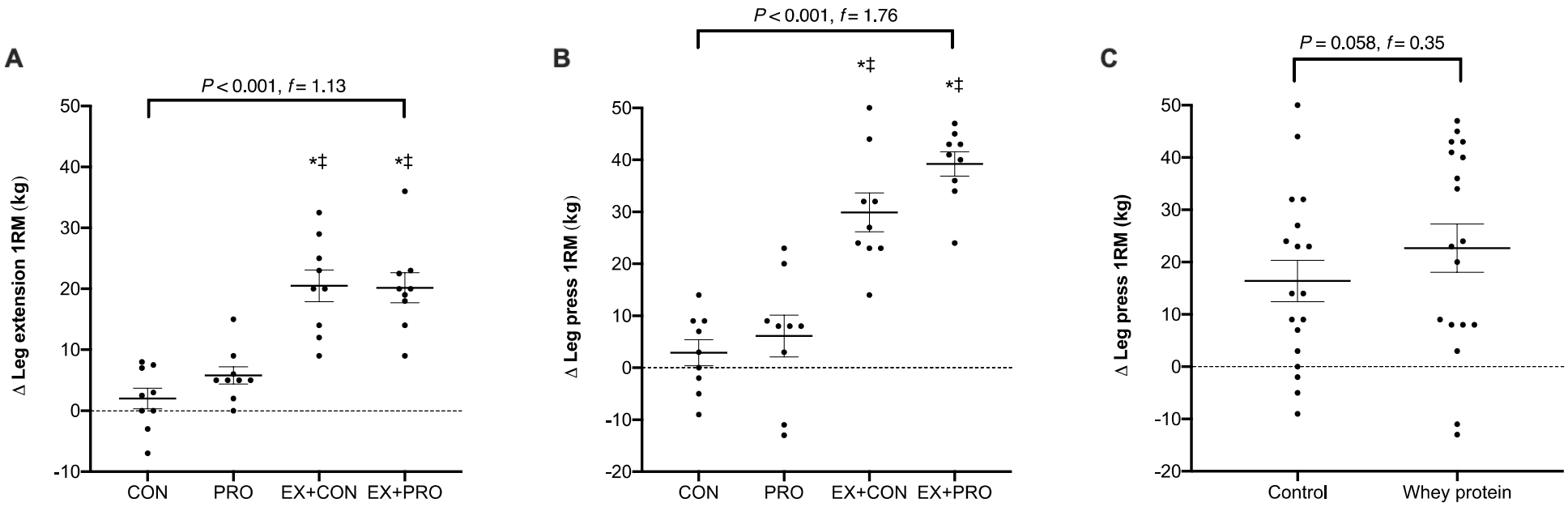


Figure 3 Changes in (A) leg extension 1RM (kg) and (B) leg press 1RM (kg) between intervention groups (CON, *n* = 9; PRO, *n* = 9; EX+CON, *n* = 9; EX+PRO, *n* = 9); and (C) change in leg press 1RM (kg) between pooled whey protein (*n* = 18) and control supplement groups (*n* = 18). Data are presented as means ± SE with circles representing individual data points. Analyses were performed using a mixed-model ANCOVA with baseline value only included as a covariate (panels A and B) and baseline value and exercise/non-exercise included as covariates (panel C). 1RM, one repetition maximum. *Significantly ($P < 0.05$) greater than CON group. ‡Significantly greater than PRO group.

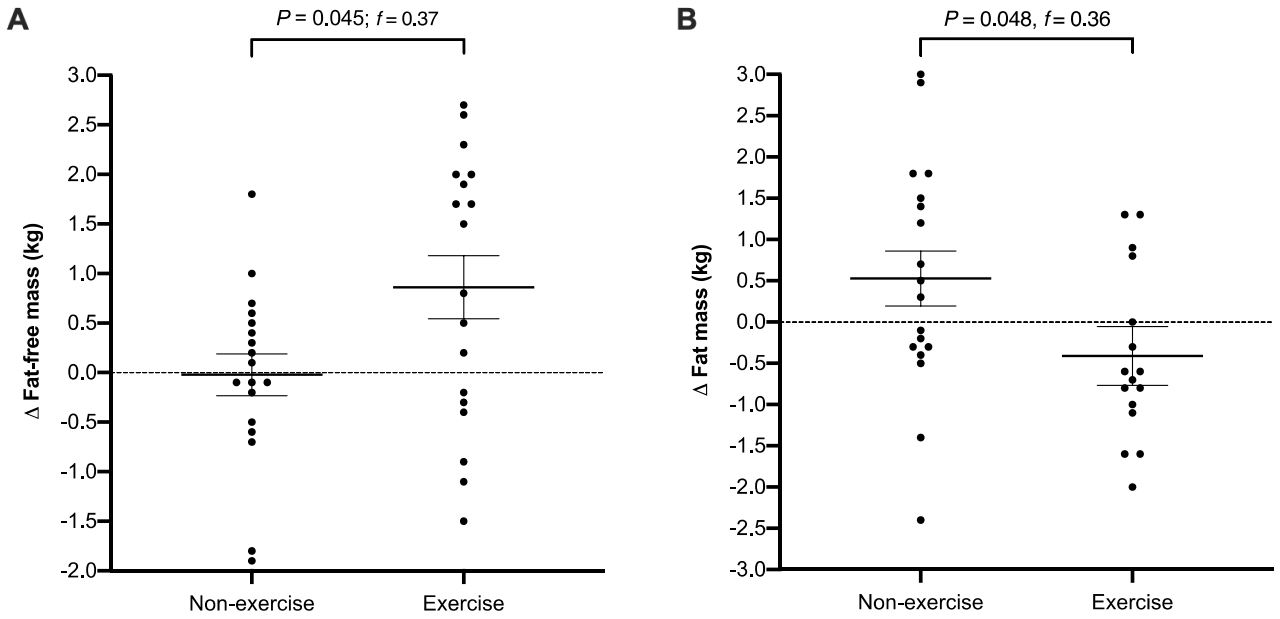
494 *3.6 Body composition*

495 No significant within- or between-group differences occurred over time for skeletal muscle or
496 FFM (Table 4); however, when exercise groups were pooled, FFM increased over time greater
497 than non-exercise groups pooled (P = 0.045, f = 0.37; Fig. 4A). Fat mass and BMI significantly
498 increased over time in the CON group (P < 0.05, d = 0.07-0.13), and FM decreased, but not
499 significantly, by -0.9 ± 0.5 kg (P = 0.09, d = 0.20) in the EX+PRO group. When expressed as
500 a percentage, significant differences in FM over the course of the study were observed
501 between the CON and EX+PRO groups (P = 0.03, d = 0.67). Also, when exercise groups were
502 pooled, FM significantly decreased compared to non-exercise groups pooled (P = 0.048, f =
503 0.36; Fig. 4B). In only the EX+PRO group, waist circumference significantly decreased over
504 time (P = 0.01, d = 0.12).

Table 4 Body composition outcomes for each treatment group at baseline and 12 weeks[1]

	CON		PRO		EX+CON		EX+PRO		*P* value	
	Baseline	12 weeks	Baseline	12 weeks	Baseline	12 weeks	Baseline	12 weeks	Time	Group x time
Body mass (kg)	79.0 ± 3.4	79.8 ± 3.3[#]	78.0 ± 3.1	78.3 ± 3.3	78.2 ± 3.9	79.0 ± 3.8	80.9 ± 4.0	81.0 ± 4.1	0.48	0.74
BMI (kg/m^2)	25.1 ± 1.0	25.3 ± 0.9[#]	25.0 ± 0.6	25.1 ± 0.7	25.1 ± 0.9	25.3 ± 0.9	26.6 ± 0.8	26.6 ± 0.8	0.45	0.80
FFM (kg)	59.8 ± 1.5	59.8 ± 1.6	60.0 ± 1.7	60.0 ± 1.9	58.5 ± 2.6	59.2 ± 2.5	60.5 ± 2.9	61.5 ± 2.9	0.92	0.23
SMM (kg)	26.7 ± 0.6	26.7 ± 0.7	27.2 ± 0.7	27.5 ± 0.8	25.9 ± 1.1	26.4 ± 1.1	26.9 ± 1.3	27.5 ± 1.3	0.99	0.35
SMI (kg/m^2)	8.5 ± 0.2	8.5 ± 0.2	8.8 ± 0.2	8.9 ± 0.2	8.3 ± 0.2	8.4 ± 0.2	8.9 ± 0.3	9.1 ± 0.2	0.25	0.23
Fat mass (kg)	19.2 ± 2.4	20.1 ± 2.2[#]	18.0 ± 1.7	18.3 ± 1.9	19.6 ± 2.0	19.8 ± 2.1	20.4 ± 1.5	19.5 ± 1.7	0.97	0.08
Fat mass (%)	23.8 ± 2.0	24.7 ± 1.9[#$]	22.7 ± 1.5	22.9 ± 1.7	24.8 ± 1.7	24.6 ± 2.0	25.1 ± 1.2	23.8 ± 1.5	0.72	0.04
FMI (kg/m^2)	6.1 ± 0.7	6.4 ± 0.7[#]	5.7 ± 0.5	5.9 ± 0.6	6.3 ± 0.6	6.3 ± 0.6	6.7 ± 0.5	6.4 ± 0.5	0.89	0.07
Waist circumference (cm)	92.5 ± 2.6	92.8 ± 2.4	92.8 ± 3.0	93.1 ± 3.1	91.3 ± 3.5	91.7 ± 3.7	98.1 ± 3.4	97.0 ± 3.3[#]	0.82	0.44
Waist:hip ratio	0.93 ± 0.02	0.92 ± 0.02	0.91 ± 0.02	0.91 ± 0.02	0.91 ± 0.02	0.91 ± 0.02	0.97 ± 0.02	0.96 ± 0.02	0.53	0.65

[1]Values are means ± SE. BMI, body mass index; FFM, fat-free mass; FMI, fat mass index; SMI, skeletal muscle index; SMM, skeletal muscle mass. [$]Significantly ($P < 0.05$) greater than EX+PRO group. [#]$P < 0.05$ from baseline.



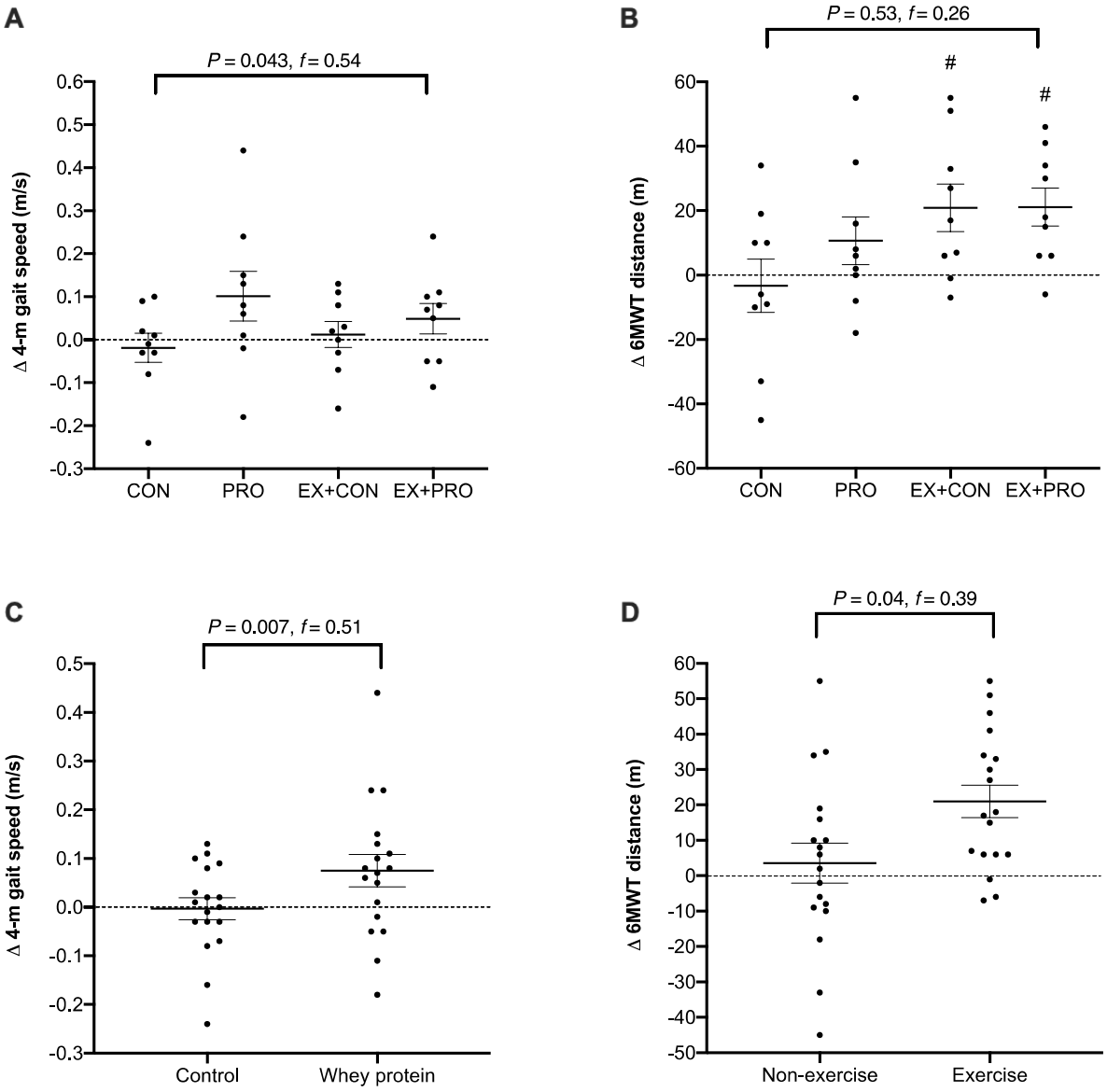
505

506 **Figure 4** Changes in (A) fat-free mass (kg) and (B) fat mass (kg) between pooled exercise (*n*
507 = 18) and non-exercise groups (*n* = 18) (means ± SE). Circles represent individual data points.
508 Data were analysed by mixed-model ANCOVA with baseline value and supplement consumed
509 (whey protein or control) included as covariates.

510

511 *3.7 Physical function*

512 A significant group-by-time interaction was observed for 4-m gait speed (P = 0.043, f = 0.54;
513 Fig. 5A) but not SPPB (P = 0.84, f = 0.17) or 6MWT distance (P = 0.53, f = 0.26; Fig. 5B). In
514 the PRO group, gait speed increased by 0.11 ± 0.06 m/s (d = 0.65), which tended to increase
515 over time greater than the CON group (P = 0.06, d = 0.64). When whey protein supplement
516 groups were pooled, 4-m gait speed increased greater than control supplement groups pooled
517 (P = 0.007, f = 0.51; Fig. 5C). Significant within-group increases in 6MWT distance occurred
518 in both the EX+CON (+3.3%; P = 0.02, d = 0.23) and EX+PRO groups (+3.6%; P = 0.007, d
519 = 0.28). When RE groups were pooled, 6MWT distance increased greater than non-exercise
520 groups pooled (P = 0.04, f = 0.39; Fig. 5D). No significant within-group differences were
521 observed for the SPPB.

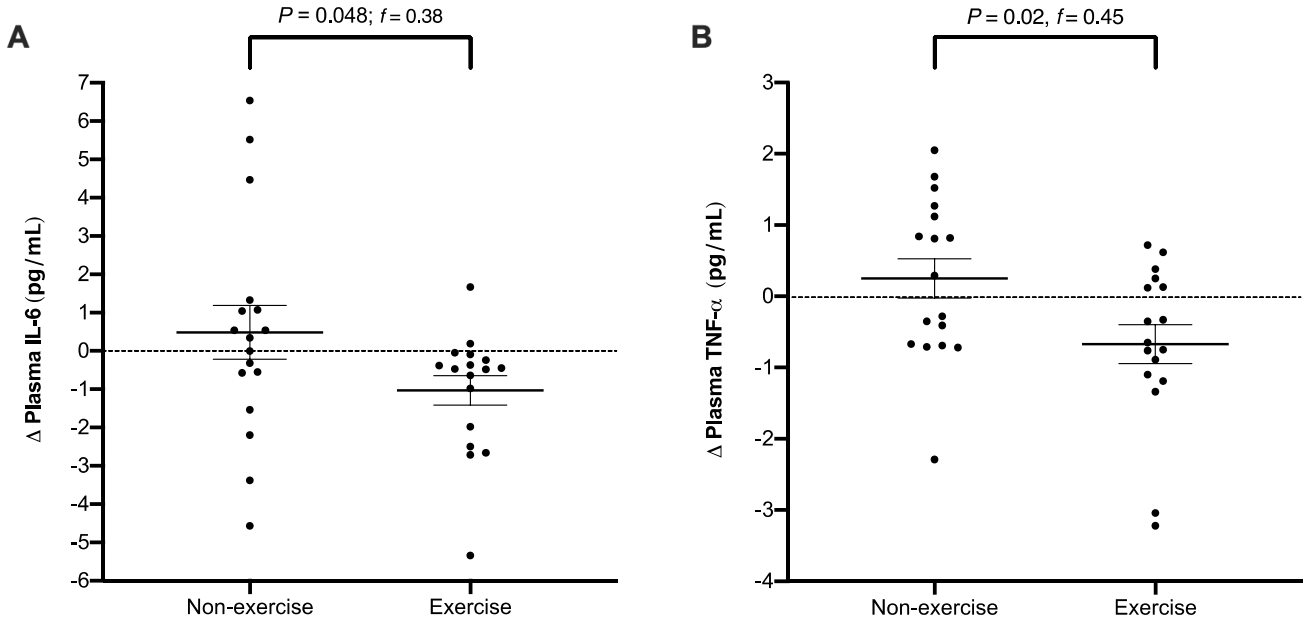


522

523 **Figure 5** Changes in (A) 4-m gait speed (m/s) and (B) 6MWT distance (m) between
524 intervention groups (CON, *n* = 9; PRO, *n* = 9; EX+CON, *n* = 9; EX+PRO, *n* = 9); (C) change
525 in 4-m gait speed (m/s) between pooled whey protein (*n* = 18) and control supplement groups
526 (*n* = 18); and (D) change in 6MWT distance (m) between pooled exercise (*n* = 18) and non-
527 exercise groups (*n* = 18). Data are presented as means ± SE with circles representing
528 individual data points. Data were analysed using a mixed-model ANCOVA with baseline value
529 only included as a covariate (panels A and B), baseline value and exercise/non-exercise
530 included as covariates (panel C), and baseline value and supplement consumed (whey protein
531 or control) included as covariates (panel D). 6MWT, 6-min walk test. $^{\#}P < 0.05$ from baseline.

3.8 Hormonal and inflammatory biomarkers

Plasma insulin significantly decreased in only the EX+CON group (-13.9%; $P = 0.04$, $d = 0.31$), but no differences occurred between groups ($P = 0.54$, $f = 0.29$; Table 5). Plasma IL-6 and TNF-α significantly decreased over time in the EX+PRO group (-21%; $P = 0.01$, $d = 0.26$; -20%; $P = 0.03$, $d = 0.65$, respectively). In the EX+CON group, similar but non-significant decreases were observed (-25%, $P = 0.15$, $d = 0.38$; -21%, $P = 0.21$, $d = 0.51$, respectively). No differences occurred between groups for either variable ($P = 0.13$, $f = 0.46$; $P = 0.11$, $f = 0.48$, respectively); however, when RE groups were pooled, both IL-6 ($P = 0.048$, $f = 0.38$; Fig. 6A) and TNF-α ($P = 0.02$, $f = 0.45$; Fig. 6B) significantly decreased over time greater than non-exercise groups pooled. Salivary cortisol slope increased in only the EX+PRO group (+91%; $P = 0.02$, $d = 1.00$), which was driven by an increase in concentration upon waking (0650 h) (+84.9%; $P = 0.06$, $d = 0.84$). When whey protein supplement groups were pooled, awakening salivary cortisol concentration significantly increased greater than control supplement groups pooled ($P = 0.049$, $f = 0.37$; Fig. 7A). Serum myostatin concentration also significantly increased greater in the pooled whey protein compared to control supplement group ($P = 0.01$, $f = 0.51$; Fig. 7B). No significant within- or between-group differences were observed for any other salivary cortisol index, or for plasma CRP, annexin A1 or IL-10, or serum IGF-1. No significant correlations were observed between changes in SMM, strength or physical function and any hormonal or inflammatory biomarker.



551

552 **Figure 6** Changes in fasting plasma (A) IL-6 (pg/mL) and (B) TNF-α (pg/mL) concentration
553 between pooled exercise (*n* = 17) and non-exercise groups (*n* = 17) (means ± SE). Circles
554 represent individual data points. Data were analysed by mixed-model ANCOVA with baseline
555 value and supplement consumed (whey protein or control) included as covariates. IL-6,
556 interleukin-6; TNF-α, tumor necrosis factor-alpha.

Table 5 Fasting hormonal and inflammatory biomarkers and salivary cortisol indices for each treatment group at baseline and 12 weeks[1]

	CON		PRO		EX+CON		EX+PRO		*P* value	
	Baseline	12 weeks	Baseline	12 weeks	Baseline	12 weeks	Baseline	12 weeks	Time	Group x time
Serum IGF-1 (ng/mL)[2]	152 ± 34	130 ± 29	119 ± 17	110 ± 14	137 ± 16	119 ± 12	118 ± 15	100 ± 10	0.07	0.86
Serum myostatin (ng/mL)[3]	2.2 ± 0.5	2.0 ± 0.4	1.9 ± 0.3	2.2 ± 0.3	1.8 ± 0.3	1.6 ± 0.1	2.3 ± 0.2	2.4 ± 0.3	0.06	0.15
Plasma insulin (mU/L)[2]	10.5 ± 1.6	10.5 ± 2.0	10.3 ± 1.5	8.9 ± 2.1	11.5 ± 2.9	9.9 ± 2.8[#]	8.8 ± 1.8	6.7 ± 1.3	0.93	0.54
Plasma IL-6 (pg/mL)[2]	4.9 ± 1.2	5.8 ± 1.3	4.0 ± 1.0	4.0 ± 1.2	3.2 ± 0.9	2.4 ± 0.6	5.8 ± 1.8	4.6 ± 1.2[#]	0.09	0.13
Plasma TNF-α (pg/mL)[2]	3.2 ± 0.3	3.0 ± 0.3	2.4 ± 0.3	3.0 ± 0.3	3.4 ± 0.6	2.7 ± 0.3	3.0 ± 0.4	2.4 ± 0.2[#]	< 0.001	0.11
Plasma CRP (ng/mL)[2]	2.4 ± 0.6	1.7 ± 0.3	1.6 ± 0.4	1.1 ± 0.5	0.8 ± 0.2	0.8 ± 0.2	2.0 ± 0.4	1.8 ± 0.2	0.18	0.18
Plasma IL-10 (pg/mL)[2]	6.4 ± 0.8	7.0 ± 1.0	6.1 ± 0.7	5.7 ± 0.6	7.0 ± 1.4	6.1 ± 0.9	7.7 ± 0.9	7.0 ± 0.8	0.008	0.61
Plasma annexin A1 (pg/mL)[2]	444 ± 28	434 ± 29	447 ± 36	537 ± 127	561 ± 70	509 ± 51	568 ± 101	569 ± 101	0.33	0.71
Salivary cortisol (0650 h) (nmol/L)[3]	8.2 ± 1.6	9.4 ± 1.3	9.3 ± 1.5	14.8 ± 2.7	11.1 ± 1.7	13.0 ± 3.2	9.3 ± 1.9	17.2 ± 4.0	< 0.001	0.21
Salivary cortisol (2000 h) (nmol/L)[3]	2.1 ± 0.5	1.9 ± 0.5	1.9 ± 0.4	2.2 ± 0.6	2.2 ± 0.6	1.8 ± 0.7	2.1 ± 0.7	2.6 ± 0.6	0.009	0.64
Salivary cortisol AUC (nmol/L x 790 min)[3]	4067 ± 551	4349 ± 528	4088 ± 196	45450 ± 425	5588 ± 969	5326 ± 778	4127 ± 587	4530 ± 414	0.001	0.99
Salivary cortisol slope (nmol/L)[3]	9.6 ± 1.1	9.1 ± 1.4	10.6 ± 0.8	13.6 ± 2.4	12.5 ± 2.5	15.7 ± 2.7	8.8 ± 1.4	16.9 ± 3.5[#]	0.004	0.15

[1]Values are means ± SE. [2]*n* = 34 (CON, *n* = 8; PRO, *n* = 9; EX+CON, *n* = 8; EX+PRO, *n* = 9). [3]*n* = 33 (CON, *n* = 8; PRO, *n* = 8; EX+CON, *n* = 8; EX+PRO, *n* = 9). AUC, area under the curve; CRP, C-reactive protein; IGF-1, insulin-like growth factor 1; IL-6, interleukin-6; IL-10, interleukin-10; TNF-α, tumor necrosis factor-alpha. [#]*P* < 0.05 from baseline value.

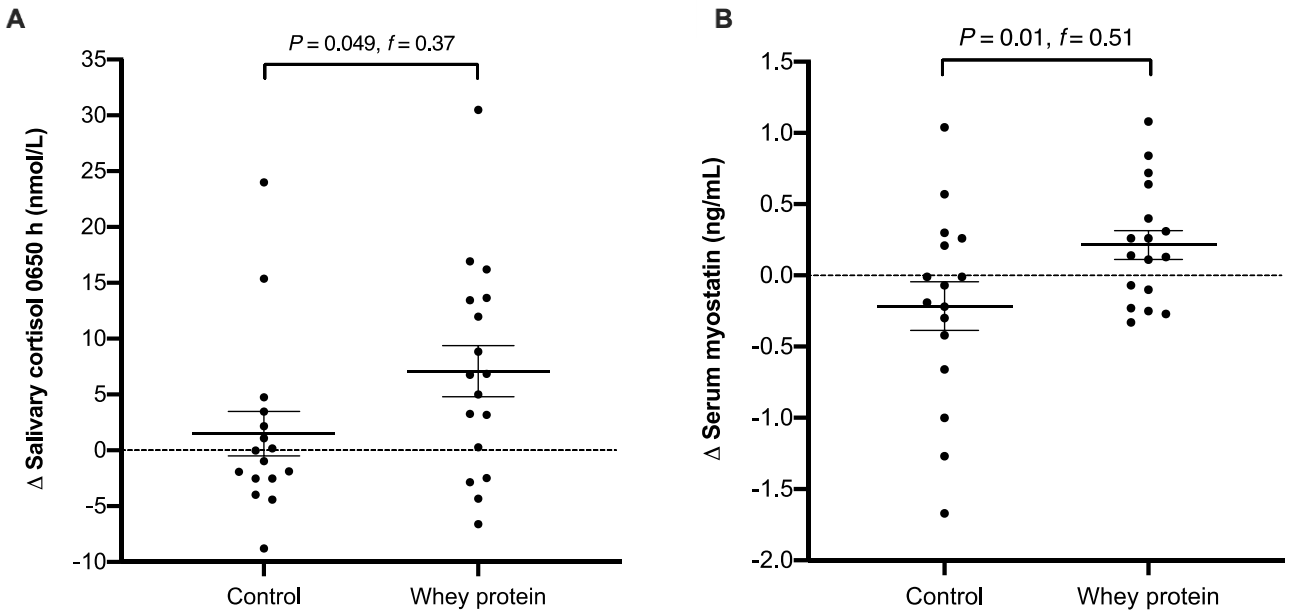


Figure 7 Changes in (A) 0650 h salivary cortisol (nmol/L) and (B) fasting serum myostatin concentration (ng/mL) between pooled whey protein (*n* = 17) and control supplement groups (*n* = 16) (means ± SE). Circles represent individual data points. Awaking salivary cortisol concentration data was analysed using the Scheirer-Ray-Hare two-way ANOVA of ranks test with baseline rank and exercise or non-exercise included as covariates. Fasting serum myostatin concentration data was analysed by mixed-model ANCOVA with baseline value and exercise or non-exercise included as covariates.

4. Discussion

To our knowledge, the present study is the first to investigate both the individual and combined effects of RE and whey protein supplementation using recent recommendations for protein dosing (>0.4 g/kg/meal; 1.6 g/kg/d), employing a double-blind, placebo-controlled design, on sarcopenia outcomes and hormonal and inflammatory biomarkers, including measurement of diurnal salivary cortisol under highly controlled conditions, in healthy active older men. The main findings were: i) RE significantly increased muscle strength, FFM and physical function, and decreased markers of systemic inflammation and fat mass compared to non-exercise; ii) whey protein supplementation significantly increased 4-m gait speed and increased muscle

strength (leg press 1RM) by a medium effect compared to an isocaloric carbohydrate control; however, increased awakening salivary cortisol and serum myostatin concentrations; iii) no synergistic effects occurred for any sarcopenia outcome compared to RE or whey protein supplementation alone; and iv) changes in sarcopenia outcomes did not correlate with changes in hormonal or inflammatory biomarkers.

Twelve weeks of progressive whole-body RE resulted in a combined mean increase in FFM of 0.9 ± 0.3 kg (+1.2%), of which 0.6 ± 0.2 kg (+2.3%) was estimated to be an increase in SMM. The magnitude of FFM increase is in line with previous studies that observed increases of ~1 kg following 12 weeks of RE in older adults (Campbell et al., 1995; Holwerda et al., 2018; Leenders et al., 2013; Verdijk et al., 2009). Accompanying the observed increase in FFM, the present study observed 36%, 31%, and 3.4% increases in leg extension and leg press 1RM, and 6MWT distance, respectively. These findings add to the current body of literature that have reported similar increases in muscle strength (Arnarson et al., 2013; Bell et al., 2017; Holwerda et al., 2018; Kirk et al., 2019; Leenders et al., 2013; Verdijk et al., 2009) and physical function as measured by the 6MWT following ≥12 weeks of RE training alone (Arnarson et al., 2013; Oesen et al., 2015) or combined with aerobic exercise in older adults (Bell et al., 2017; Kirk et al., 2019).

The present study observed a greater increase in 4-m gait speed and a medium, albeit non-significant effect towards a greater increase in muscle strength (leg press 1RM) following ingestion of whey protein supplementation compared to a carbohydrate control twice daily. These outcomes are in agreement with others that reported increases in muscle strength and/or physical function following increased dietary protein intake in older adults (Bauer et al., 2015; Bell et al., 2017; Kang et al., 2020; ten Haaf et al., 2019; Tieland et al., 2012b). Nevertheless, the novelty of data presented in the present compared to these studies is that protein supplementation, without additional nutrients known to stimulate hypertrophy and in healthy active non-sarcopenic older adults with habitual protein intakes >1.0 g/kg/d, is an

effective strategy to improve physical function and mitigate sarcopenia. It is hypothesised that a more evenly distributed dietary protein intake produced by ingestion of whey protein supplementation at breakfast and lunch daily, which led to a protein intake of >0.4 g/kg/meal, the required dose to maximally stimulate rates of MPS in older adults (Moore et al., 2015), may partly explain these beneficial effects in this healthy active population. In support, previous cross-sectional studies have reported an association between evenly distributed dietary protein intake and increased muscle strength and physical function in older adults (Loenneke et al., 2016; ten Haaf et al., 2018). Whilst the effect of whey protein supplementation on muscle function was far inferior to that of RE (~10-30% of the effect of RE), as not all older adults are able or willing to perform RE (Dismore et al., 2020), these findings suggest that higher intakes of dietary protein, which is evenly distributed across the day, may be of clinical importance to attenuate age-related declines in these individuals.

Although whey protein supplementation aided muscle function, no effect was observed on skeletal muscle or FFM, which is in agreement with some (Björkman et al., 2020; Cramer et al., 2016; de Carvalho Bastone et al., 2020; Kim et al., 2012; Kirk et al., 2020; Kukuljan et al., 2009; Verreijen et al., 2017; Zhu et al., 2015) but not all previous studies (Bauer et al., 2015; Bell et al., 2017; Bo et al., 2019; Kang et al., 2020; Mitchell et al., 2017; Negro et al., 2019; Norton et al., 2016; ten Haaf et al., 2019). It has been suggested that disparities between previous studies may be explained by differences in the increase of dietary protein intake from baseline (≥0.4 vs. <0.4 g/kg/d) (Park et al., 2018). However, the findings of this study oppose this hypothesis as dietary protein intake was increased by 0.6 g/kg/d. These data contrast others that increased dietary protein intake by 0.4-0.6 g/kg/d (Bell et al., 2017; Norton et al., 2016). Of note, the whey protein-based multi-ingredient supplement investigated by Bell and colleagues (2017) contained creatine, vitamin D and omega-3 polyunsaturated fatty acids, all of which have been shown to aid muscle hypertrophy (Devries and Phillips, 2014; Rosendahl-Riise et al., 2017; Smith et al., 2015). Also, the study by Norton et al. (2016) was double the duration of the present study, which might have provided a greater timeframe for protein-

induced increases in FFM. Therefore, inconsistencies between studies may be explained by differences in the instrumentation used to measure FFM, as most studies that report beneficial effects used dual x-ray absorptiometry (DXA), which is associated with less error than BIA (Achamrah et al., 2018).

This study tested the hypothesis that twice daily ingestion of a leucine-rich whey protein supplement would augment the effects of RE on SMM, strength and physical function. Despite gains in these outcomes following RE training alone and improved muscle function following whey protein supplementation, no augmented effects were observed. These findings are consistent with the majority (Arnarson et al., 2013; Candow et al., 2006; Chalé et al., 2013; de Carvalho Bastone et al., 2020; Dulac et al., 2020; Englund et al., 2018; Fielding et al., 2017; Gryson et al., 2014; Hofmann et al., 2016; Holm et al., 2008; Holwerda et al., 2018; Kim et al., 2012; Kirk et al., 2020, 2019; Krause et al., 2019; Kukuljan et al., 2009; Leenders et al., 2013; Maesta et al., 2007; Maltais et al., 2016; Oesen et al., 2015; Ottestad et al., 2017; Shahar et al., 2013; Thomson et al., 2016; Verdijk et al., 2009; Verreijen et al., 2017) but not all previous studies in older adults (Bell et al., 2017; Daly et al., 2014; Huschtscha et al., 2021; Junior et al., 2018; Kang et al., 2019; Rondanelli et al., 2020, 2016; Tieland et al., 2012b; Verreijen et al., 2015; Yamada et al., 2019; Zdzieblik et al., 2015). Similar to that of many studies that did not observe synergistic effects, the population used in this study were non-frail, i.e., displayed high baseline physical function scores, were physically active, and consumed sufficient but not optimal amounts of dietary protein at baseline according to consensus groups (Bauer et al., 2013; Deutz et al., 2014). In contrast, most studies that observed synergistic effects recruited sarcopenic or frail older adults, or, as previously highlighted, supplemented participants with multi-ingredient supplements (Bell et al., 2017; Kang et al., 2019; Rondanelli et al., 2020, 2016; Tieland et al., 2012c; Verreijen et al., 2015; Yamada et al., 2019; Zdzieblik et al., 2015). Nevertheless, the originality of the present study design adds a significant contribution to the literature that in healthy active older adults with a sufficient (~1 g/kg/d) but not optimal habitual protein intake, using recent recommendations for protein dosing (>0.4

g/kg/meal; 1.6 g/kg/d) without additional nutrients known to stimulate muscle hypertrophy is ineffective at augmenting RE-induced improvements in sarcopenia outcomes. However, it is important to note that as an effect of whey protein supplementation on muscle function when whey protein groups were pooled was observed in this study, the lack of synergistic effects may also be due to the present study being underpowered to detect post-hoc differences between the EX+CON and EX+PRO groups. For example, the post-hoc effect size for leg press 1RM (*d* = 0.53) indicated 57 participants per group would have been required to determine a significant difference between the EX+CON and EX+PRO groups. Larger RCTs are therefore required to determine whether increased dietary protein intake in isolation [at a dose of ~1.6 g/kg/d (>0.4 g/kg/meal)] augments RE-induced effects in healthy older adults habitually consuming adequate amounts of dietary protein.

An interesting observation from the present study was the significant increase in awakening salivary cortisol and fasting plasma myostatin concentrations following termination of whey protein supplementation. Previously in this cohort, a significant increase in nocturnal protein oxidation and decreased protein balance have been reported following whey protein supplementation (Griffen, 2020). Together, these data indicate an increase in protein breakdown during the overnight fasting period, which has also been observed in older adults by others following termination of a high protein diet (Højfeldt et al., 2020). Glucocorticoids (e.g., the endogenous glucocorticoid cortisol) have been demonstrated to upregulate myostatin gene expression (Wang et al., 2016), an effect that may be mediated via glucocorticoid response elements in the promoter region of the myostatin gene (Qin et al., 2013). Furthermore, stress-induced catabolism by cortisol is thought to be myostatin dependent (Allen et al., 2010), suggesting a mechanistic link between cortisol concentration and regulation of myostatin. These novel findings highlight the importance of older individuals refraining from significantly reducing their dietary protein intake once commenced on a high protein diet to mitigate rises in nocturnal protein breakdown.

687 A key finding of this study was the significant reduction in markers of systemic inflammation
688 following RE training. Age-related, low-grade systemic inflammation, termed inflammaging
689 (Franceschi et al., 2006), is associated with numerous adverse health outcomes, including
690 cardiovascular disease, insulin resistance, and higher mortality risk (Calder et al., 2017).
691 Inflammation is also often cited in the aetiology of sarcopenia (Beyer et al., 2012). In the
692 present study, the pro-inflammatory cytokines IL-6 and TNF-α decreased by ~20% following
693 RE training alone and combined with whey protein supplementation. These findings are
694 consistent with others (Bell et al., 2018; Rondanelli et al., 2016; Sardeli et al., 2018); however,
695 these studies used multimodal exercise interventions (Bell et al., 2018), multi-ingredient
696 supplements which contained nutrients with anti-inflammatory properties (Bell et al., 2018;
697 Rondanelli et al., 2016), and only studies who employed a thrice weekly RE programme in the
698 meta-analysis by Sardeli et al. (2018) reported reductions in markers of systemic
699 inflammation. Consequently, the present data is original in that it highlights that as little as
700 twice weekly RE performed at a high load either alone, or in combination with increased dietary
701 protein intake without additional nutrients with known anti-inflammatory properties, is an
702 effective strategy to offset inflammaging in healthy older adults.
703
704 Whilst RE decreased markers of systemic inflammation, changes in sarcopenia outcomes did
705 not explain these reductions, which is in agreement with some (Hangelbroek et al., 2018) but
706 not all studies (Grosicki et al., 2019). Instead, the changes seen may be explained in part by
707 the differential change in fat mass observed between exercise and non-exercise groups.
708 Specifically, the reduction in central adiposity observed in the EX+PRO group, which is a well-
709 known causative factor of inflammaging (Beyer et al., 2012). In contrast to the effects on pro-
710 inflammatory cytokines, RE did not alter concentrations of the anti-inflammatory markers IL-
711 10 or annexin A1, suggesting the effects may be pro-inflammatory pathway specific. Thus,
712 given that elevated IL-6 in particular is strongly associated with advancing age, morbidity and
713 mortality (Beavers et al., 2010; Ershler, 1993), the findings of this study highlight the

714 importance of regular RE training in older age as a strategy to offset age-related increases in
715 pro-inflammatory cytokines and to prolong healthy ageing.
716
717 The present study is only one of few that has investigated both the independent and combined
718 effects of RE and increased dietary protein intake over a ≥10 week period on SMM, strength
719 and physical function in older adults (de Carvalho Bastone et al., 2020; Gryson et al., 2014;
720 Huschtscha et al., 2021; Kim et al., 2012; Kirk et al., 2020; Krause et al., 2019; Kukuljan et al.,
721 2009; Maesta et al., 2007; Shahar et al., 2013; Verreijen et al., 2017). As such, the current
722 research extends our understanding of this topic. In contrast to the above cited studies, the
723 present study incorporated all of the following: i) a randomised, double-blind, placebo-
724 controlled design; ii) the optimal dietary protein intake (>0.4 g/kg/meal; 1.6 g/kg/d) to
725 maximally augment RE-induced accretion of SMM (Moore et al., 2015; Morton et al., 2018);
726 and iii) measurement of multiple hormonal and inflammatory biomarkers related to sarcopenia,
727 including measurement of diurnal salivary cortisol under highly controlled conditions whilst
728 participants resided in respiration chambers. These are novel aspects of this study. Limitations
729 of this study include estimation of SMM using BIA, the small sample size per group and lack
730 of statistical power to determine post-hoc differences between whey protein and control
731 groups, inclusion of only men, and lack of familiarisation of physical function measures prior
732 to baseline testing. The sample size is, however, coherent with a recently published
733 exercise/protein 4-arm RCT in older adults (Huschtscha et al., 2021). Women were excluded
734 based on reported sex differences in the magnitude of adaptation to RE previously reported
735 in older adults (Da Boit et al., 2016). Nevertheless, as women account for a large proportion
736 of older adults, future studies should address this aspect. Whilst a learning effect over time
737 may have occurred for physical function measures that observed significant differences
738 between pooled groups (i.e., 6MWT and 4-m gait speed), previous work has indicated no
739 learning effect in older adults on these outcomes (Simonsick et al., 2000).
740
741

5. Conclusion

Twelve weeks of twice weekly RE significantly increased muscle strength, FFM and physical function and decreased circulating concentrations of pro-inflammatory biomarkers in healthy older men. Whey protein supplementation, which led to a protein intake of >0.4 g/kg/meal and 1.6 g/kg/d, was ineffective at increasing skeletal muscle or FFM and increased awakening salivary cortisol and serum myostatin concentrations; however, led to a greater increase in 4-m gait speed and a medium effect towards a greater increase muscle strength (leg press 1RM) compared to control supplements pooled. Despite these increases following RE and whey protein supplementation independently, no synergistic effects were observed for any sarcopenia outcome. Finally, data from this study suggests that changes in sarcopenia outcomes are not related to changes in hormonal or inflammatory biomarkers.

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Authors' Contributions

CG designed the study, conducted data collection and analysed data, and wrote the manuscript; JH provided support in the design, conduct and analysis of the study and contributed to writing and critical review of the manuscript; DR provided support in the design

of the study, and contributed to writing and critical review of the manuscript; MD provided support in the design of the study, and contributed to writing and critical review of the manuscript; AD critically reviewed the manuscript; MOW served as primary clinical advisor and critically reviewed the manuscript. All authors have read and approved the final version of the manuscript and agree with the order of author presentation.

Competing Interests

The whey protein supplement used in this study (Instantized BiPRO) was supplied by Agropur, Quebec, Canada. Agropur provided the supplement free of charge but had no involvement in data collection or analysis of this study. The authors declare no other conflicts of interest.

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