

# Skeletal muscle disuse atrophy is not attenuated by dietary protein supplementation in healthy, older men<sup>1,2,3</sup>

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This manuscript contains four (4) figures and three (3) tables.

<sup>2</sup>Online supporting material is available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

<sup>3</sup>**Abbreviations used:** CSA, cross-sectional area; CT, Computed Tomography; DEXA, Dual Energy X-ray Absorptiometry; FAK, Focal Adhesion Kinase; FOXO1, Forkhead box protein O1; FT, fiber typing; HOMA-index, Homeostatic Model Assessment Index; LAT1, Large Neutral Amino Acid Transporter 1; MAFBx, Muscle Atrophy F-box/Atrogen-1; mTOR, mammalian target of rapamycin; MuRF1, Muscle RING-finger protein-1; SC, satellite cell; PAT1, Proton-coupled amino acid transporter 1; PBS, phosphate-buffered saline; P70S6K, P70S6 kinase; 1RM, one-repetition maximum

1 **Abstract**

2 Short successive periods of muscle disuse, due to injury or illness, can contribute significantly to the loss  
3 of muscle mass with aging (sarcopenia). It has been suggested that increasing the protein content of the diet  
4 may be an effective dietary strategy to attenuate muscle disuse atrophy. We hypothesized that protein  
5 supplementation twice-daily would preserve muscle mass during a short period of limb immobilization.

6 Twenty-three healthy, elderly ( $69\pm 1$  y) males were subjected to 5 days of one-legged knee immobilization  
7 by means of a full leg cast with (PRO group;  $n=11$ ) or without (CON group;  $n=12$ ) administration of a  
8 dietary protein supplement (20.7 g protein, 9.3 g carbohydrate, and 3.0 g fat) twice daily. Two days prior  
9 to and immediately after the immobilization period, single slice CT-scans of the quadriceps and single leg  
10 1-Repetition Maximum (1RM) strength tests were performed to assess muscle cross-sectional area (CSA)  
11 and leg muscle strength, respectively. Additionally, muscle biopsies were collected to assess muscle fiber  
12 characteristics, and mRNA and protein expression of selected genes.

13 Immobilization decreased quadriceps CSA by  $1.5\pm 0.7\%$  ( $P<0.05$ ) and  $2.0\pm 0.6\%$  ( $P<0.05$ ), and muscle  
14 strength by  $8.3\pm 3.3\%$  ( $P<0.05$ ) and  $9.3\pm 1.6\%$  ( $P<0.05$ ) in the CON and PRO groups, respectively; without  
15 differences between groups. Skeletal muscle myostatin, myogenin, and *MuRF1* mRNA expression  
16 increased following immobilization in both groups ( $P<0.05$ ), while muscle *MAFbx* mRNA expression  
17 increased in the PRO group only ( $P<0.05$ ). In conclusion, dietary protein supplementation (~20 g twice  
18 daily) does not attenuate muscle loss during short-term muscle disuse in healthy older men. **Clinical trial**

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20

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22

23 **Introduction**

24 A period of prolonged (i.e. several weeks) muscle disuse, due to illness or injury, can lead to substantial  
25 loss of skeletal muscle mass and strength in otherwise healthy individuals. The resulting negative health  
26 consequences, such as impaired functional capacity (1-3), decreased muscle strength (4), the onset of insulin  
27 resistance (5), and a decline in basal metabolic rate (6, 7), are of particular concern to elderly individuals,  
28 who are already functionally and/or metabolically compromised. Recently, we (8, 9) as well as others (10)  
29 have shown that even a few days of disuse can already lead to significant losses of muscle mass and strength  
30 in young and old men. These findings are of particular clinical relevance as hospitalization of elderly due  
31 to acute illness generally results in an average 5-7 day hospital stay (11). It has been hypothesized that such  
32 short successive periods of muscle disuse occurring throughout the lifespan may be instrumental in the  
33 progressive loss of muscle mass that occurs with aging (12, 13).

34 Any substantial loss of skeletal muscle mass due to muscle disuse must be attributed to a chronic imbalance  
35 between muscle protein synthesis and breakdown rates. A decline in basal (post-absorptive) muscle protein  
36 synthesis rates has been reported following both bed-rest (14-16) as well as limb immobilization (17-19).  
37 Furthermore, recent work from our laboratory (20) as well as others (17, 21, 22) has shown that the muscle  
38 protein synthetic response to protein or amino acid administration becomes blunted following a period of  
39 disuse. Additionally, there is some indirect evidence that increases in muscle protein breakdown rates occur  
40 during the initial first few days of muscle disuse only (23-25). As such, it is now widely thought that  
41 declines in both post-absorptive and post-prandial muscle protein synthesis rates play the major causal role  
42 in the loss of muscle mass during a period of disuse (26, 27). Dietary protein intake stimulates muscle  
43 protein synthesis rates and inhibits muscle protein breakdown, and thereby allows net muscle protein  
44 accretion (28). Accordingly, it has been speculated that maintaining or even increasing dietary protein  
45 intake can attenuate muscle loss during a period of disuse (12, 27). In support, intervention studies have  
46 shown high-dose, essential amino acid supplementation to attenuate muscle loss during prolonged bed-rest  
47 in young (29-31) and elderly individuals (32). However, the potential for a practical dietary protein feeding

48 strategy to alleviate muscle loss during short-term disuse in the elderly population remains to be  
49 investigated.

50 In the present study, we investigated our hypothesis that dietary protein supplementation attenuates muscle  
51 loss during a short period of muscle disuse in older men. To test this hypothesis, 23 healthy elderly men  
52 were selected to participate in a study during which they were subjected to 5 days of one-legged knee  
53 immobilization with or without dietary protein supplementation (~20 g protein twice daily). Muscle mass  
54 and strength were assessed prior to and immediately after immobilization, and muscle biopsy samples were  
55 collected to assess muscle fiber characteristics and associated myocellular signaling.

56

57 **Methods**

58

59 *Subjects*

60 Twenty-three healthy elderly men (mean age  $69\pm 1$  y) were included in the present study. Prior to inclusion,  
61 a general health questionnaire was filled in by the subjects and a routine medical screening was completed  
62 to exclude individuals with BMI below 18.5 or above  $30 \text{ kg/m}^2$ ; any back, knee or shoulder complaints that  
63 could interfere with the use of crutches; a (family) history of thrombosis; type 2 diabetes mellitus  
64 (determined by HbA1c values  $>7.0\%$ ); severe cardiac problems; or a history of performing prolonged  
65 resistance-type exercise in the six months preceding the start of the study. All subjects were informed on  
66 the nature and risks of the experiment before written informed consent was obtained. The present study was  
67 approved by the Medical Ethical Committee of Maastricht University Medical Centre+ in accordance with  
68 the Declaration of Helsinki.

69

70 *Experimental outline*

71 An overview of the experimental protocol is depicted in **Figure 1**. After inclusion into the study, subjects  
72 were randomly allocated to either the control (CON,  $n=12$ ) or the protein (PRO,  $n=11$ ) group. Both groups  
73 were subjected to five days of muscle disuse induced by way of a full leg cast. The immobilized leg was  
74 randomly allocated and counter-balanced between left and right. Two days prior to casting and directly  
75 after cast removal, a series of measurements was performed. Single slice computed tomography (CT) scans  
76 were performed at the mid-thigh of both legs, whole body dual energy x-ray absorptiometry (DEXA) scans  
77 were taken, a single muscle biopsy from the immobilized leg and venous blood sample were collected, and  
78 one-legged knee extension strength (1RM, one-repetition maximum) was assessed for both legs.

79

80 *Muscle mass and function tests*

81 Forty eight hours prior to, and directly after the casting period, subjects visited the laboratory for two  
82 identical test days (i.e. test days 1 and 2). During these test days, multiple measurements of muscle mass

83 and function were performed. Firstly, the anatomical cross-sectional area (CSA) of *m. quadriceps femoris*  
84 and whole thigh were assessed via a single slice computed tomography (CT) scan (Philips Brilliance 64,  
85 Philips Medical Systems, Best, The Netherlands) as done before (8). With subjects placed in a supine  
86 position, their legs extended and their feet secured, a 3 mm thick axial image was taken 15 cm proximal to  
87 the top of the patella. On test day 1 the exact scanning position was marked with semi-permanent ink for  
88 replication on test day 2. ImageJ software (version 1.46r, National Institute of Health, Bethesda, MD, USA)  
89 was used to analyze CT scan images for the cross-sectional area of all thigh muscles as well as the  
90 quadriceps muscle separately. Secondly, a DEXA scan (Hologic, Discovery A, QDR Series, Bradford, MA,  
91 USA) was used to determine body composition and bone mineral content. Leg lean mass was determined  
92 using the system's software package Apex version 2.3. Maximal muscle strength was determined for each  
93 leg individually by 1RM strength tests on a leg extension machine (Technogym, Rotterdam, the  
94 Netherlands) as done before (8, 33).

95

#### 96 *Blood and muscle sampling*

97 Fasting venous blood samples were collected for determination of basal plasma glucose and insulin  
98 concentrations on test day 1 and 2. Blood (10 mL) was collected in EDTA-containing tubes and  
99 immediately centrifuged at 1,000g for 10 min at 4°C. Aliquots of plasma were snap frozen in liquid nitrogen  
100 and stored at -80°C until further analysis. Plasma glucose, free fatty acids, and triglyceride concentrations  
101 were analyzed with a ABX Pentra 400 analyzer (Horiba Diagnostics, Montpellier, France) with test kits  
102 from ABX Diagnostics (Montpellier, France), whereas plasma insulin concentrations were determined by  
103 radioimmunoassay (Millipore, ref. HI-14K , Billerica, MA, USA). Plasma amino acid concentrations were  
104 measured using ultra-performance liquid chromatography tandem mass spectrometry as described  
105 previously (34).

106 Muscle biopsies were taken from *m. vastus lateralis* of the immobilized leg prior to casting and immediately  
107 after cast removal, prior to performing any weight bearing activities. Biopsies were taken at the same time  
108 (08.30 AM) in the morning after an overnight fast and the same standardized meal was provided the evening

109 prior to muscle biopsy collection. Percutaneous muscle biopsies were taken from *m. vastus lateralis* with  
110 the Bergstrom technique, approximately 15 cm above the patella. The collected muscle was freed from any  
111 visible non-muscle tissue, processed immediately, and stored at -80°C until further analysis.

112

### 113 *Leg immobilization*

114 Two days after performing test day 1, at 8:00 in the morning, a full leg cast (randomized and  
115 counterbalanced for left and right leg) was applied in the casting room of the Academic Hospital in  
116 Maastricht. This marked the start of the 5 day immobilization period that always contained 3 week days  
117 and 2 weekend days. The cast extended from ~5 cm above the ankle until ~25 cm above the patella. A ~30  
118 degree angle of flexion of the knee joint was established in order to prevent subjects from performing  
119 weight-bearing activities with the immobilized leg. Subjects received crutches and were instructed on the  
120 correct usage before being provided with transportation home. The cast was removed at 8:00 on the morning  
121 of test day 2, after exactly 5 days of immobilization.

122

### 123 *Protein supplementation*

124 Subjects were randomly allocated to the group receiving a high whey protein leucine-enriched oral  
125 nutritional supplement (PRO) or the control group receiving no supplement (CON). Subjects allocated to  
126 the PRO group consumed the first drink in the laboratory on the morning of casting and were instructed to  
127 consume one drink directly after breakfast and one drink immediately prior to sleep on each day during  
128 immobilization (i.e. twice-daily, 10 drinks in total). Each drink provided 635 kJ, 21 g protein, 9 g  
129 carbohydrates, 3 g fat, and a mixture of vitamins, minerals and fibers. **Supplemental Table 1** depicts the  
130 composition of the study product.

131

### 132 *Dietary intake*

133 Standardized meals, containing 2.9 MJ and providing 51 Energy% (En%) as carbohydrate, 32 En% as fat,  
134 and 17 En% as protein, were consumed on the evening prior to both test days. Weighted dietary intake



135 records were completed by the subjects for the 5 day duration of the immobilization period as well as on a  
136 separate consecutive 5 day occasion either before or after (randomly allocated to avoid recording bias) the  
137 immobilization period. The same 5 days of the week were selected for both recording periods. DieetInzicht  
138 software (35), based on the NEVO table 2011, was used to analyze dietary intake records.

139

#### 140 *Muscle analysis*

141 Muscle samples were freed from any visible non-muscle tissue and separated into two sections. The first  
142 part (~30 mg) was imbedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, the Netherlands), frozen on  
143 liquid nitrogen cooled isopentane and used to determine muscle fiber-type specific cross-sectional area  
144 (CSA) and satellite cell content as done previously (8). The second part (~15 mg) was snap frozen in liquid  
145 nitrogen and used for real time-PCR analysis to determine mRNA expression of selected genes as described  
146 before (8, 20). A detailed overview of the muscle analyses is presented in the online supporting material.

147

#### 148 *Statistics*

149 All data are expressed as mean±SEM. Baseline values between groups were compared by means of an  
150 independent samples t-test. Pre- versus post-immobilization data were analyzed using Repeated Measures  
151 ANOVA with treatment (CON vs PRO) as between-subjects factor and time (pre- vs post-immobilization)  
152 as within-subjects factor. Fiber type (type I vs type II) was added to the test as a within-subjects factor when  
153 performing the statistical analyses for the muscle data. In case of a significant main effect, paired-samples  
154 t tests were executed to determine time effects within treatment groups or within fiber types, and  
155 independent-samples t tests were performed to determine group differences in pre- and post-immobilization  
156 values. When a significant main effect was detected, Bonferroni's post hoc test was applied to locate the  
157 differences. A *P*-value of <0.05 was used to determine statistical significance. All data were analyzed using  
158 SPSS version 20.0 (SPSS Inc., Chicago, IL, USA).

159 **Results**

160

161 *Subjects*

162 Subjects' characteristics are provided in **Table 1**. No baseline differences between the control (CON) and  
163 protein (PRO) groups were observed for age, height, weight, BMI, glucose, insulin, HOMA, or HbA1c  
164 levels at baseline. Glucose, insulin, and HOMA were measured pre- and post-intervention, and did not  
165 change over time in either group.

166

167 *Muscle mass and strength*

168 Quadriceps muscle cross-sectional area (CSA) is displayed in **Figure 2A**. At baseline, no differences were  
169 observed in quadriceps or whole leg muscle CSA between groups ( $P>0.05$  for both parameters). Five days  
170 of immobilization caused significant muscle atrophy of the quadriceps (time effect,  $P<0.001$ ; see **Figure**  
171 **2A**) and the whole leg (time effect,  $P<0.05$ ; from  $13.3\pm 5.4$  to  $13.2\pm 5.3$  cm<sup>2</sup> ( $-0.7\pm 0.6\%$ ) in CON and from  
172  $12.6\pm 4.2$  to  $12.4\pm 4.6$  cm<sup>2</sup> ( $-1.6\pm 0.6\%$ ) in PRO) with no differences between groups ( $P$ -interaction $>0.05$  for  
173 both parameters). Immobilization did not affect whole-body or leg lean mass in either group (data not  
174 shown; both  $P>0.05$ ). Leg muscle strength data are presented in **Figure 2B**. Maximal leg muscle strength  
175 had decreased following immobilization in the CON and PRO group (time effect,  $P<0.001$ ), with no  
176 differences between groups ( $P$ -interaction $>0.05$ ).

177

178 *Dietary intake*

179 **Table 2** shows data for subjects' habitual diet for 5 days under free living conditions and during the 5 day  
180 immobilization period. No differences in habitual diet were observed between groups (all measured  
181 parameters  $P>0.05$ ). Habitual diet did not change due to immobilization in the CON group ( $P>0.05$ ),  
182 whereas in the PRO group, twice-daily ingestion of the protein drink significantly increased protein intake  
183 (expressed as g·day<sup>-1</sup>, g·kg<sup>-1</sup>·day<sup>-1</sup>, and En%) compared with baseline ( $P<0.05$ ) and the CON group  
184 ( $P<0.05$ ). Habitual protein intake averaged  $1.1$  g·kg<sup>-1</sup>·day<sup>-1</sup> and was increased to  $1.6$  g·kg<sup>-1</sup>·day<sup>-1</sup> during the

185 immobilization period in the PRO group. Energy intake in the PRO group was maintained during  
186 immobilization; a relatively higher amount of energy was received from protein, at the expense of energy  
187 from fat ( $P<0.05$ ).

188

#### 189 *Plasma analyses*

190 Plasma amino acid concentrations (**Supplemental Table 2**) were increased in both groups for alanine,  
191 cysteine, phenylalanine, threonine, and tryptophan (all  $P<0.05$ ). For valine ( $P$ -interaction $<0.05$ ), an  
192 increase following immobilization was observed in the PRO group only ( $P<0.05$ ). All other measured  
193 amino acids were not changed following immobilization (all  $P>0.05$ ). Immobilization, with or without  
194 protein supplementation, did not influence plasma free fatty acid (CON: from  $384\pm33$  to  $354\pm33$   $\mu\text{mol/L}$ ;  
195 PRO: from  $446\pm48$  to  $404\pm46$   $\mu\text{mol/L}$ ) or triglyceride (CON: from  $1190\pm210$  to  $1270\pm92$   $\mu\text{mol/L}$ ; PRO:  
196 from  $968\pm88$  to  $1110\pm118$   $\mu\text{mol/L}$ ) concentrations (both  $P>0.05$ ).

197

#### 198 *Muscle fiber characteristics*

199 Muscle fiber characteristics are displayed in **Table 3**. At baseline, no differences between groups were  
200 observed for any of the variables. No measurable decline in muscle fiber CSA was observed following  
201 immobilization in either group ( $P>0.05$ ). Although no changes in myonuclear content were observed  
202 following immobilization ( $P>0.05$ ), myonuclear domain size decreased in both fiber types in both CON  
203 and PRO (time effect,  $P<0.05$ ). At baseline, satellite cell (SC) content expressed per muscle fiber, per  
204 millimetre squared, and as a percentage of the total number of myonuclei was higher in type I compared  
205 with type II fibers ( $P<0.05$  for all three parameters). No changes over time or differences between groups  
206 were observed ( $P>0.05$ ).

207

#### 208 *mRNA expression*

209 **Figure 3** and **Supplemental Figure 1** display the skeletal muscle mRNA expression of the selected genes  
210 of interest. Muscle mRNA expression of *myostatin* (**Figure 3A**) and *myogenin* (**Figure 3C**) increased

211 following immobilization in both groups ( $P<0.05$ ), whereas *myoD* (**Figure 3B**) tended towards an increase  
212 in both groups ( $P=0.07$ ). *MAFBx* mRNA expression (**Figure 3D**) showed a significant time\*treatment  
213 interaction ( $P<0.05$ ) with a significant increase only detected in the PRO group ( $P<0.05$ ) following  
214 immobilization. Muscle *MuRF1* mRNA (**Figure 3E**) significantly increased in both groups (time effect,  
215  $P<0.01$ ). There was a trend for an interaction effect ( $P=0.07$ ) such that *MuRF1* mRNA expression was  
216 increased to a greater extent in PRO ( $P<0.05$ ) compared with CON ( $P>0.05$ ). For the mRNA expression of  
217 both amino acid transporters *LATI* (**Supplemental Figure 1D**) and *PATI* (**Supplemental Figure 1E**), a  
218 significant time effect was found ( $P<0.01$  for both genes) such that expression was upregulated following  
219 immobilization in both groups. All other genes showed no significant changes between or within groups.

## 220 **Discussion**

221 In the present study, we demonstrate that merely 5 days of one-legged knee immobilization leads to  
222 substantial skeletal muscle mass and strength loss in healthy, elderly men. Increasing dietary protein intake  
223 by supplementing ~20 g protein twice-daily did not attenuate the loss of muscle mass or strength during 5  
224 days of muscle disuse in older males.

225 A period of prolonged muscle disuse and the associated muscle atrophy causes numerous negative health  
226 consequences (1, 2, 4, 6), and the occurrence of successive periods of muscle disuse likely represents a key  
227 factor responsible for the loss of muscle mass during the later decades of our lifespan (13). In the present  
228 study, we report that only 5 days of muscle disuse already leads to substantial loss of muscle mass (-  
229  $1.5\pm 0.7\%$ ; **Figure 2A**) and strength ( $-8.3\pm 3.3\%$ ; **Figure 2B**) in older individuals. These data are in line with  
230 recent data from our group in which we observed similar muscle mass and strength losses in younger  
231 individuals (8). Furthermore, Suetta *et al.* reported significant muscle fiber atrophy after 4 days of  
232 immobilization in both young and older individuals (10). The rapid muscle atrophy observed in our older  
233 subjects after merely 5 days of leg immobilization is of important clinical significance, as successive short  
234 periods of muscle disuse due to illness or injury are highly prevalent during the later stages of our lifespan  
235 (36). In line, the average length of hospitalization for elderly patients admitted with acute illness is 5–7  
236 days (11). The observed muscle loss is of particular relevance as the older population has difficulty to regain  
237 skeletal muscle mass and strength following a period of disuse (37). Even when applying rehabilitative  
238 resistance-type exercise training after a period of disuse, muscle mass does not seem to be restored after 4  
239 weeks of intense supervised training (37). For these reasons, it is presently believed that the impact of short  
240 successive episodes of muscle disuse may be of key relevance in the development of sarcopenia (13).

241 Practical and effective interventional strategies are needed to prevent or attenuate muscle mass and strength  
242 loss during short periods of muscle disuse in healthy elderly as well as more clinically compromised  
243 subpopulations. It has been proposed that simply increasing the protein content of the diet may alleviate the  
244 loss of muscle tissue during a period of disuse (12, 27). Indeed, studies focusing on mimicking prolonged  
245 hospitalization (i.e. >2-3 weeks bed-rest under tightly controlled dietary conditions) have shown that

246 supplementation with high doses of crystalline essential amino acids (~50 g, equivalent to ~100-150 g intact  
247 protein) attenuates the loss of muscle mass (29-31). Given the clinical relevance of short, successive periods  
248 of muscle disuse, we assessed the efficacy of a more practical and feasible dietary strategy to attenuate  
249 muscle loss during a short period of limb immobilization under free living conditions. Increasing dietary  
250 protein intake from 1.1 to 1.6 g·kg body weight<sup>-1</sup>·day<sup>-1</sup> did not rescue the loss of muscle mass or strength  
251 observed during a 5 day period of leg immobilization (**Figure 2**). The apparent discrepancy between the  
252 outcome of the present study and previous work in prolonged bed rest studies may be attributed to  
253 differences in protein intake in the control group. In the present study the control group retained normal  
254 habitual energy and protein intake (1.1 g·kg<sup>-1</sup>·day<sup>-1</sup>) whereas the protein group received additional  
255 supplementation (1.6 g·kg<sup>-1</sup>·day<sup>-1</sup>). In contrast, in previous bed rest studies that show benefits of amino acid  
256 supplementation on muscle mass maintenance, the control groups generally consumed dietary protein at a  
257 level no higher than 0.8 g·kg<sup>-1</sup>·day<sup>-1</sup> (29-31). Consequently, we speculate that maintaining dietary protein  
258 intake is required to prevent muscle loss during disuse, but that increasing dietary protein intake above  
259 habitual levels does not further alleviate muscle loss during disuse (38, 39). This would be of particular  
260 relevance in institutionalized or hospitalized elderly who are unable to maintain habitual dietary protein  
261 consumption during more prolonged periods of muscle disuse due to illness or injury. Additional  
262 considerations of the present nutritional intervention include the type and timing of protein administered.  
263 We selected whey protein in the present study as we have previously shown it leads to greater post-prandial  
264 muscle protein accretion compared with casein protein in healthy elderly men (40). We chose to supplement  
265 volunteers at breakfast time since we have previously shown that community dwelling elderly individuals  
266 generally consume inadequate amounts of protein at breakfast (41). Specifically, the supplement was  
267 consumed directly *after* breakfast to avoid volunteers compensating for the supplement by consuming less  
268 breakfast and therefore ensuring adequate protein was consumed. This was achieved given that the PRO  
269 group consumed (36±2 g at this meal compared to the CON group who only consumed 13±1 g, the latter  
270 being an amount insufficient to properly stimulate muscle protein synthesis rates (42). We opted to deliver  
271 the second supplement immediately prior to sleep, since we have recently shown that such a strategy

272 effectively stimulates overnight muscle protein synthesis rates (43). However, it is also true that these  
273 beneficial effects on nocturnal muscle protein synthesis were obtained with the ingestion (or intragastric  
274 administration) of large amounts of casein protein, to ensure a more sustained hyperaminoacidemia  
275 throughout the night (44). Accordingly, it could be speculated that future nutritional strategies aimed at  
276 attenuating muscle disuse atrophy may wish to consider incorporating large boluses of casein as a pre-  
277 bedtime meal. In contrast, it could also be hypothesized that ingestion of a large bolus of dietary protein  
278 prior to sleep increases both muscle protein synthesis and breakdown rates, without net muscle protein  
279 accretion (45). Though previous work has shown improvements in overnight whole-body protein balance  
280 following protein administration in healthy older men (43) and in young adults during overnight recovery  
281 from exercise (46), we cannot exclude that such improvements in overnight protein balance may not occur  
282 in a setting of muscle disuse.

283 Besides assessing the impact of protein supplementation on muscle mass and strength during short-term  
284 disuse, we wished to gain insight into the underlying myocellular mechanisms involved in muscle disuse  
285 atrophy and/or muscle mass maintenance. Muscle loss during short-term muscle disuse is thought to be, at  
286 least partly, mediated by accelerated rates of muscle protein breakdown (13). Myostatin is known as a  
287 negative regulator of muscle growth *in vivo* (47), and acts through multiple pathways including the  
288 stimulation of muscle protein breakdown (48). Consistent with this role, we observed increases in myostatin  
289 mRNA expression (**Figure 3**) and in markers of muscle protein breakdown (i.e. increased gene expression  
290 of MAFBx and MuRF1; **Figure 3**). This is in line with previous findings (10) and our own work in young  
291 men (8, 9), and supportive of a role for muscle protein breakdown in short-term muscle atrophy, possibly  
292 mediated through increased myostatin transcription. Given the lack of effect of protein supplementation on  
293 muscle mass in the present study, it is not surprising that we observed no attenuation of the rise in myostatin  
294 and markers of proteolysis. In fact, we actually observed that MAFBx and MuRF1 gene expression  
295 increased to a greater extent in the PRO group (**Figure 3**), supporting the idea that increasing dietary protein  
296 intake beyond the habitual dietary protein intake level may strongly stimulate overall protein turnover rates.

297 Myostatin is also reported to regulate muscle size by acting via the inhibition of myogenesis through its  
298 inhibitory action on the myogenic regulatory factors (49). However, in line with our previous work (8, 50),  
299 we report that the disuse-induced increase in myostatin expression does not coincide with impaired  
300 expression of the myogenic regulatory factors (i.e. MyoD and myogenin, **Figure 3**). Moreover, no  
301 alterations in muscle satellite cell content were observed, suggesting that the mechanisms underlying short-  
302 term disuse atrophy do not require alterations in myogenesis or satellite cell content. Recent data have  
303 suggested that the expression of specific amino acid transporters within skeletal muscle provide a site of  
304 regulation for muscle protein synthesis (51). As such, we analyzed the gene expression of Large Neutral  
305 Amino Acid Transporter 1 (LAT1/SLC) and Proton-coupled amino acid transporter 1 (PAT1) which are  
306 thought to be the key transporters facilitating intramuscular transport of BCAAs particularly in response to  
307 nutrition (52). Interestingly, LAT1 and PAT1 mRNA expression (**Supplemental Figure 1**) increased  
308 following immobilization in both groups, possibly indicating a compensatory mechanism by which  
309 atrophying muscle attempts to ‘scavenge’ circulating amino acids as a substrate for muscle protein  
310 synthesis.

311 In the present study we show that protein supplementation on top of a diet containing ample protein (1.1  
312 g·kg<sup>-1</sup>·day<sup>-1</sup>) does not alleviate muscle loss during short-term single leg disuse. This shows that besides  
313 maintaining dietary protein intake, other strategies are warranted to help maintain muscle mass. Where  
314 possible, performing some degree of exercise should be considered during disuse (39). In conditions where  
315 exercise is not feasible due to injury or illness, low-volume physical activity (53) or even exercise surrogates  
316 (8) could be suggested. Furthermore, other nutritional compounds, such as creatine or omega-3 fatty acids,  
317 may support muscle maintenance during disuse (27). An often under-appreciated consideration is how  
318 dietary strategies could support rehabilitation following a period of disuse. This area has been  
319 comparatively under studied (54-57) but, given the opportunity to combine nutrition with re-ambulation  
320 and/or physical exercise, future research should address how dietary protein and/or other nutritional  
321 strategies could best be used to facilitate the rapid and complete restoration of muscle mass following a  
322 period of disuse.



323 In short, we conclude that short-term muscle disuse results in a substantial decline in both muscle mass and  
324 strength in older individuals. Increasing dietary protein intake during short-term muscle disuse on top of a  
325 diet providing  $>1.0 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  does not alleviate muscle disuse atrophy in healthy, elderly men.  
326

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331

332 **Author contributions**

333 The study was performed at Maastricht University, Maastricht, the Netherlands. M.L.D., B.T.W. and  
334 L.J.C.v.L. designed the study; M.L.D., B.T.W., R.N. and D.H.J.M.W. conducted the research; M.L.D.,  
335 B.T.W. and R.N. analyzed the data; M.L.D., B.T.W., R.N., D.H.J.M.W. L.B.V. and L.J.C.v.L. wrote the  
336 paper. M.L.D. had primary responsibility for the final content. All authors have read and approved the final  
337 manuscript.

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## Tables

**Table 1:** Subjects' characteristics of healthy older men in the control (CON) and protein supplemented (PRO) groups<sup>1,2,3</sup>

	<b>CON (n=12)</b>	<b>PRO (n=11)</b>
<b>Age, y</b>	70 ± 1	68 ± 1
<b>Body mass, kg</b>	82.9 ± 3.0	79.6 ± 2.4
<b>Height, m</b>	1.74 ± 0.02	1.74 ± 0.02
<b>BMI, kg/m<sup>2</sup></b>	27.3 ± 0.6	26.4 ± 0.8
<b>Leg volume, L</b>	7.96 ± 0.28	7.90 ± 0.35
<b>Plasma glucose, mmol/L</b>	5.6 ± 0.1	5.7 ± 0.1
<b>Plasma insulin, uU/mL</b>	11.7 ± 1.4	9.9 ± 1.0
<b>HOMA-index</b>	3.0 ± 0.4	2.6 ± 0.3
<b>Glycated hemoglobin, %</b>	5.4 ± 0.1	5.7 ± 0.1
<b>Glycated hemoglobin, mmol/mol</b>	35.9 ± 1.2	38.4 ± 1.3

<sup>1</sup> Values are means±SEM, n=23.

<sup>2</sup> Abbreviations: HOMA-index, Homeostatic Model Assessment Index.

<sup>3</sup> No differences were observed between groups ( $P>0.05$  for all variables).

**Table 2:** Dietary intake of healthy elderly subjects under free-living conditions and during a 5-day period of leg immobilization, with (PRO) or without (CON) supplementation.<sup>1,2,3</sup>

Variable	CON (n=12)		PRO (n=11)	
	Free living	Immobilization	Free living	Immobilization
Energy intake, $MJ \cdot day^{-1}$	8.82 ± 0.62	9.03 ± 0.46	8.73 ± 0.54	9.50 ± 0.49
Protein intake, $g \cdot day^{-1}$	85 ± 9	86 ± 4	90 ± 4	125 ± 6 *
Protein, $g \cdot kg^{-1} \cdot day^{-1}$	1.04 ± 0.12	1.05 ± 0.06	1.14 ± 0.07	1.60 ± 0.11 *
Protein, En%	16.7 ± 1.2	16.4 ± 0.7	18.0 ± 0.9	22.9 ± 1.0 *
Fat, En%	31.6 ± 1.3	32.9 ± 2.3	29.5 ± 1.8	25.8 ± 1.2 *
Carbohydrate, En%	51.7 ± 2.0	50.6 ± 2.1	52.5 ± 2.1	51.3 ± 1.5

<sup>1</sup> Data represent means±SEM, n=23.

<sup>2</sup> Data in the PRO group are expressed including twice-daily intake of the protein supplement.

<sup>3</sup> \* Significantly different from free living value ( $P<0.05$ )



**Table 3:** Muscle fiber characteristics of healthy elderly individuals before (pre) and after (post) 5 days of leg immobilization, with (PRO) or without (CON) supplementation.<sup>1,2,3,4</sup>

	Fiber type	CON (n=12)		PRO (n=11)	
		Pre	Post	Pre	Post
<b>Muscle fiber CSA, <math>\mu\text{m}^2</math></b>	I	5654 $\pm$ 391	5037 $\pm$ 487	5646 $\pm$ 469	5370 $\pm$ 379
	II	5592 $\pm$ 564	5000 $\pm$ 525	5131 $\pm$ 390	5027 $\pm$ 356
<b>Fiber, %</b>	I	49 $\pm$ 3	44 $\pm$ 3	48 $\pm$ 5	48 $\pm$ 5
	II	51 $\pm$ 3	56 $\pm$ 3	52 $\pm$ 5	52 $\pm$ 5
<b>Fiber, area %</b>	I	50 $\pm$ 4	45 $\pm$ 3	51 $\pm$ 5	49 $\pm$ 6
	II	50 $\pm$ 4	55 $\pm$ 3	49 $\pm$ 5	51 $\pm$ 6
<b>Nuclei, n/fiber</b>	I	2.8 $\pm$ 0.1	2.9 $\pm$ 0.3	2.8 $\pm$ 0.2	2.9 $\pm$ 0.2
	II	2.8 $\pm$ 0.1	2.8 $\pm$ 0.2	2.8 $\pm$ 0.2	2.8 $\pm$ 0.2
<b>Myonuclear domain, <math>\mu\text{m}^2</math></b>	I	2026 $\pm$ 86	1716 $\pm$ 106 *	2035 $\pm$ 89	1914 $\pm$ 97 *
	II	2072 $\pm$ 112	1770 $\pm$ 126 *	1843 $\pm$ 111	1791 $\pm$ 112 *
<b>SC, n/fiber</b>	I	0.101 $\pm$ 0.014	0.091 $\pm$ 0.013	0.099 $\pm$ 0.010	0.099 $\pm$ 0.007
	II	0.056 $\pm$ 0.008 #	0.055 $\pm$ 0.009 #	0.062 $\pm$ 0.006 #	0.060 $\pm$ 0.006 #
<b>SC, n/mm<sup>2</sup></b>	I	18.1 $\pm$ 2.5	18.0 $\pm$ 2.3	17.7 $\pm$ 2.1	19.1 $\pm$ 1.6
	II	10.1 $\pm$ 1.5 #	10.3 $\pm$ 1.4 #	11.8 $\pm$ 1.1 #	12.7 $\pm$ 1.6 #
<b>SC, n/myonuclei, %</b>	I	3.6 $\pm$ 0.4	3.3 $\pm$ 0.5	3.7 $\pm$ 0.5	3.6 $\pm$ 0.3
	II	1.9 $\pm$ 0.2 #	2.0 $\pm$ 0.3 #	2.2 $\pm$ 0.2 #	2.2 $\pm$ 0.2 #

<sup>1</sup> Data represent means $\pm$ SEM, n=23.

<sup>2</sup> Abbreviations: CSA, Cross-sectional area; SC, satellite cell; SC, n/myonuclei (%), the number of SCs as a percentage of the total number of myonuclei (i.e. number of myonuclei + number of SCs).

<sup>3</sup> # Significantly different from values in type I fiber ( $P < 0.05$ )

<sup>4</sup> \* Significantly different from pre-immobilization values ( $P < 0.05$ )

## Figure legends

**Figure 1:** Outline of the experimental protocol. Two groups of healthy elderly males were included to undergo 5 days of one-legged knee-immobilization, with (PRO;  $n=11$ ) or without (CON;  $n=12$ ) protein supplementation (~20 g protein twice daily).

**Figure 2: A** Cross-sectional area (CSA) of *m. quadriceps femoris* in healthy elderly participants in the CON ( $n=12$ ) and PRO ( $n=11$ ) groups, measured by single-slice CT scan 48h prior to and immediately following 5 days of leg immobilization. **B** Leg muscle strength as measured by 1RM, in both the CON and PRO group. Data are expressed as means $\pm$ SEM. \* $P<0.05$ ; significantly different when compared with pre-immobilization values.

**Figure 3:** Skeletal muscle mRNA expression of selected genes involved in myogenesis (**A-B-C**) and muscle proteolysis (**D-E-F**), measured 48h prior to and immediately following 5 days of one-legged knee immobilization in healthy elderly men in the CON ( $n=12$ ) and PRO ( $n=11$ ) group. \* $P<0.05$ ; significantly different when compared with pre-immobilization values. Data are expressed as means $\pm$ SEM. Abbreviations: *MAFbx*, Muscle Atrophy F-box; *MuRF1*, Muscle RING-finger protein-1; *FOXO1*, Forkhead box protein O1.

**Supplemental Table 1:** Composition of the study product<sup>1,2</sup>

<b>Component</b>	<b>Unit</b>	<b>PRO</b>
Energy	kcal / kJ	150 / 635
Protein	%	55
Carbohydrates	%	25
Fat	%	18
Fiber	%	2
Protein		
Total	g	20.7
Total EAA	g	10.6
Total leucine	g	2.8
Total phenylalanine	g	0.6
Carbohydrates		
Total	g	9.4
Sugars	g	4.2
Fat		
Total	g	3.0
Saturated	g	0.8
Fiber		
Total	g	1.3
Soluble	g	1.3
Minerals		
Sodium	mg	150
Potassium	mg	279
Chloride	mg	70
Calcium	mg	500
Phosphorus	mg	250
Magnesium	mg	37
Trace elements		
Iron	mg	2.4
Zinc	mg	2.2
Copper	µg	270
Manganese	mg	0.50
Fluoride	mg	0.15
Molybdenum	µg	15
Selenium	µg	15
Chromium	µg	7.5
Iodine	µg	20
Vitamins		
Vitamin A	µg-RE	152
Cholecalciferol	µg	20
Vitamin E	mg α-TE	7.5
Phylloquinone	µg	12
Thiamin	mg	0.23

## Online supporting material (OSM)

<b>Component</b>	<b>Unit</b>	<b>PRO</b>
Riboflavin	mg	0.25
Niacin	mg NE	8.8
Pantothenic acid	mg	0.81
Vitamin B6	mg	0.76
Folic acid	µg	203
Vitamin B12	µg	3.0
Biotin	µg	6.1
Vitamin C	µg	32
Extra additions		
Carotenoids	mg	0.30
Choline	mg	56

<sup>1</sup> Data are presented as mean values,  $n=23$

<sup>2</sup> Abbreviations used: EAA, essential amino acid; NE, niacin equivalents; RE, retinol equivalents;  $\alpha$ -TE,  $\alpha$ -tocopherol equivalents

### *Muscle analyses*

Muscle samples that were mounted and frozen in Tissue-Tek were cut into 5µm thick cryosections using a cryostat at -20°C. Samples were carefully aligned for cross-sectional fiber analyses. Pre and post immobilization samples from one PRO and one CON subject were mounted together on uncoated, pre-cleaned glass slides. All biopsies were stained for muscle fiber type (FT) and satellite cell (SC) content. At the start of the staining procedure, glass slides were incubated with primary antibodies against myosin heavy chain (MHC)-I (A4.840, dilution 1:25, Developmental Studies Hybridoma Bank, Iowa City, IA), laminin (polyclonal rabbit anti-laminin, dilution 1:50; Sigma, Zwijndrecht, the Netherlands) and CD56 (dilution 1:40; BD Biosciences, San Jose, CA). CD56 has been used in previous research by ourselves (33, 58, 59) and others (60) for determination of SC content in human skeletal muscle. After washing, slides were incubated with the appropriate secondary antibodies: goat anti-rabbit IgG AlexaFluor647, goat anti-mouse IgM AlexaFluor555, and Streptavidin Alexa 488 (dilution 1:400, 1:500, and 1:200, respectively; Molecular Probes, Invitrogen, Breda, the Netherlands). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, 0.238 µM; Molecular Probes). All incubations steps were done at room temperature. Both primary and secondary antibodies were diluted in 0.1% Bovine Serum Albumin (BSA) in 0.1% Tween- phosphate-buffered saline (PBS). The staining procedure was done as follows. After slides were fixated in acetone for 5 min, slides were air dried and incubated for 30 min with 3% BSA in 0.1% Tween-PBS. After a 5 min washing step with PBS, slides were incubated with CD56 in 0.1% BSA in 0.1% Tween-PBS for 2 h. Afterwards slides were washed (standard washing protocol: 5 min 0.1% Tween-PBS, 2 x 5 min PBS) and incubated with goat anti-mouse Biotin (dilution 1:133, Vector Laboratories, Inc., Burlingame, CA) for 60 min. After washing, slides were incubated with Steptavidin for 30 min. Thereafter, slides were washed and incubated with primary antibodies against MHC-1 and laminin for 30 min. Slides were washed and the appropriate secondary antibodies were applied, diluted together with DAPI. After a final washing step, all slides were mounted with cover glasses using Mowiol (Calbiochem, Amsterdam, the Netherlands). Staining procedures resulted in nuclei stained in blue, CD56 in green, MHC-I in red, and laminin in far-red. Images were visualized and automatically captured at 10x magnification with a fluorescent microscope equipped with an automatic stage (IX81 motorised inverted microscope, Olympus, Hamburg, Germany) and EXi Aqua CCD camera (QImaging, Surrey, BC, Canada). Image acquisition was performed by Micro-Manager 1.4 software as done before (50). Analysis of the recorded images was performed by an investigator blinded to subject coding. To assess fiber circularity, form factors were calculated by using the following formula:  $(4\pi \cdot \text{CSA})/(\text{perimeter})^2$ . Fiber circularity did not change over time or between groups. Mean numbers of  $148 \pm 12$  and  $151 \pm 12$  fibers were analyzed in pre- and post-immobilization samples, respectively.

## Online supporting material (OSM)

The part of the muscle that was directly frozen in liquid nitrogen was used to determine mRNA expression of several genes of interest. Total RNA was isolated by using Tri Reagent (Sigma-Aldrich) on 10-20 mg of frozen muscle, according to the manufacturer's protocol. Quantification of total RNA was carried out spectrophotometrically at 260 nm (NanoDrop ND-1000 Spectrophotometer, Thermo Fisher Scientific, USA), and RNA purity was determined as the ratio of readings at 260/280 nm. Subsequently, first strand cDNA was synthesized from 1 µg RNA sample using random primers (Promega) and PowerScript Reverse Transcriptase (AppliedBiosystems, USA). Taqman PCR was carried out using an ABI Prism 7000 sequence detector (AppliedBiosystems, USA), with 2 µL of cDNA, 18 µL<sup>-1</sup> of each primer, 5 µL<sup>-1</sup> probe, and Universal Taqman 2 × PCR mastermix (Eurogentec) in a final volume of 25 µL. Each sample was run in duplicate, in duplex reactions, with a separate standard curve included for each gene (serial dilutions of cDNA synthesized in parallel with the study sample). 18S was used as a housekeeping gene as an internal control, and similarly to previous human immobilization studies (8, 20) it seemed unaffected by treatment (i.e. mean *Ct* values did not change over time in each of the intervention groups; *data not shown*). Taqman primer/probe sets (Applied Biosystems, Foster City, USA) were obtained for the following genes of interest: mammalian target of rapamycin (*mTOR*), P70S6 kinase (*P70S6K*), myogenic factor 4 (*myogenin*), *MyoD*, *myostatin*, Atrogin-1/Muscle Atrophy F-box (*MAFbx*), Muscle RING-finger protein-1 (*MuRF1*), Forkhead box protein O1 (*FOXO1*), Focal Adhesion Kinase (*FAK*), large neutral amino acid transporter 1 (*LATI*) and Proton-coupled amino acid transporter 1 (*PATI*). All genes of interest were labelled with the fluorescent reporter FAM. The thermal cycling conditions used were: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. *Ct* values of the genes of interest were normalized to *Ct* values of the housekeeping gene, and final results were calculated as relative expression against the standard curve.

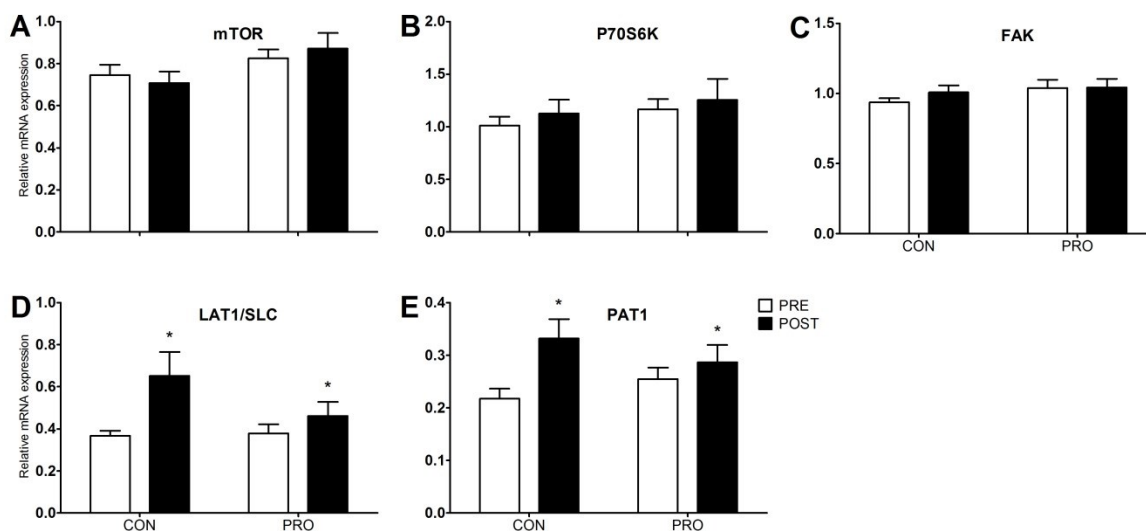
**Supplemental Table 2:** Plasma amino acid concentrations measured 48h prior to and immediately following 5 days of one-legged knee immobilization in healthy elderly men with (PRO;  $n=11$ ) or without (CON;  $n=12$ ) twice-daily protein supplementation.<sup>1,2</sup>

	CON ( $n=12$ )		PRO ( $n=11$ )	
	Pre	Post	Pre	Post
	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$
<b><math>\alpha</math>-aminobutyric acid</b>	30 $\pm$ 4	25 $\pm$ 2	27 $\pm$ 2	30 $\pm$ 4
<b>Alanine</b>	393 $\pm$ 36	485 $\pm$ 36 *	379 $\pm$ 25	423 $\pm$ 22 *
<b>Arginine</b>	88 $\pm$ 5	90 $\pm$ 4	84 $\pm$ 3	81 $\pm$ 4
<b>Asparagine</b>	47 $\pm$ 2	47 $\pm$ 2	45 $\pm$ 2	45 $\pm$ 2
<b>Aspartic acid</b>	5 $\pm$ 1	5 $\pm$ 1	4 $\pm$ 1	4 $\pm$ 1
<b>Citrulline</b>	39 $\pm$ 3	36 $\pm$ 2	35 $\pm$ 1	38 $\pm$ 3
<b>Cysteine</b>	42 $\pm$ 3	46 $\pm$ 2 *	44 $\pm$ 2	50 $\pm$ 2 *
<b>Glutamic acid</b>	69 $\pm$ 10	68 $\pm$ 7	58 $\pm$ 9	59 $\pm$ 9
<b>Glutamine</b>	605 $\pm$ 29	622 $\pm$ 27	575 $\pm$ 39	540 $\pm$ 31
<b>Glycine</b>	217 $\pm$ 11	237 $\pm$ 12	204 $\pm$ 10	194 $\pm$ 13
<b>Histidine</b>	86 $\pm$ 5	86 $\pm$ 2	83 $\pm$ 4	84 $\pm$ 3
<b>Isoleucine</b>	70 $\pm$ 5	73 $\pm$ 4	66 $\pm$ 4	76 $\pm$ 8
<b>Leucine</b>	138 $\pm$ 8	137 $\pm$ 5	128 $\pm$ 7	154 $\pm$ 16
<b>Lysine</b>	190 $\pm$ 10	191 $\pm$ 7	177 $\pm$ 9	212 $\pm$ 14
<b>Methionine</b>	28 $\pm$ 2	29 $\pm$ 1	27 $\pm$ 1	30 $\pm$ 2
<b>Ornithine</b>	60 $\pm$ 4	62 $\pm$ 3	58 $\pm$ 4	60 $\pm$ 4
<b>Phenylalanine</b>	59 $\pm$ 3	60 $\pm$ 2 *	56 $\pm$ 3	64 $\pm$ 2 *
<b>Proline</b>	218 $\pm$ 27	223 $\pm$ 22	169 $\pm$ 14	192 $\pm$ 12
<b>Serine</b>	90 $\pm$ 6	93 $\pm$ 5	85 $\pm$ 5	89 $\pm$ 7
<b>Taurine</b>	81 $\pm$ 8	89 $\pm$ 6	96 $\pm$ 10	82 $\pm$ 7
<b>Threonine</b>	129 $\pm$ 7	135 $\pm$ 8 *	120 $\pm$ 7	154 $\pm$ 15 *
<b>Tryptophan</b>	56 $\pm$ 4	59 $\pm$ 3 *	53 $\pm$ 3	62 $\pm$ 4 *
<b>Tyrosine</b>	67 $\pm$ 4	69 $\pm$ 3	65 $\pm$ 4	71 $\pm$ 3
<b>Valine</b>	261 $\pm$ 17	259 $\pm$ 9	240 $\pm$ 12	288 $\pm$ 19 *

<sup>1</sup> Data are presented as means  $\pm$  SEM,  $n=23$ .

<sup>2</sup> \* Significantly different from pre-immobilization value ( $P<0.05$ ).

## Supplemental Figure 1



**Supplemental Figure 1:** mRNA expression of anabolic genes of interest in the CON ( $n=12$ ) and PRO ( $n=11$ ) group 48h prior to and immediately following 5 days of leg immobilization.  $*P<0.05$ ; significantly different when compared with pre-immobilization values. Data are expressed as means $\pm$ SEM,  $n=23$ . Abbreviations: *FAK*, Focal Adhesion Kinase; *LAT1*, large neutral amino acid transporter; *mTOR*, mammalian target of rapamycin; *PAT1*, proton-coupled amino acid transporter 1; *P70S6K*, P70S6 kinase.

No significant time\*treatment nor time effects were found for *mTOR* (Supplemental Figure 1A), *P70S6K* (Supplemental Figure 1B), and *FAK* (Supplemental Figure 1C: all  $P>0.05$ ).