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The impact of dairy protein during limb immobilization and recovery on muscle size and protein synthesis; a randomized controlled trial.

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Abstract

Muscle disuse results in the loss of muscular strength and size, due to an imbalance between protein synthesis (MPS) and breakdown (MPB). Protein ingestion stimulates MPS, although it is not established if protein is able to attenuate muscle loss with immobilization (IM) or influence the recovery consisting of ambulatory movement followed by resistance training (RT). Thirty men (49.9±0.6 years) underwent 14 days of unilateral leg IM, 14 days of ambulatory recovery (AR) and a further 6 RT sessions over 14 days. Participants were randomized to consume an additional 20g of dairy protein or placebo with a meal during the intervention. Isometric knee extension strength was reduced following IM (-24.7 ± 2.7 %), partially recovered with AR (-8.6 ± 2.6%) and fully recovered after RT (-0.6 ± 3.4 %), with no effect of supplementation. Thigh muscle cross sectional area decreased with IM (-4.1 ± 0.5 %), partially recovered with AR (-2.1 ± 0.5 %), and increased above baseline with RT (+2.2 ± 0.5 %), with no treatment effect. Myofibrillar MPS, measured using deuterated water, was unaltered by IM, with no effect of protein. During AR MPS was increased only with protein supplementation. Protein supplementation did not attenuate the loss of muscle size and function with disuse or potentiate recovery, but enhanced myofibrillar MPS during AR.

New & Noteworthy

Twenty grams of daily protein supplementation does not attenuate the loss of muscle size and function induced by two weeks of muscle disuse or potentiate recovery in middle-age men. Average mitochondrial but not myofibrillar muscle protein synthesis was attenuated during immobilization with no effect of supplementation. Protein supplementation increased myofibrillar protein synthesis during a two week period of ambulatory recovery following disuse but without group differences in phenotype recovery.

Introduction

Periods of muscle disuse are a common consequence of illness, disability, and injury, including bone fractures. These episodes of extreme inactivity result in rapid muscle atrophy and loss of contractile function (7, 19, 34). Thus, immobility is a risk for falls (66), loss of independence (60), prolonged health care requiring hospitalization (71), and may further lead to premature mortality (44). There are currently limited therapeutic options for the management of disuse atrophy (20, 56), with significantly delayed or incomplete recovery a frequent consequence of even short periods of muscle disuse in the elderly (42, 74). Thus repeated periods of disuse may accelerate the age related loss of muscle mass (26) which begins in middle age (79).

Whilst only a few high intensity muscle contractions have been shown to effectively attenuate disuse atrophy, ongoing health needs and complications may limit the introduction of adequate mobilization and exercise (18, 20, 56). Nutritional interventions therefore may present a practical countermeasure to limit muscle loss during muscular disuse and immobility, and further aid recovery (17, 25, 29, 57, 81). Limited analysis in animal models have shown protein supplementation can be effective in attenuating muscle loss during extreme models of muscle disuse (47, 63). However, clinically the evidence is not well established. A recent study demonstrated that protein supplementation failed to attenuate muscle loss during a five day period of muscle disuse in older individuals (19). Conversely, high dose leucine supplementation may attenuate the loss of muscle size and strength following longer periods of bedrest (25) however, a lower dose of leucine contained within a high quality protein might have a similar effect (13). The potential ability of supplemental protein to enhance recovery following a period of muscle disuse has not been investigated. Therefore it needs to be established whether protein supplementation during a follow up period of habitual activity or structured exercise may accelerate recovery.

Fundamentally, muscle mass is regulated by the balance between synthesis and degradation of contractile (myofibrillar) proteins. Disuse atrophy is likely the result of both an increase in myofibrillar protein breakdown (MPB) and a suppression of myofibrillar protein synthesis (MPS) (67). However, the relative importance of these two processes has not yet been defined (59, 61). It is well established that a period of muscle disuse results in 'anabolic resistance' to protein feeding such that the MPS response to protein consumption is blunted after immobilization or inactivity compared to when the same dose of protein is ingested after normal muscle activity (7, 19, 34). This is in part responsible for the observed atrophy following muscle disuse (59). A similar anabolic resistance to feeding is often observed in older adults and can be partially overcome through the consumption of larger protein doses (16, 53). In the resting fasted state, anabolically resistant older adults are not able to further stimulate MPS with doses of protein beyond 20g of dairy protein (85) or the equivalent dose of essential amino acids (16). However, some authors have suggested that ~32g of protein would be required to maximally stimulate MPS in older men (53). In anabolically resistant muscle that is sensitized to feeding by prior resistance exercise, larger protein doses (~40g) may further stimulate MPS (85). This quantity of protein is not frequently achieved at morning or midday meals (11), but would be achieved when a standard meal is supplemented with 20g of diary protein. Therefore it is hypothesized that the consumption of additional high quality protein, with morning meals, during a period of limb immobilization might partially ameliorate anabolic resistance and attenuate the degree of muscle atrophy induced by disuse.

The purpose of the present study is to determine whether daily supplementation with 20g of high quality dairy protein, consumed with morning meals, is able to attenuate the reduction in muscle size and function, induced by unilateral lower limb immobilization in middle aged men. This model of disuse has previously been shown to result in significant but reversible

muscle atrophy in 14 days (33). The secondary aim was to determine if dairy protein supplementation would increase the rate of muscle recovery during both ambulatory recovery (AR) and resistance training (RT) follow ups. This study also analyzed the integrated MPS response to immobilization and recovery using deuterium labelling in order to determine the regulation of MPS in disuse atrophy and recovery.

Methods

Participants

Thirty healthy middle aged men between the ages of 45 and 60 years were recruited from Auckland, New Zealand through local newspaper advertisements. The participants were free from metabolic conditions such as diabetes, musculoskeletal impairments, and a history of heart disease, or cancer or a family history of blood clots. Smokers were excluded from the study. Participants taking medications such as statins, steroids or nonsteroidal anti-inflammatory drugs which may affect muscle biology were excluded. Plasma biochemistry was measured at the familiarization visit using a Roche C311 autoanalyzer, (Roche, Mannheim, Germany) by enzymatic colorimetric assay. Plasma insulin concentrations were analyzed using electrochemiluminescence immunoassay on a Cobas e11 (Roche, Mannheim, Germany) and are shown along with participant's demographic information in Table 1. All 30 participants randomized into the study completed the intervention. All participants who received the intervention or control supplements completed the trial. One participant in the protein group had an abdominal injury during the resistance training phase but was able to complete the full protocol. Participants provided informed oral and written consent before the commencement of the study. This study was approved by the Northern Health and Disability Ethics Committee (New Zealand) and is in compliance with the most recent declaration of Helsinki. The trial was registered with the Australia New Zealand Clinical Trial Registry# ACTRN12615000454572 on May 11, 2015.

Experimental design

The study consisted of a total of four phases over the course of seven weeks (Figure 1) during which time all participants underwent a 2 week period of unilateral lower limb immobilization. Participants were randomized to either the placebo or dairy protein

supplement groups in a parallel design, the leg to be immobilized was counter balanced based on dominance. Random sequences were generated with www.random.org and the dairy protein supplement was dispensed in a double blind manner. Allocation was conducted with a one to one ratio between groups by investigator MC and concealed using a locked spreadsheet. All testing took place at the Liggins Institute Clinical Research Unit. The *a priori* primary outcomes of the study were changes in knee extensor isometric strength and thigh muscle cross sectional area (CSA) at 50% of femur length. Prior to the beginning of the baseline (BL) phase a biopsy was obtained from the vastus lateralis muscle with a Bergström needle modified for manual suction under local anaesthetic (1% lidocaine). Participants were then familiarized with the muscle function testing procedure (described below). They were then asked to maintain their normal lifestyle and physical activity patterns for one week while wearing a wristband accelerometer (Fitbit Charge, San Francisco, California, United States) to count daily steps. After one week participants returned to the laboratory and underwent a Dual-energy X-ray absorptiometry (DXA) scan, peripheral quantitative computed tomography (pQCT) scan of the lower leg and thigh, and a second muscle biopsy from the same leg followed by muscle function testing. Afterwards a rigid knee brace (DonJoy, IROM, Vista, California, United States) was worn for 14 days on the contralateral leg from the first two biopsies. The brace was locked at 60º during the immobilization (IM) phase (33). Participants were provided with a set of crutches and asked to avoid any weight bearing on the immobilized leg. The brace was removed and re-adjusted every two days by an investigator who sealed the brace with tape and then signed the seal to ensure compliance (34). After two weeks of immobilization the brace was removed and the scans were repeated before a muscle biopsy was obtained from the immobilized leg and muscle function measurements were repeated. Participants were then asked to resume their normal lifestyle (ambulatory recovery, [AR]) for two weeks while physical activity and daily steps were

recorded. Following the AR, the biopsy, scans and muscle function testing were repeated, and a one repetition maximum (1-RM) for leg extension and leg press was estimated independently for each leg using the Brzycki equation (8). The final two week stage of the study consisted of thrice weekly resistance training (RT) sessions. Each RT session included four sets at 80% of 1-RM of both leg press and extension (Sygeum, Gym80, Gelsenkirchen, Germany). Each exercise was performed unilaterally with the previously immobilized leg always training first. Ten repetitions were performed for the first three sets of each exercise while the last set of each exercise was performed until the point of failure, this protocol has previously been shown to acutely increase MPS (14). The final scans, muscle biopsy and muscle function testing were performed 48 hours after the last RT session in order to minimize the effects of fatigue on functional tests and edema on the imaging measures, while still allowing 6 RT sessions to be completed in 14 days with at least one days rest between sessions.

Dairy protein supplement and dietary intake

Beginning at the start of the BL phase participants were prescribed a partially controlled diet with the aim of controlling protein intake at 1 g/kg/day, excluding the supplements. Dietary control was intended to reduce potential effects of variability in habitual diet on muscle mass (41) and MPS changes (85). Protein intake of 1g/kg/day was chosen because it has been recommended as the minimum to maintain lean mass and function in older adults (4), although the participants in the present study were middle aged. Previous work has suggested the adults within the study age range require more than the current recommended daily allowance of protein in order to maintain muscle mass (10). Energy requirements were estimated using the Harris-Benedict equation (65) to predict basal metabolic rate and an activity factor of 1.3 based on the requirements of men with low activity levels (1.4-1.6) and then adjusted slightly for under reporting of dietary intake (30). Breakfasts (25% of energy

and protein requirements, ~20g of protein) and evening meals (50% of energy and protein requirements, ~42g of protein) were provided to participants, which accounted for ~75% of protein and energy requirements, and individual dietary advice was provided for lunch meals to ensure participants met their protein and energy intake targets. The unequal balance of protein intake throughout the day is not optimal for maximizing MPS (55) but is reflective of normal eating habits (11). Once daily during the IM, AR, and RT phases, participants consumed either 20g of milk protein concentrate (MPC 485 [Fonterra Co-operative Group Ltd., Auckland, New Zealand], containing 2.1g of leucine)(52) or an isoenergetic carbohydrate placebo (maltodextrin); both supplements were vanilla flavored and artificially sweetened. Sachets containing the powdered supplements were provided in identical packaging and participants were instructed to add the powder to 250ml of water. The milk protein concentrate used in the study consisted of 80% casein and 20% whey protein and has previously been demonstrated to induce a similar MPS response to whey protein in middle age men (52). Supplements (protein or placebo) were consumed with a meal immediately after resistance training in order to stimulate post exercise MPS (15) or with breakfast on non-training days because morning meals often contain less protein than midday or evening meals (11). Twenty four hour urine collections were completed at the end of each phase of the study and protein intake was estimated based on urine urea nitrogen excretion as described by Maroni and colleagues (48). The average total protein intake for the three study phases when the dairy supplement was consumed were averaged for analysis. Dietary intake was analyzed using Foodworks software (Version 8, Xyris, Australia). To confirm that participants were in energy balance over the seven week period of diet control, changes in whole body DXA derived fat and lean mass were used to calculate average daily energy deficit or surplus (24). Chemical energy equivalents for changes in fat mass of 39.5 MJ/kg and lean mass of 7.6 MJ/kg were used (38).

Imaging

Whole body dual energy x-ray absorptiometry (DXA, Lunar Prodigy, GE, Waltham, MA, USA) scans were performed at the beginning and end of each study phase to assess body composition. The whole body scan was automatically segmented by the software, and segmentation of tissue regions were defined by lines positioned on the image. These lines were adjusted where necessary to include all of the tissue within the appropriate regions. Segmentation of all scans were completed by a single investigator who was blinded to treatment allocation.

Muscle CSA of the immobilized leg was assessed using a Stratec XCT 3000 peripheral quantitative computed tomography (pQCT) with software version 6.20C (Stratec Medizintechnik, Pforzheim, Germany). Participants were positioned supine with the immobilized leg centered within the machine's gantry and anchored by a foot rest with straps to limit movement during each scan. Muscle CSA was measured at 20% and 50% of femur length and at 66% of tibia length. Femur length was measured from the lateral knee joint space to the greater trochanter, and tibia length from the lateral malleolus to the lateral knee joint space. For the upper leg scans, a scout view scan was used to position the anatomical reference line at the distal femur joint surface. For the lower leg scan, the CT position was defined manually. The following measurements were obtained: total area with subcutaneous fat removed (mm^2), and cortical area (mm^2). Muscle CSA was determined as the difference in these measures. For analysis of the total area, threshold was set at 40 $mg \cdot cm^{-3}$ (contour mode 1/peel mode 1). For cortical area, the threshold was set at 280 $mg \cdot cm^{-3}$ (contour mode 1/peel mode 1). All pQCT scans were analyzed by the same operator.

Muscle function

230 Single leg vertical jump height was used as a measure of muscle power production.
231 Participants performed four maximal single leg vertical jumps on a force platform (Leonardo
232 Mechanograph GRFP, Novatech medical, Pforzheim, Germany) with a 5 s rest between
233 jumps, and the average of four jumps was reported. Isometric muscle strength of the knee
234 flexors and extensors was tested using a Biodex dynamometer (Shirley, New York, United
235 States) with the knee angle set to 90º of flexion. Five maximal contractions were performed
236 for each movement with 30 s of rest in-between and the highest values were used for
237 analysis. Muscle aerobic capacity was measured using an incremental single leg cycling test.
238 Participants were seated on a cycle ergometer (Velotron, Racemate, Seattle, Washington,
239 United States), with one foot attached to the pedal with tape, while the other pedal was
240 removed and in its place was an 8.5 kg counter weight. Participants cycled at 50 W for 1 min
241 and then the resistance was increased 2 W every 12 s until their cadence dropped below 50
242 RPM; motivation and encouragement was provided throughout the test.

243 *Real time polymerase chain reaction (RT-PCR)*

244 Gene expression of atrogenes (MuRF1/*TRIM63* and Atrogin-1/*FBXO32*) and myostatin
245 (*MSTN*) was analyzed by RT-PCR. RNA was extracted from ~15 mg of muscle tissue using
246 the AllPrep® DNA/RNA/miRNA Universal Kit (QIAGEN GmbH, Hilden, Germany)
247 following the manufacturer's instructions. High-Capacity RNA-to-cDNA™ kit (Life
248 Technologies, Carlsbad, CA, USA) was used to synthesise cDNA. RT-PCR was performed
249 using SYBR Green I Master Mix (Roche Applied Science, Penzberg, Germany) on a
250 LightCycler 480 II (Roche Applied Science). Primers used in this study are listed in Table 2.
251 For normalization purposes, several proposed stable reference genes, (*C1orf43*, *CHMP2A*,
252 *EMC7*, and *VCP*) (23), and genes commonly used as reference genes in the muscle literature
253 (*TBP*, *PPIA*, and *HPRT*) were analyzed. The geometric mean of the three most stable genes
254 across the whole population set for all timepoints (78) were used for normalization. The

levels of *TBP*, *HPRT* and *CHMP2A* mRNAs were identified as the three least variable, and were therefore used as reference genes. Standard and melting curves were performed for every target to confirm primer efficiency and single product amplification. The $2^{-\Delta CT}$ method was used to compare difference in raw gene expression (45).

Muscle protein synthesis

For two days prior to the first study visit participants consumed three 50ml aliquots of 70% deuterium oxide (D_2O, Isowater, Collingwood, Ontario, Canada) each day, and they continued to consume thrice daily aliquots (morning, midday, evening) of deuterium oxide for the first five days of the study. For the remainder of the study participants consumed two daily (morning, evening) 50ml aliquots of 70% D_2O. This protocol has previously been demonstrated to rapidly achieve a precursor pool enrichment of 1-2% (64, 69). Plasma samples were collected weekly to measure isotopic enrichment of total body water.

Skeletal muscle tissue was fractionated according to previously published procedures (21, 22, 49, 50, 64). Muscle (30–50 mg) was homogenized 1:10 with a bead homogenizer (Next Advance, Averill Park, NY) in isolation buffer (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, pH 7.5) with phosphatase and protease inhibitors (HALT, Thermo Scientific, Rockford, IL). After homogenization, subcellular fractions of myofibrillar (containing primarily contractile elements and nuclei), and mitochondrial proteins were isolated via differential centrifugation as previously described (21, 22, 49, 50, 64, 69). After muscle fractions were isolated and purified, 250 µl 1 M NaOH was added, and pellets were incubated for 15 min at 50°C and centrifuged at 900 rpm. Protein was hydrolyzed by incubation for 24 h at 120°C in 6 N HCl. The hydrolysates were ion exchanged, dried under vacuum, and resuspended in 1 ml molecular biology grade H_2O. 500 µl of suspended samples were derivatized [500 µl acetonitrile, 50 µl 1 M K_2HPO_4 (pH = 11),

and 20 µl of pentafluorobenzyl bromide (Pierce Scientific, Rockford, IL)], sealed, and incubated at 100°C for 1 h. Derivatives were extracted into ethyl acetate. The organic layer was removed and dried by N_2 followed by vacuum centrifugation. Samples were reconstituted in 1 ml ethyl acetate then analyzed. The pentafluorobenzyl-N,N-di(pentafluorobenzyl) derivative of alanine was analyzed on an Agilent 7890B GC coupled to an Agilent 5977A MS as previously described (21, 22, 49, 50, 64, 69). To determine body water enrichment, 125 µl of plasma was placed into the inner well of an O-ring screw cap and inverted on a heating block overnight. Two microliters of 10 M NaOH and 20 µl of acetone were added to all samples and to 100 µl 0–20% D_2O standards then capped immediately. Samples were vortexed at low speed and left at room temperature overnight. Extraction was performed by the addition of 200 µl hexane. The organic layer was transferred through anhydrous Na_2SO_4 into GC vials and analyzed via EI mode with a DB-17MS column.

The newly synthesized fraction of proteins was calculated from the true precursor enrichment with plasma analyzed for D_2O enrichment and adjusted by mass isotopomer distribution analysis (MIDA) (9). Protein synthesis was calculated as the ratio of deuterium-labelled to unlabelled alanine (9) in proteins over the entire labelling period and then converted to fractional synthesis rate by dividing by time (day) and multiplying by 100.

Citrate Synthase (CS) activity

CS activity was measured from a separate piece of tissue. Briefly, tissue was weighed and homogenized in ice-cold homogenization buffer (25mM TRIS-HCL pH7.8, 1mM EDTA, 2mM MgCL2, 50mM KCL, 0.50% Triton X-100) using a TissueLyser II (Qiagen, Dusseldorf, Germany). Homogenates were centrifuged at 14,000 x g for 10 minutes at 4^{o}C, and the supernatant was frozen at -80^{o}C until further use. All assays were performed using the Molecular Devices Spectramax-340 96-well microplate reading spectrophotometer at

303 25°C. CS activity was determined by measuring absorbance at 412nm in 50mM Tris-HCL
304 (pH 8.0) with 0.2mM DTNB, 0.1mM acetyl-coA and 0.25mM oxaloacetate. Rate of change
305 of absorbance and path length of each well was determined using SoftMax pro version 3.1.1
306 (Molecular Devices, Sunnyvale, CA), and CS activity was calculated using an extinction
307 coefficient of 13.6$mM^{-1}cm^{-1}$.

308 *Statistical analysis*

309 Sample size was based on a 20% decrease in knee extension torque following immobilization
310 and was powered to detect a 50% attenuation in torque loss with 80% power, resulting in 15
311 participants per group. Estimated variability in torque loss was based on Dirks et al. (19) and
312 Oates et al.(56). Differences in muscle size, function and fractional synthetic rate were
313 assessed using a mixed models approach to repeated measures analysis of variance (SAS,
314 SAS Institutes, Cary, NC, version 9.4, [proc mixed function]). Baseline values were used as a
315 covariate, time was used as a within subjects fixed factor and supplement was used as a
316 between subject factor. The subject was added to the model as a random factor. Where
317 appropriate pairwise comparisons were examined using Tukey's post hoc test. PCR data was
318 analyzed using two-way ANOVA as a fold change from baseline. Two-way random effects
319 model interclass correlation coefficient (ICC) $_{(2,1)}$ was used to test the reliability of
320 measurements of muscle function and size between the familiarization visit (week 0) and the
321 baseline visit (week 1) (82). Least square means are shown as ± standard error of the mean
322 (SEM) in the figures and text, and ± standard deviation (SD) in the tables. Alpha was set at
323 $P<0.05$.

324

Results

Muscle strength and function

Reliability of all physical function measurements were excellent (knee extension ICC=0.92, keen flexion ICC=0.87), single leg cycling peak power ICC=0.96) with the exception of isometric plantar flexion (ICC=0.64) and single leg jump height (ICC=0.60) which were classified as good. Baseline knee extension torque was 231 ± 14 and 212 ± 19 Nm in the placebo and protein groups respectively, there were no group or group-by-time effects (P=0.839) but there was a main effect of time (P<0.0001). Knee extension torque decreased with IM, was partially recovered after AR and fully recovered after RT (Figure 2a). Baseline knee flexion torque was 89 ± 4 Nm in the placebo group and 90 ± 6 Nm. There were no group or group-by-time effects (P=0.542) but there was a main effect of time (P<0.0001). Knee flexion torque was also reduced with IM, and returned to baseline levels with AR (Figure 2b), and increased above baseline following RT. Baseline plantar flexion strength was 59 ± 7 Nm and 69 ± 8 Nm in the placebo and protein groups respectively. There were no group or group-by-time effects (P=0.257) but there was a main effect of time (P=0.019). Plantar flexion strength decreased with IM, increased above baseline with AR and increased further following RT (Figure 2c).

Single leg jump height was used as a measure of muscular power and at baseline was 25.7 ± 0.1 cm in the placebo group and 23.6 ± 0.1 cm in the protein group. There were no group or group-by-time effects (P=0.479) but there was a main effect of time (P<0.0001). Jump height decreased with IM, was partially recovered following AR and fully recovered following RT (Figure 3a). Peak power production during the incremental cycling test was 149 ± 9 W and 148 ± 9 W in the placebo and protein groups respectively at baseline. Peak power decreased with IM, was partially recovered following AR and fully recovered following RT (Figure 3b).

There were no group or group-by-time effects (P=0.314) but there was a main effect of time (P<0.0001).

Muscle size

Reliability of all imaging measures was excellent (ICC=0.99). Muscle CSA was measured using pQCT. Thigh muscle CSA at 50% femur length was 16469 ± 637 mm^2 and 16033 ± 831 mm^2 at baseline in the placebo and protein groups' respectively. There were no group or group-by-time effects (P=0.582) but there was a main effect of time (P<0.0001). Muscle CSA at 50% femur length was reduced with IM, partially recovered with AR and increased above baseline with RT (Figure 4a). Muscle CSA at 20% femur length was 8687 ± 426 mm^2 and 8726 ± 512 mm^2 at baseline in the placebo and protein group respectively. There were no group or group-by-time effects (P=0.943) but there was a main effect of time (P<0.0001). Thigh muscle CSA at 20% femur length reduced with IM, recovered with AR and increased above baseline with RT (Figure 4b). Calf muscle CSA at 66% tibia length was 9330 ± 345 mm^2 in the placebo group at baseline and 8867 ± 388 mm^2 in the protein group at baseline. Calf muscle CSA reduced with IM, recovered with AR and increased above baseline with RT (Figure 4c). There were no group or group-by-time effects (P=0.648) but there was a main effect of time (P<0.0001).

DXA was also used to estimate changes in muscle mass throughout the study. There were no changes in fat or lean mass of the control (non-immobilized) leg throughout the study with the exception of a main effect for an increase in lean mass from the post IM time point to the post RT time point (P=0.043) (data not shown). Fat mass of the immobilized leg did not change during the study. The immobilized leg decreased in lean mass from 10510 ± 339 g and 10412 ± 528 g in the placebo and protein group respectively by 127 ± 256 g in the protein group and 199 ± 256 g in the placebo group, however this change did not reach

significance (P=0.076). Lean leg mass increased above baseline after RT 116 ± 177 g and 123 ± 177 g in the protein and placebo groups respectively, although significance was not reached (P=0.060). However, there was a significant difference between the post IM and post RT time points (P=0.002)

Protein intake and activity level

Urea values from 24h urine collections were used to estimate different protein intakes of 67.6 ± 6.3 g/day in the placebo group and 91.1 ± 7.4 g/day in the supplement group (P=0.023). When expressed relative to body weight estimated protein intakes were 0.79 ± 0.09 g/kg/day in the control group and 1.08 ± 0.11 g/kg/day in protein group (P=0.06). Dietary intake (Table 3) was unchanged in fat or carbohydrate content across the study phases. Protein consumption increased in the protein supplementation group after the onset of supplementation (IM phase) and was maintained for the AR and RT phases. The pattern was apparent when consumption was expressed as total protein (P=0.004), relative to body weight (P=0.005) and as a percentage of energy intake (P=0.002). Participants in both groups self-reported to slightly exceeding their lunch protein targets resulting in an average daily protein intake of ~1.1g/kg/day in the diets of both groups exclusive of the supplements provided. Dietary protein intake at the midday meal was prescribed as ~20g however participants in both groups consumed ~30g of protein at the midday meal. Daily steps were tracked with a wrist worn accelerometer which counted baseline steps per day of 10060 ± 1042 and 11427 ± 1389 in the placebo and protein groups respectively. Daily steps were not recorded during the IM phase because participants were ambulating using crutches. Steps per day during the AR phase were 11825 ± 726 and 11677 ± 901 in the placebo and protein groups respectively. During the RT phase steps per day were 11778 ± 722 and 12300 ± 1130 in the placebo and protein groups respectively. There were no differences in daily steps between diet groups or study phases. The average energy balance over the 7 week diet control period was a deficit of

42.3 ± 81.0 and 38.5 ± 90.6 kcal/day in the placebo and protein groups respectively. These values were not different than zero (P=0.501) and were not different between groups (P=0.971).

Muscle protein synthesis

Mitochondrial protein synthesis rate was measured using D_2O as a tracer and was 1.45 ± 47 %/day and 1.03 ± 0.13 %/day in the placebo and protein groups respectively; there were no group effects or group by time interactions (P=0.879). However there was a main effect of time such that the fractional synthetic rate of mitochondrial proteins decreased during the IM phase compared to baseline (P=0.030) (Figure 5a). Myofibrillar protein synthesis rate at baseline was 1.18 ± 0.34 %/day and 1.22 ± 0.39 %/day in the placebo and protein groups respectively. There were effects of both supplement group (P=0.010) and phase of the study (P=0.040) but the group by time interaction did not reach significance (P=0.150). IM had no effect on myofibrillar protein synthesis whereas myofibrillar protein synthesis was increased above baseline during the AR phase only in the protein group (P=0.049) (figure 5b). Total body water deuterium enrichment was not different between groups and increased from 1.02 ± 0.06% during the baseline phase to 1.36 ± 0.08% during the IM phase (P<0.001). Enrichment was then maintained at 1.53 ± 0.11% and 1.58 ± 0.10% in the AR and RT phases respectively (P=0.524).

Gene expression and citrate synthase activity

Atrogin gene expression increased following IM (P<0.001) with no difference between groups. Expression returned to baseline after AR and remained at baseline levels following RT (Figure 6a). *MuRF-1* gene expression also increased following IM (P=0.003) with no difference between groups and returned to baseline following AR and remained at baseline (Figure 6b) after RT. mRNA expression of *myostatin* was not increased above baseline after

IM or AR with no difference between groups. Myostatin mRNA was greater after IM when compared to after RT (P=0.001) (Figure 6c). CS activity was 13.15 ± 1.3 nM/min/µg and 11.56 ± 1.21 nM/min/µg protein in the protein and control group respectively, which did not change over the course of the study (P=0.124) and was not different between groups.

Discussion

The consumption of 20g of daily supplemental dairy protein, consumed along with a morning meal, did not attenuate the decline of any measures of muscle size or function in this group of middle aged men consuming 1.1g total protein per kilogram of body weight per day, during a two week period of single leg immobilization. Despite supplemental protein increasing the rate of synthesis of contractile proteins during recovery from immobilization, the recovery of muscle size or function were not improved. Restoration of muscle function and size was not fully achieved with 2 weeks of normal activity, but full recovery was attained with an additional two weeks of RT.

The ingestion of 20g of milk protein is known is stimulate MPS in middle aged men and this dose of protein can increase resistance training mediated gains in muscle mass (12). However, this finding is not consistent across all studies (62). Given the actions of 20g of high quality protein on MPS, it was hypothesized that this dose of supplemental dairy protein could potentially attenuate muscle loss during immobilization. Muscle disuse or immobilization are known to decrease the sensitivity of muscle to protein feeding in both younger and older adults (33, 80). No dose response studies have been conducted to identify the optimal dose of protein to overcome disuse induced anabolic resistance. However, it has been suggested the 40g of protein is sufficient to maximally simulate MPS in anabolically resistant older adults (53). Similarly 40g of protein ingested after exercise is more effective at simulating MPS in anabolically resistant older adults than the 20g required to maximize MPS in those without anabolic resistance (54, 85). During disuse, the present study provided 20g of dairy protein along with a breakfast containing 20g of protein. The consumption of 40g of protein was likely sufficient to result in a maximal but transient stimulation of MPS following in the morning meal. The provided evening meals also contained ~42g of protein which again was likely sufficient to maximally simulate MPS however, it is possible that inclusion of

some lower quality plant based proteins reduced the total anabolic potential of the provided mixed meals (77). It is also possible that an attenuation of the muscle loss during immobilization might have been achieved by providing high quality supplemental protein with the lunch meal and before bed (76) in order to maximally simulate MPS four times daily rather than the twice daily that was likely achieved in the current study (46).

Recently Dirks and colleagues (19) reported a lack of efficacy for 20g of dairy based protein ingested twice daily to attenuate muscle loss during just five days of immobilization in men aged over 60 years. Our study agrees with these findings over a 2 week period of muscle disuse. A number of studies have examined the ability of protein or amino acid supplementation to attenuate atrophy during longer periods of bed rest (reviewed in (72)). The findings of these studies are variable, but suggests that a of benefit of supplemental protein is evident more frequently when compared to control participants consuming at or below the RDA (0.8 g/kg/day) for dietary protein (72). In the present study participants consumed 1.1 g/kg/day of protein daily, exclusive of the supplement. This habitual protein ingestion exceeding the RDA may partially explain the lack of efficacy of the supplement. A lower habitual protein intake might have increased the potential of the dairy protein to attenuate disuse atrophy. Yet relevance would be limited, as less than 10% of men in the study age range consume protein at or below the RDA (31).

In the present study, protein supplementation did not alter the rate at which muscle size and function recovered following immobilization, yet supplementation increased the aggregate myofibrillar MPS rate, during the first two weeks of recovery. Given the lack of effect of protein supplementation on muscle size during this period, the observed increase in MPS might represent a higher turnover with protein supplementation compared to the placebo group. This higher turnover could suggest a greater rate of remodelling (39). Yet, in the absence of a measurable phenotype adaptation definitive conclusions cannot be made.

476 Speculatively, either the changes in MPS could have been unrelated to the gains in mass and
477 strength, or that the changes were below the limits of detection. It is then possible that a more
478 sensitive measure of muscle size such as magnetic resonance imaging derived muscle volume
479 or a longer AR period may have resulted in a measurable difference in muscle size recovery
480 with protein supplementation.

481 The relative contributions of blunted MPS and increased MPB on muscle mass during human
482 disuse atrophy is unknown. Animal studies suggest that MBP is likely a major contributor to
483 disuse atrophy (5). However, it is has been argued that because the magnitude of atrophy
484 observed in animal models is much larger than is observed even with severe human disuse
485 models such as bed rest, findings from animal studies might not directly translate to human
486 physiology (59). In humans multiple studies have shown that disuse lasting longer than five
487 days decreases both postprandial and postabsorptive MPS (28, 32, 58, 80), whilst *in vivo*
488 human measurements of muscle MPB are rarely performed. Of the available data, fasted
489 MPB in six men did not to change after 14 days of bedrest (27). Further, MPB has also been
490 inferred not to change with muscle disuse, based on declining MPS and changes in muscle
491 size (32). Thus the consensus of existing literature suggests that accelerated MPB is unlikely
492 to exert a predominant role in the loss of muscle mass with immobilization (2). Yet, in the
493 present study, baseline myofibrillar MPS rates were ~1.2%/day, and the mean change in
494 muscle CSA was -4.5% following immobilization. Thus a ~27% daily increase in MPB,
495 decrease in MPS or combination of the two would be required to explain the observed muscle
496 loss. It is likely that that this imbalance, at least in part, was due to increased MPB. To date
497 the only available measures of muscle protein turnover during disuse have been over the
498 course of several hours in the postprandial and postabsorptive periods, measured following
499 weeks of prior disuse (58, 80). Long term protein turnover measurements have not previously
500 been reported, so no direct comparisons can be made. Animal studies (35, 68) along with

measures of human catabolic gene expression (19, 37, 73) and breakdown products (75) suggest MPB may transiently increase at the onset of disuse. In the present study catabolic gene expression (*MurF-1*, *Atrogin-1* and *Myostatin*) were also elevated after two weeks of disuse supporting at least some elevation of MPB. Given that MPB in humans has not been measured at the beginning of disuse, it is possible an increase in MPB during the first several days of immobilization is in part contributing to the ~27% imbalance between MPS and MPB measured over the 2 week intervention. However, it also possible that true suppression of MPS was simply not detected.

Loss of mitochondrial mass and function are common consequences of muscle disuse (1, 36). We developed an incremental single leg cycling protocol to evaluate muscular aerobic function (6). Along with the commonly observed decreases in strength and power, the present study shows a decline in aerobic power that was not fully recovered after two weeks of AR. This finding was accompanied by a decrease in mitochondrial MPS but no change in CS activity, a marker of mitochondrial content (43). The depressed mitochondrial MPS is the result of a slower turnover of mitochondrial proteins, and this may result in a decline in mitochondrial function resulting from a slower clearance of damaged mitochondrial proteins (36).

The present study was conducted in middle-aged males, an inadequately-defined life stage (51) which marks a transition between the ability of young adults to rapidly recover from muscle disuse to the impaired recovery observed in older adults (74). Suetta et al. (74) have shown that even when structured RT is commenced immediately after immobilization and continued for four weeks, older men do not fully recover muscle lost after two weeks of immobilization. Numerous animal studies have also shown an impaired ability to recover from disuse in older animals (3, 84). Due to the design of the current study it unclear if the middle-aged participants would have recovered their pre-immobilization muscle strength and

size without the RT phase or how long this recovery would take. Based on the findings it seems prudent to recommend resistance exercise following periods of disuse such as those induced by casting and hospital stays in middle-aged and older adults to ensure the recovery of muscle function and morphology.

The study design that was employed enabled analysis of the actions of protein supplementation during muscle disuse and recovery on muscle size, function and protein synthesis. Because of the complexity of the design it is important to acknowledge a number of limitations. Firstly, it is not possible to separate the effects of resistance training from the effects of an additional two weeks of active recovery so it is not known if the recovery observed after RT would have also occurred with an additional two weeks of free living recovery. It is also unknown if a longer period of immobilization or RT would have been able to differentiate any actions of the protein supplementation on muscle mass and function. Secondly, it was not technically possible to measure MPB or protein net balance at the same time as MPS, so definitive quantification of MPB is not possible. Further research using a tracer dilution technique will be required to draw firm conclusions regarding the extent of MPB during muscle disuse (40). The present study only recruited men which limits the generalizability of the findings. Pre-menopausal young women display similar resting and post exercise MPS responses to men of the same age (83) whereas older postmenopausal women have very different muscle protein metabolism compared to men of the same age (70). Middle aged women may respond differently both disuse and protein intake compared to men of the same age and thus should be the subject of further research.

Short term muscle disuse resulted in a rapid decline in muscle function and size which was not recovered after two weeks of normal activity, however an additional two weeks of RT fully normalized muscle size and function. The addition of 20g of high quality protein to the controlled diets of middle aged men did not attenuate the loss of muscle function or size

during immobilization, possibly due in part to the participants consuming adequate protein. During disuse, MPS of contractile proteins was not altered. This maintenance of MPS during immobilization suggests that MPB may be important for the loss of muscle mass during disuse. Interestingly during recovery, dairy protein supplementation increased myofibrillar MPS, in the absence of measurable differences in size and function, making the physiological significance unclear. Importantly, for those in middle-age, even a brief period of muscle disuse results in skeletal muscle atrophy and functional decline. This is not modified with 20g of daily supplemental protein and is not fully normalized by two weeks of ambulatory recovery.

The age related loss of muscle mass and strength begins in middle age and may be exacerbated by incomplete recovery from periods of muscle disuse. Resistance training should be prescribed after periods of casting, immobilization, bedrest and reduced activity in order to support the full normalization of muscle function. Protein supplementation does not appear to be beneficial in attenuating the loss of muscle mass or strength during immobilization in middle aged men consuming adequate protein. Further nutritional research should focus on the potential use of protein to enhance recovery from disuse and the use of higher dose more frequent protein supplementation during disuse.

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Figure 1. Study timeline. The study consisted of four phase over seven weeks. The timing of each phase, diet control and deuterium oxide ingestion protocol are shown. The timing of sample collection, muscle function testing and scanning is also shown. Muscle function testing consisted of single legged vertical jump, isometric strength testing and a single leg incremental cycling test.

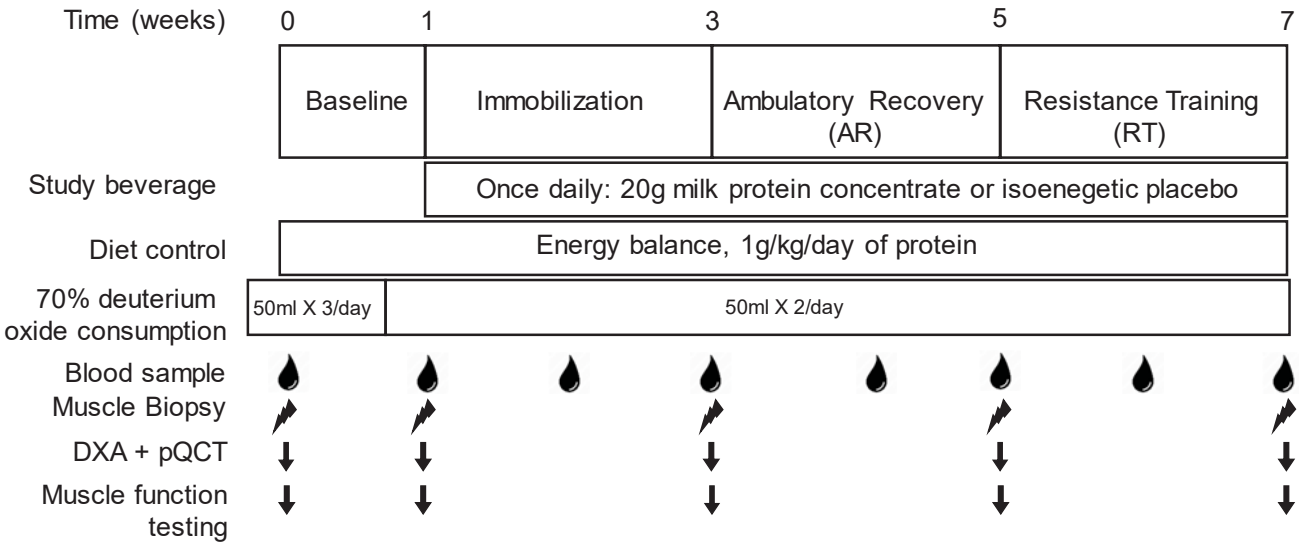
Figure 2. Muscle torque. Changes from baseline in maximal voluntary muscle torque for knee extension (A), knee flexion (B), and plantar flexion (C). Horizontal line represents a main effect for time, *= significantly different than baseline, $P<0.05$. #= significantly different from immobilization, $P<0.05$. Φ= significantly different from ambulatory recovery, $P<0.05$. Bars are mean ± SEM.

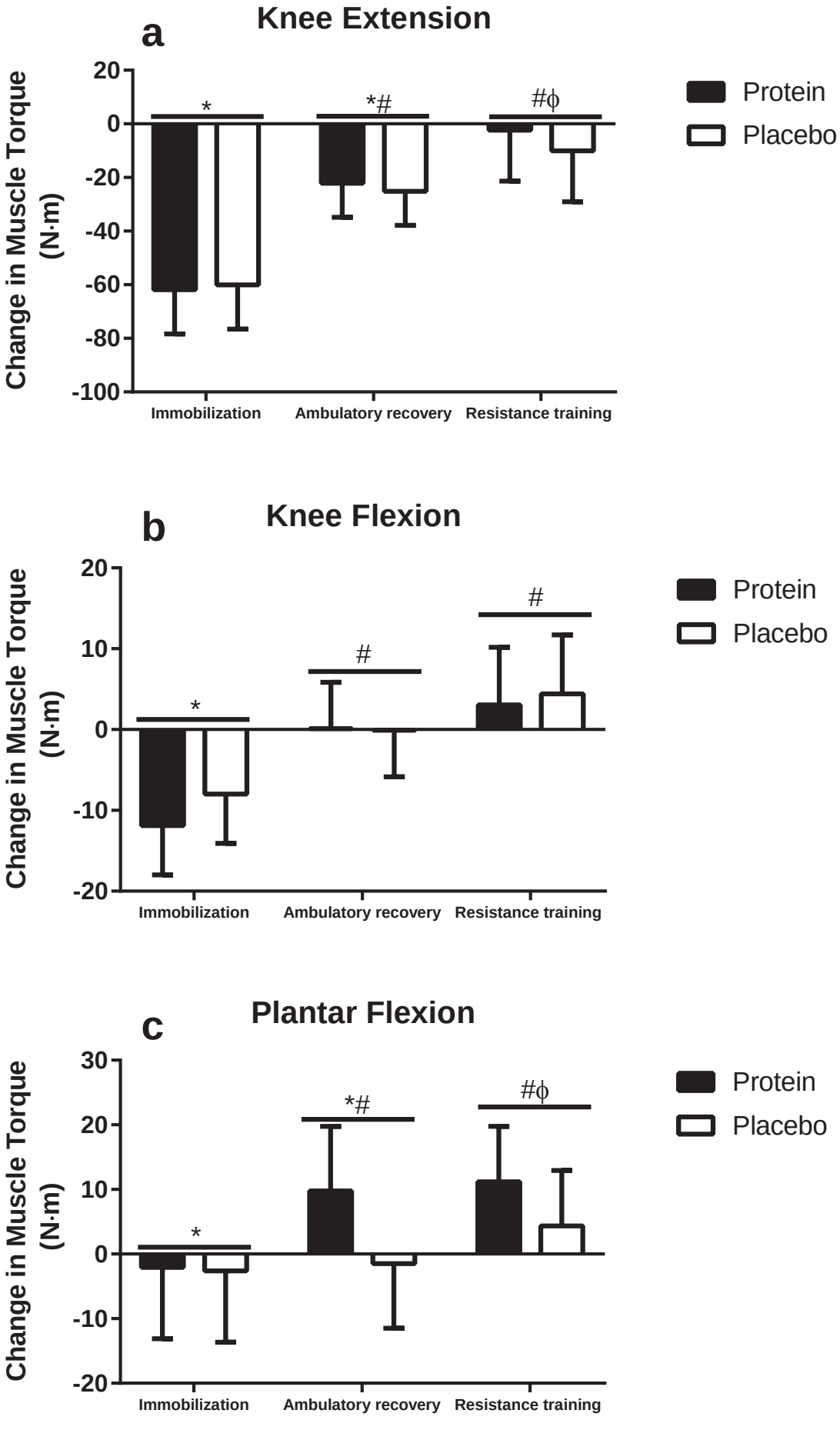
Figure 3. Muscle function. Changes in single leg jump height (A), peak power during an incremental single leg cycling test performed until exhaustion (B). Horizontal line represents a main effect for time, *= significantly different than baseline, $P<0.05$. #= significantly different from immobilization, $P<0.05$. Bars are mean ± SEM.

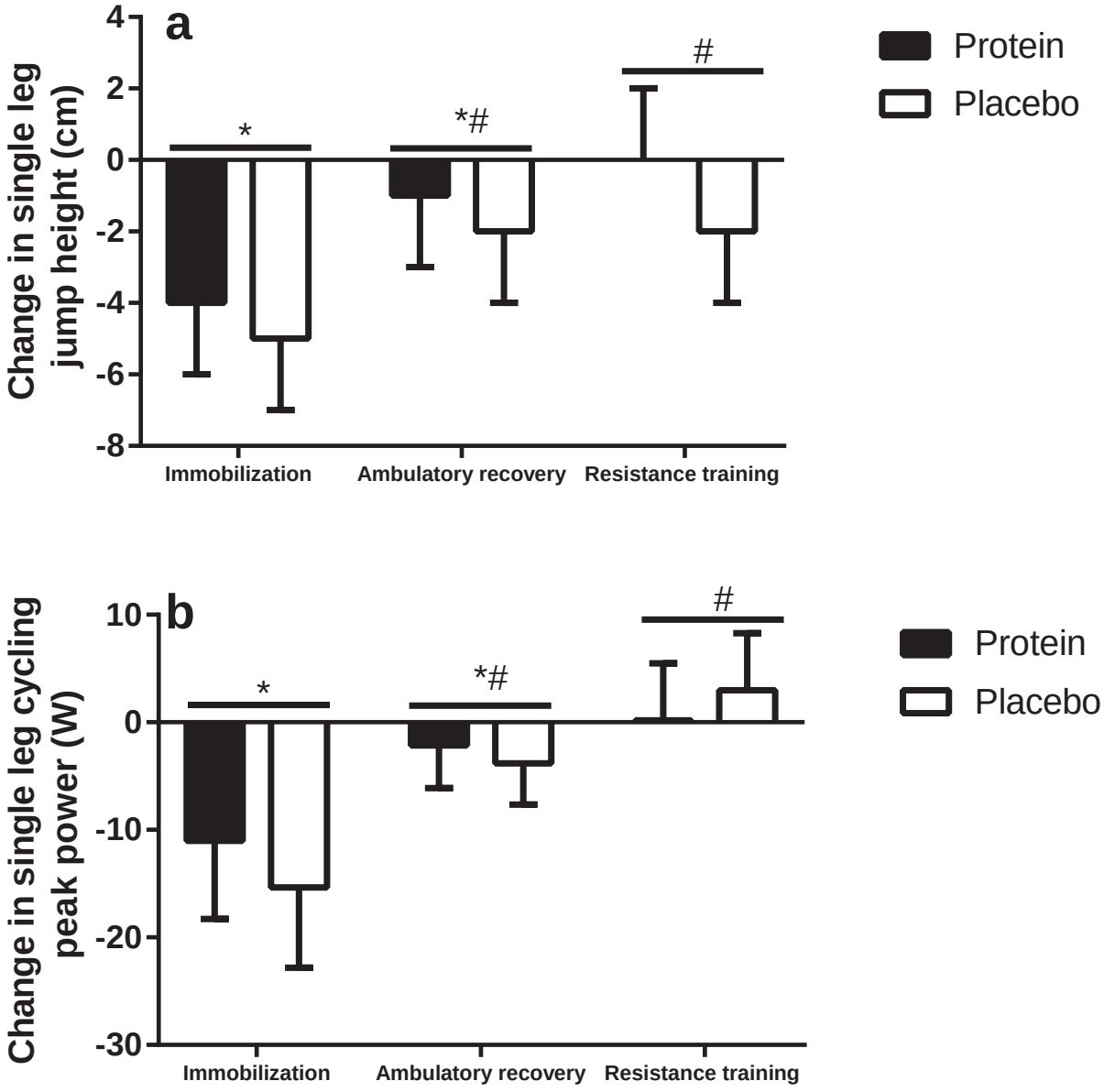
Figure 4. Muscle cross sectional area (CSA). Changes from baseline in thigh muscle CSA at 50% (A) and 20% of femur length as well as calf CSA at 66% of tibia length (C). Horizontal line represents a main effect for time, *= significantly different than baseline, $P<0.05$. #= significantly different from immobilization, $P<0.05$. Φ= significantly different from ambulatory recovery, $P<0.05$. Bars are mean ± SEM.

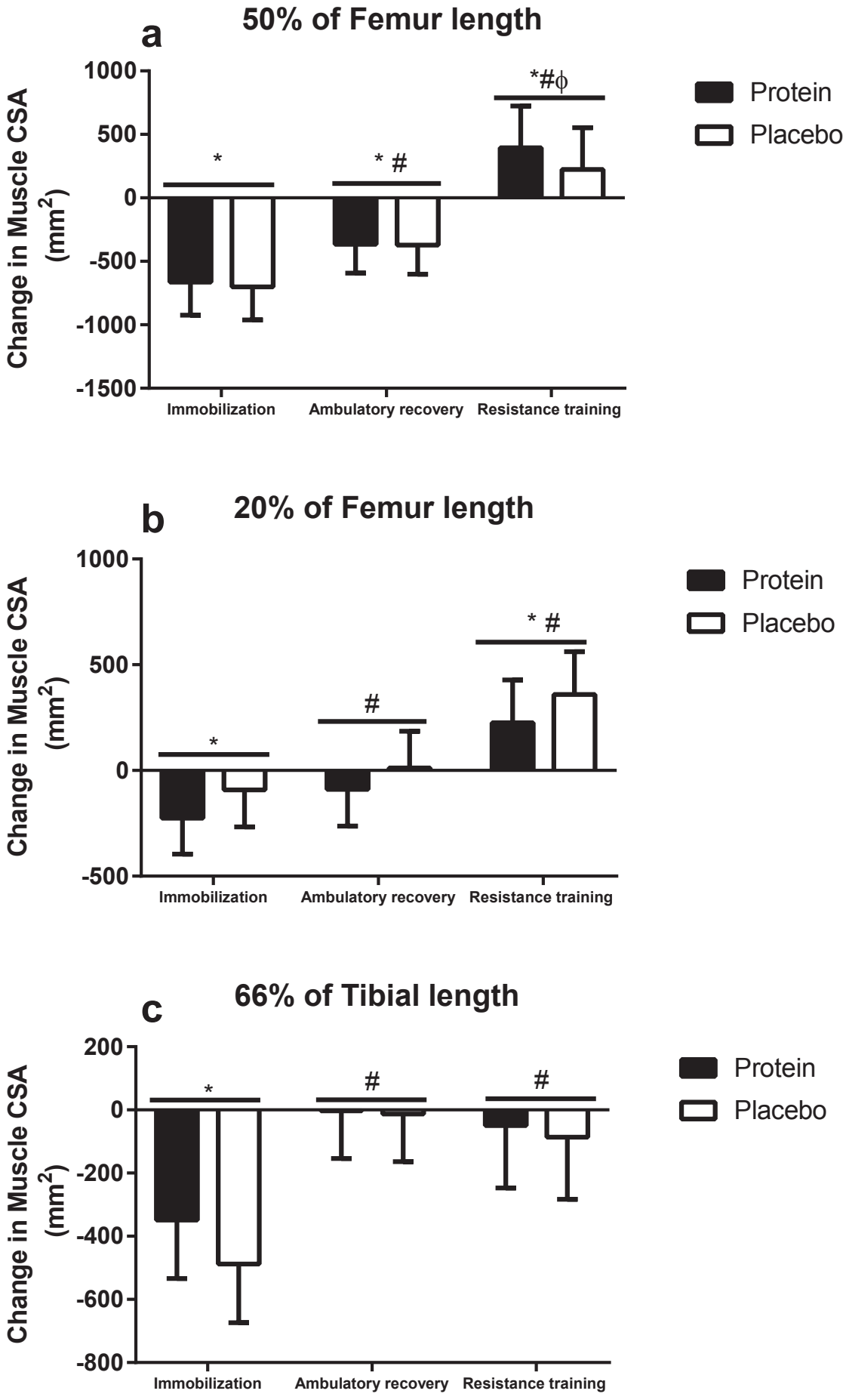
Figure 5. Muscle protein synthesis. The changes from baseline in fractional synthetic rate of mitochondrial (A), and myofibrillar (B) protein fractions. Horizontal line represents a main effect for time, *= significantly different than baseline, $P<0.05$. #= significantly different from immobilization, $P<0.05$. Bars are mean ± SEM.

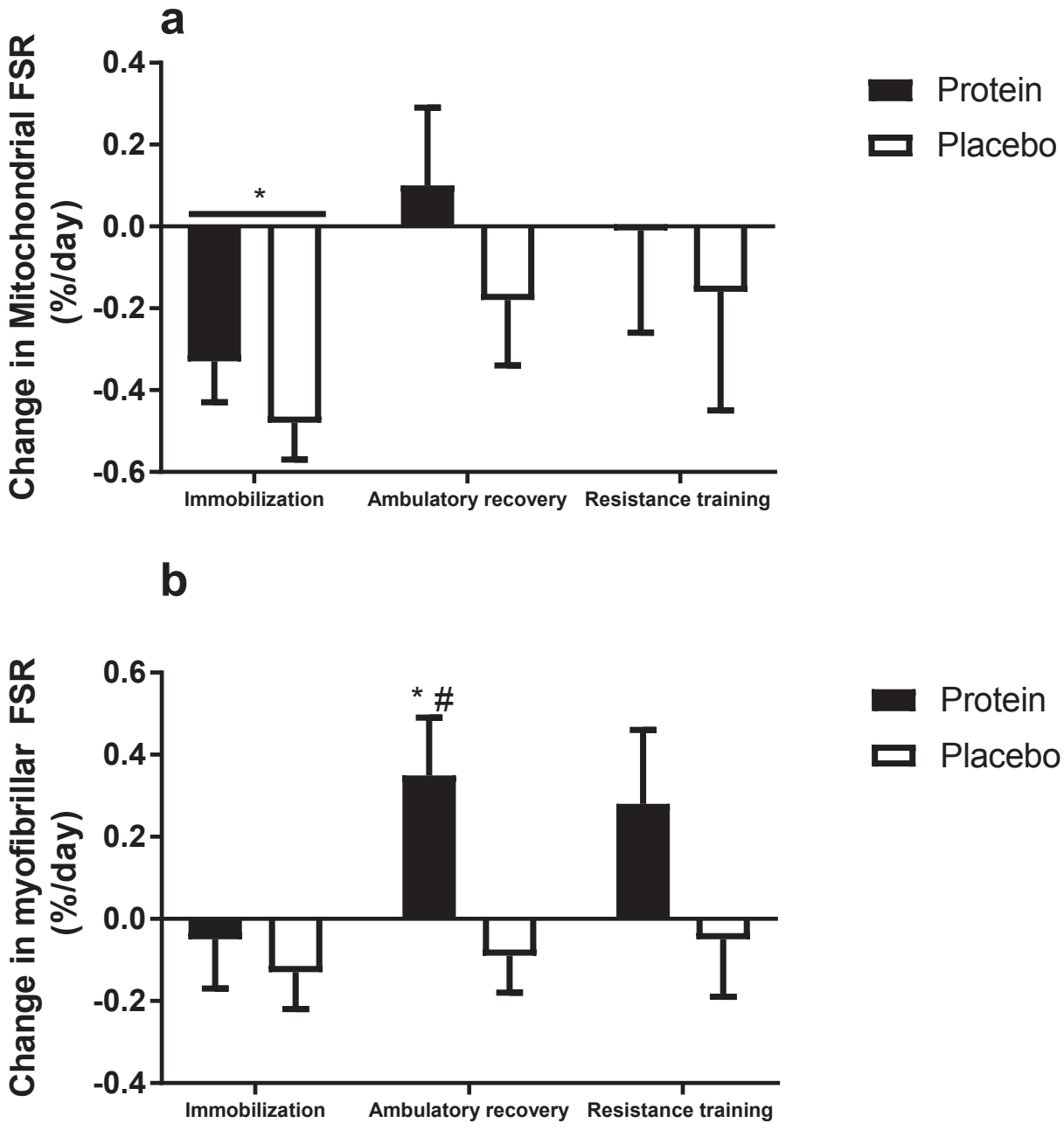
Figure 6. Expression of genes regulating muscle catabolism. Changes in mRNA expression of *Atrogin1* (A), *MuRF-1* (B) and *Myostatin* (C). Data are expressed as fold change from baseline for each subject. Horizontal line represents a main effect for time, *= significantly different than baseline, $P<0.05$. #= significantly different from immobilization, $P<0.05$. Bars are mean ± SEM.











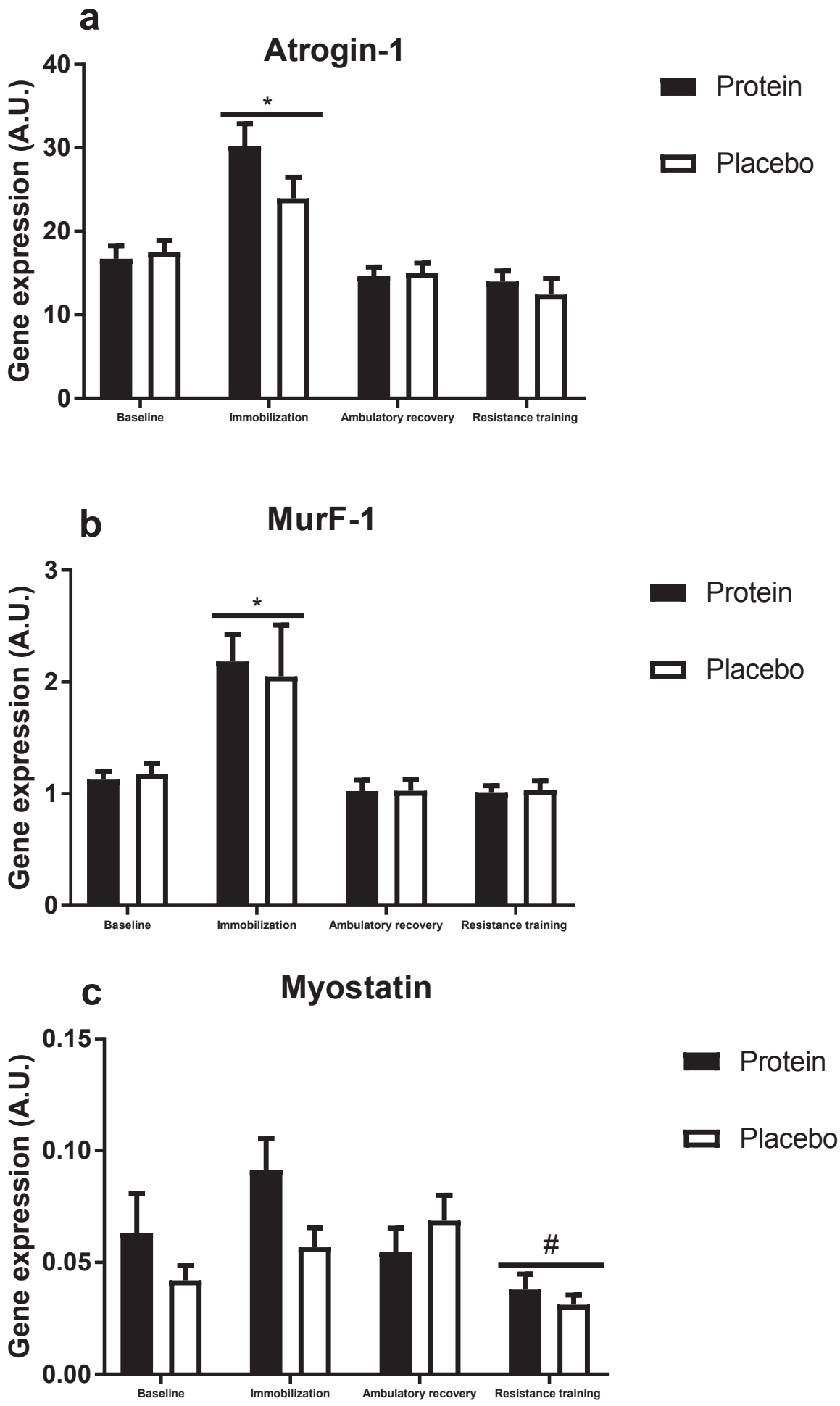


Table 1. Participant characteristics

	Dairy Protein (n=15)	Placebo (n=15)
Age (y)	51.5 ± 3.8	48.5 ± 2.4
Height (cm)	177.5 ± 7.4	176.3 ± 7.5
Weight (kg)	87.0 ± 14.2	87.9 ± 11.5
BMI (kg/m^2)[a]	27.5 ± 3.2	28.3 ± 3.2
HDL-cholesterol (mmol/L)	1.23 ± 0.40	1.18 ± 0.48
LDL-cholesterol (mmol/L)	3.50 ± 0.85	3.23 ± 0.77
Triglycerides (mmol/L)	1.89 ± 1.62	1.61 ± 1.41
Total cholesterol (mmol/L)	5.80 ± 0.86	5.36 ± 0.77
Insulin (pmol/L)	60.42 ± 40.14	79.1 ± 42.9
Glucose (mmol/L)	5.59 ± 0.49	5.53 ± 0.55
HOMA-IR[b]	1.81 ± 0.98	1.80 ± 0.90
Body fat (%)	25.1 ± 7.4	25 ± 6.1

[a] Body mass Index, [b] Homeostatic model assessment of insulin resistance. Means are shown ± SD.

Table 2. RT-PCR primer sequences

Target	Primer Sequence
Atrogin-1, Forward	AATAAGGAGAATCTTTTCAACAGCC
Atrogin-1, Reverse	TCCATGGCGCTCTTTAGTACTTC
MuRF1, Forward	GGGACAAAAGACTGAACTGAATAAC
MuRF1, Reverse	GGCTCAGCTCTTCCTTTACCT
MSTN, Forward	CTACAACGGAAACAATCATTACCA
MSTN, Reverse	GTTTCAGAGATCGGATTCCAGTAT
TBP, Forward	TGTGCTCACCCACCAACAAT
TBP, Reverse	TCTGCTCTGACTTTAGCACCTG
CHMP2A, Forward	CGCTATGTGCGCAAGTTTGT
CHMP2A, Reverse	GGGGCAACTTCAGCTGTCTG
HPRT, Forward	CCTGGCGTCGTGATTAGTGAT
HPRT, Reverse	TCGAGCAAGACGTTCAGTCC

Table 3. Dietary intake during the 4 phases of the intervention

	Baseline		Immobilization		Ambulatory recovery		Resistance Training	
	Protein	Placebo	Protein	Placebo	Protein	Placebo	Protein	Placebo
Protein (g/kg/day)	1.1 ± 0.2	1.1 ± 0.1	1.3 ± 0.3*	1.1 ± 0.1	1.2 ± 0.2*	1.1 ± 0.2	1.3 ± 0.3*	1.1 ± 0.1
Protein (g/day)	93.6 ± 10.3	92.0 ± 15.5	115.0 ± 21.4*	97.5 ± 17.6	109.1 ± 13.4*	92.0 ± 12.0	113.9 ± 14.4*	92.7 ± 13.9
Protein (% energy)	15.5 ± 2.0	16.2 ± 1.8	18.4 ± 1.7*	15.3 ± 1.9	17.7 ± 1.4*	14.9 ± 1.3	17.8 ± 1.5*	15.4 ± 1.6
Carbohydrate (g/day)	281.9 ± 27.7	280.0 ± 55.3	279.6 ± 52.3	319.7 ± 61.4	282.2 ± 37.8	313.3 ± 67.4	288.4 ± 32.2	303.0 ± 43.0
Carbohydrate (% energy)	46.5 ± 3.0	49.0 ± 3.7	44.6 ± 2.6	50.0 ± 2.7	45.8 ± 2.2	50.2 ± 2.8	45.2 ± 3.1	50.2 ± 3.6
Fat (g/day)	95.8 ± 19.5	80.1 ± 11.1	98.4 ± 31.9	93.0 ± 20.6	94.6 ± 24.9	85.3 ± 21.0	100.2 ± 25.0	87.4 ± 17.0
Fat (% energy)	35.2 ± 3.3	31.8 ± 2.5	34.7 ± 3.6	32.6 ± 2.8	34.0 ± 2.9	30.7 ± 5.2	34.8 ± 3.6	32.4 ± 3.7
Total energy (kcal)	2434 ± 291	2280 ± 368	2523 ± 555	2559 ± 461	2476 ± 406	2493 ± 468	2568 ± 374	2420 ± 329

* Different from baseline within the same group P<0.05. All values include both intake of meals and supplements (protein or placebo) Means are shown ± SD.