

Dietary feeding pattern does not modulate the loss of muscle mass or the decline in metabolic health during short-term bed rest

Marlou L. Dirks¹, Joey S.J. Smeets¹, Andrew M. Holwerda¹, Imre W.K. Kouw¹, Gabriel N. Marzuca-Nassr¹, Annemie P. Gijsen¹, Graham P. Holloway², Lex B. Verdijk¹, and Luc J.C. van Loon¹

¹*Department of Human Biology, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre⁺, the Netherlands*

²*Human Health & Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada*

Address for correspondence:

Prof. L.J.C. van Loon, PhD
Maastricht University Medical Centre⁺
P.O. Box 616
6200 MD, Maastricht, the Netherlands
Phone: +31 43 3881397
Email: L.vanLoon@maastrichtuniversity.nl

Short running head: Dietary feeding pattern during bed rest

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Abbreviations: BMD, bone mineral density; BMI, body mass index; BW, body weight; CSA, cross-sectional area; CT, computed tomography; DXA, dual-energy X-ray absorptiometry; en%, energy percentage; FoxO1, Forkhead box protein O1; GIR, glucose infusion rate; HbA_{1c}, glycated hemoglobin; MAFbx, Atrogen-1/Muscle Atrophy F-box; MJ, Mega Joule; mTOR, mammalian target of rapamycin; MuRF1, Muscle RING-finger protein-1; P70S6K, ribosomal protein 70-kDa S6 kinase; RMR, resting metabolic rate

1 **Abstract**

2 Short periods of bed rest lead to the loss of muscle mass and quality. It has been speculated
3 that dietary feeding pattern may impact upon muscle protein synthesis rates and, therefore,
4 modulate the loss of muscle mass and quality. We subjected 20 healthy men (age: 25 ± 1 y, BMI:
5 23.8 ± 0.8 kg·m⁻²) to one week of strict bed rest with intermittent (4 meals/day) or continuous
6 (24 h/day) enteral tube feeding. Participants consumed deuterium oxide for 7 days prior to bed
7 rest and throughout the 7-day bed rest period. Prior to and immediately after bed rest, lean body
8 mass (DXA), quadriceps cross-sectional area (CSA; CT), maximal oxygen uptake capacity
9 (VO₂peak), and whole-body insulin sensitivity (hyperinsulinaemic-euglycaemic clamp) were
10 assessed. Muscle biopsies were collected 7 days prior to, 1 day prior to, and immediately after
11 bed rest to assess muscle tracer incorporation. Bed rest resulted in 0.3 ± 0.3 vs 0.7 ± 0.4 kg lean
12 tissue loss and a 1.1 ± 0.6 vs $0.8\pm 0.5\%$ decline in quadriceps CSA in the intermittent vs
13 continuous feeding group, respectively (both $P<0.05$), with no differences between groups
14 (both $P>0.05$). Moreover, feeding pattern did not modulate the bed rest-induced decline in
15 insulin sensitivity ($-46\pm 3\%$ vs $39\pm 3\%$; $P<0.001$) or VO₂peak (-2.5 ± 2.2 vs $-8.6\pm 2.2\%$;
16 $P<0.010$)(both $P>0.05$). Myofibrillar protein synthesis rates during bed rest did not differ
17 between the intermittent and continuous feeding group (1.33 ± 0.07 vs $1.50\pm 0.13\%$ ·d⁻¹,
18 respectively; $P>0.05$). In conclusion, dietary feeding pattern does not modulate the loss of
19 muscle mass or the decline in metabolic health during one week of bed rest in healthy men.

20

21 **Abstract word count: 248**

22 **Introduction**

23 Periods of bed rest are often required for the recovery from illness or injury. Despite the
24 necessity of such periods of disuse for recovery, bed rest leads to substantial changes in body
25 composition, characterized by a decrease in skeletal muscle mass of 0.5-0.6% per day (64), and
26 an overall decline in metabolic health (5). The impact of bed rest on muscle mass and quality
27 is already evident after as little as 5-7 days of bed rest (20, 24, 56, 58). This is of important
28 clinical relevance, as the current overall average duration of hospitalization for all ages and
29 reasons for hospital admission is seven days (22). However, the reason for the bed rest-induced
30 decline in muscle mass and muscle quality remains to be elucidated.

31 Both physical activity and food intake are key anabolic stimuli, which are required to maintain
32 skeletal muscle tissue mass and quality. Muscle contractions as well as food intake, i.e.
33 ingestion of protein meals, strongly increase muscle protein synthesis rates and improve net
34 muscle protein balance (47, 48). Hospitalization is characterized by a strong decline or even
35 absence of physical activity due to restricted bed rest. Furthermore, in many patients food
36 intake is reduced, often due to surgical stress, anxiety, nausea, lack of appetite, and/or
37 gastrointestinal disorders. Maintaining energy balance and habitual protein consumption have
38 been shown to be requirements to attenuate muscle loss during a period of bed rest or limb
39 immobilization (7, 52). In many conditions, this is performed by nutritional supplementation
40 or even enteral (tube) feeding.

41 Previous work has shown that ingestion of 20 g of a high quality protein maximizes muscle
42 protein synthesis rates during a four hour postprandial period (67, 68). This has led to the
43 formation of guidelines advocating consumption of 20 g protein with each main meal (16). Due
44 to the stimulation of muscle protein synthesis following ingestion of each meal, an intermittent
45 feeding strategy has been suggested to be preferred over more continuous feeding.
46 Furthermore, the hormonal response to continuous feeding may be suboptimal to fully suppress

47 postprandial muscle protein breakdown (29). However, whether intermittent feeding leads to
48 an attenuated decline in skeletal muscle mass and/or quality when compared to continuous
49 feeding is far from evident. Animal work has suggested that continuous feeding leads to lower
50 rates of muscle protein synthesis (21, 26) and a more rapid decline in insulin sensitivity (54).
51 However, work in humans is inconclusive (12, 37), and the impact of dietary feeding pattern
52 on bed rest-induced muscle atrophy remains to be assessed. We hypothesized that continuous
53 enteral feeding would lead to greater loss of muscle mass and quality when compared to
54 intermittent enteral feeding during one week of bed rest in healthy volunteers fed in energy
55 balance.

56 To test this hypothesis, we subjected 20 young, healthy men to one week of bed rest while
57 being tube-fed in energy balance using either a continuous (24 h) or an intermittent (4 boluses
58 daily) enteral feeding protocol. Muscle mass (CT, DXA) and metabolic health (VO_2 peak,
59 whole-body insulin sensitivity via hyperinsulinaemic-euglycaemic clamp) were assessed prior
60 to and after one week of bed rest. Muscle protein synthesis rates were assessed for one week
61 prior to bed rest and during one week of bed rest using deuterated water administration and
62 muscle biopsy sampling. This is the first study to compare the impact of continuous versus
63 intermittent enteral feeding on changes in muscle mass and quality during one week of bed rest
64 *in vivo* in humans.

65 **Methods**

66

67 *Participants*

68 Twenty healthy, young men (age 25 ± 1 y) were included in the present study. Participants´
69 characteristics are presented in **Table 1**. Prior to inclusion, participants completed a general
70 health questionnaire and visited the University for a routine medical screening to ensure their
71 eligibility to take part. Exclusion criteria included a BMI below 18.5 or above $30 \text{ kg}\cdot\text{m}^{-2}$, a
72 (family) history of deep vein thrombosis, type 2 diabetes mellitus (determined by HbA_{1c} values
73 $>7.0\%$), and any back, knee or shoulder complaints that could be problematic during the bed
74 rest period. Additionally, participants who had been involved in progressive resistance-type
75 exercise training during the 6 months prior to the study were also excluded. All subjects were
76 informed on the nature and risks of the experiment before written informed consent was
77 obtained. During the screening visit, a fasting blood sample was taken to assess HbA_{1c} and
78 resting energy expenditure was measured with the use of a ventilated hood. The current study
79 was part of a larger project investigating the impact of short-term bed rest on muscle mass and
80 metabolic health, registered on clinicaltrials.gov as NCT02521025. The study was approved
81 by the Medical Ethical Committee of Maastricht University Medical Centre⁺ (registration
82 number MEC 15-3-035) in accordance with the latest version of the Declaration of Helsinki.

83

84 *Experimental outline*

85 Following inclusion, participants visited the University for a deuterium oxide (D_2O) loading
86 visit. On the subsequent day, on test day 1, a single muscle biopsy was taken from the *m. vastus*
87 *lateralis*. After this visit, a 7-day period of standardized nutrition was started. On day 7 of this
88 standardized diet (test day 2), a second muscle biopsy was obtained, DXA and CT scans and a
89 hyperinsulinemic-euglycemic clamp were performed. VO_2peak was assessed prior to the free-

90 living period, and on the day following bed rest. On the same evening participants arrived at
91 the University for insertion of a nasogastric tube, and subsequently stayed overnight. The
92 following morning at 8:00, a 7-day period of strict bed rest was started. During this period,
93 participants were tube-fed with an enteral food product in an intermittent ($n=10$, Intermittent,
94 4 boluses per day) or continuous ($n=10$, Continuous, 24 h per day at a constant rate) feeding
95 pattern. After exactly seven days, test day 2 was repeated and participants were allowed to go
96 home.

97

98 *One week of bed rest*

99 Participants underwent a 7-day period of strict bed rest to mimic the effects of a standard
100 hospitalization period. On the morning of day 1, at 8:00, participants started the 7-day period
101 of strict bed rest during which they were not allowed to leave the bed. During daytime,
102 participants were allowed to use a pillow and slight elevation of the bed-back to be able to
103 perform their daily activities. Washing and all sanitary activities were performed in bed.
104 Participants were woken at 7:30 and lights were switched off at 23:30 every day. Participants
105 were continuously monitored by members of the research team.

106

107 *Dietary intake*

108 During the screening visit, resting energy expenditure was measured by indirect calorimetry
109 using an open-circuit ventilated hood system (Omnical, Maastricht University, Maastricht, the
110 Netherlands; (50)). During the seven days prior to bed rest, and during the bed rest period itself,
111 dietary intake was fully controlled. During the pre-bed rest period, subjects received all food
112 products from the research team. Energy requirements were estimated based on indirect
113 calorimetry data, multiplied by an activity factor (AF) of 1.60 (free-living) and 1.35 (bed rest).
114 Energy intake was adjusted if participants reported to be hungry or felt overfed for more than

115 one day. In those situations, food provision was adjusted by decreasing or increasing the
116 activity factor by 0.1. Macronutrient composition of the diet was identical between free-living
117 and bed rest periods (**Table 2**).

118 During bed rest, food administration in both groups was performed via a nasogastric tube
119 (Flocare© PUR tube Enlock, Ch8, 110 cm, Nutricia Advanced Medical Nutrition, Utrecht, the
120 Netherlands). Correct positioning of the tube in the stomach was assessed by means of a pH
121 check directly following insertion and on every morning during the bed rest period. A standard
122 enteral food product (Nutrison Multi Fibre, Nutricia Advanced Medical Nutrition) was given,
123 composed of 47 en% carbohydrates, 34 en% fat, 16 en% protein (blend of casein, whey, soy,
124 and pea), and 3 en% fibers. Participants in the intermittent feeding group received the same
125 product provided in four daily boluses. These boluses were administered at a rate of $25 \text{ mL} \cdot \text{min}^{-1}$
126 ¹ (providing ~28 g protein per bolus) at 8:00 (30% of total daily food intake), 13:00 (30%),
127 18:00 (30%), and 23:00 (10%, representing a smaller pre-sleep meal), with the first meal
128 administered on the morning of the first day of bed rest. Participants in the continuous feeding
129 were fed in a continuous manner, using a Flocare© Infinity enteral feeding pump (Nutricia
130 Advanced Medical Nutrition) at a constant speed (i.e. $\sim 100 \text{ mL} \cdot \text{h}^{-1}$) based on daily energy
131 requirements. Continuous feeding started at 0:00 on the evening before bed rest and ended at
132 0:00 on the evening of day 7 to ensure fasting conditions on test day 3. Nasogastric tubes were
133 removed at 0:00 on the evening of day 7 in both groups.

134

135 *Body composition*

136 During test days 2 and 3 (one day prior to and immediately after bed rest, respectively), at 9:00,
137 anatomical cross-sectional area (CSA) of the quadriceps muscle was assessed via a single slice
138 CT scan (Philips Brilliance 64, Philips Medical Systems, Best, the Netherlands) as described
139 previously (20). Briefly, a 3 mm thick axial image was made at 15 cm above the patella, with

140 participants in supine position while their legs were extended and their feet secured. On test
141 day 2, the exact scanning position was marked on the skin with semi-permanent ink for
142 replication on test day 3. CT scans were analyzed for quadriceps muscle CSA by manual tracing
143 using ImageJ software (version 1.50c, National Institute of Health, Maryland, USA, (55)). On
144 the same days, a DXA-scan (Dual Energy X-Ray Absorptiometry; Hologic, Discovery A,
145 Waltham, MA, USA) was made at 14:00 to assess body composition. The system's software
146 package Apex version 4.0.2 (en-CORE 2005, version 9.15.00 Hologic, Marlborough, MA,
147 USA) was used to determine whole-body and regional lean mass, fat mass, and bone mineral
148 content.

149

150 *Metabolic health*

151 Prior to the free-living period and on the day following bed rest, maximal oxygen uptake
152 capacity was measured as VO_2peak (described previously (20)). Whole-body insulin sensitivity
153 was measured via a one-step hyperinsulinaemic-euglycaemic clamp as described previously
154 (20). In short, 20% glucose (Baxter B.V., Utrecht, the Netherlands) was co-infused with insulin
155 ($40 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$; Novorapid, Novo Nordisk Farma, Alphen aan den Rijn, the Netherlands)
156 during a 2.5 h clamp which was started at 9:30. Arterialized blood glucose concentrations were
157 measured every 5 min, and the glucose infusion rate was altered to maintain euglycaemia at
158 $5.0 \text{ mmol} \cdot \text{L}^{-1}$.

159

160 *Deuterium oxide loading and body water enrichments*

161 To increase body water deuterium oxide (D_2O , or ^2H) enrichments, participants attended the
162 University for a D_2O loading day. During that day, participants consumed 8 x 50 mL oral doses
163 of 70% D_2O (Cambridge Isotope Laboratories, Tewksbury, MA, USA) with 1.5 h in between
164 doses. To maintain body water enrichments throughout the study period, participants consumed

165 one daily 50 mL oral dose every morning of the study period. Daily saliva samples were
166 collected using a cotton swab at 18:00 on every study day, to determine body water enrichment.
167 Samples were frozen in liquid nitrogen and stored at -80°C. Body water ²H-alanine enrichments
168 were measured as described elsewhere (32). In short, samples were centrifuged at 10,000 g to
169 remove debris and subsequently diluted 70-fold with ddH₂O to achieve deuterium enrichments
170 within the detection limits of the GC-C-IRMS. Samples were prepared for analysis using the
171 protocol by Scrimgeour and colleagues (51). This involved placing small plastic cups holding
172 4 mg of catalyst (5% platinum on alumina, 325 mesh, Sigma-Aldrich, St. Louis, USA) inside
173 3 mL glass vials, after which 300 µL of diluted saliva sample was added and vials were sealed
174 using rubber septums and a screw cap. Air in each vial was evacuated and replaced by hydrogen
175 gas simultaneously, after which vials were left at 21 °C for 24 h for deuterium equilibration to
176 occur between the hydrogen gas and the saliva samples. The deuterium enrichment of the
177 hydrogen gas was then measured in duplicate on a GC-C-IRMS (Micromass Optima IRMS
178 fitted with a Multiprep and Gilson autoinjector, Micromass UK Limited, Manchester, UK).
179 Standard regression curves were applied from a series of known standard enrichment values
180 against the measured values to assess the linearity of the mass spectrometer and to account for
181 deuterium loss during equilibration.

182

183 *Myofibrillar protein synthesis*

184 On test days 1, 2, and 3, a single muscle biopsy sample was collected from *m. vastus lateralis*
185 at 8:15. After local anesthesia was induced, a percutaneous needle biopsy was taken
186 approximately 15 cm above the patella using the Bergström technique (6). The collected
187 muscle tissue was freed from any visible blood and non-muscle tissue, and rapidly frozen in
188 liquid nitrogen. Muscle samples were subsequently stored at -80°C until further analyses.
189 Myofibrillar protein enriched fractions were extracted from ~60 mg of wet muscle tissue by

190 hand-homogenizing on ice using a pestle in a standard extraction buffer ($10 \mu\text{L}\cdot\text{mg}^{-1}$). The
191 samples were spun at $2500 g$ and 4°C for 5 min. The pellet was washed with $500 \mu\text{L}$ ddH₂O
192 and centrifuged at $2500 g$ and 4°C for 10 min. The myofibrillar protein was solubilized by
193 adding 1 mL of 0.3 M NaOH and heating at 50°C for 30 min with vortex mixing every 10 min.
194 Samples were centrifuged at $9500 g$ and 4°C for 5 min, the supernatant containing the
195 myofibrillar proteins was collected and the collagen pellet was discarded. Myofibrillar proteins
196 were precipitated by the addition of 1 mL of 1 M PCA and spinning at $700 g$ and 4°C for 10
197 min. The myofibrillar protein was washed twice with 70% ethanol and hydrolyzed overnight
198 in 2 mL of 6 M HCL at 110°C . The free amino acids from the hydrolyzed myofibrillar protein
199 pellet were dried under a continuous nitrogen stream while being heated at 120°C . The free
200 amino acids were then dissolved in 25% acetic acid solution, passed over cation exchange AG
201 50W-X8 resin columns (mesh size: 100-200, ionic form: hydrogen; Bio-Rad Laboratories,
202 Hercules, CA, USA), and eluted with 2 M NH₄OH. Thereafter, the eluate was dried and the
203 purified amino acids were derivatized to their N(O,S)-ethoxycarbonyl ethyl esters (33). The
204 derivatized samples were measured using a gas chromatography-isotope ratio mass
205 spectrometer (GC-IRMS; Thermo Fisher Scientific, MAT 253; Bremen, Germany) equipped
206 with a pyrolysis oven and a 60 m DB-17MS column (no. 122-4762; Agilent, Wilmington, DE,
207 USA) and 5 m precolumn. Ion masses 2 and 3 were monitored to determine the $^2\text{H}/^1\text{H}$ ratios of
208 muscle protein bound alanine. A series of known standards was applied to assess linearity of
209 the mass spectrometer and to control for the loss of tracer.

210

211 *Skeletal muscle gene expression*

212 A second part of the obtained muscle sample ($\sim 15 \text{ mg}$) was used to measure mRNA expression
213 of target genes as described in detail elsewhere (61). Briefly, total RNA was isolated from
214 frozen muscle tissue and spectrophotometrically quantified. Next, after RNA purity was

215 determined and cDNA synthesis was performed, Taqman PCR was carried out using 18S as a
216 housekeeping gene. We have previously demonstrated that 18S expression does not change
217 with muscle disuse (63). Taqman probe sets were obtained from Applied Biosystems (Foster
218 City, CA, USA) for the following genes of interest: Atrogen-1/Muscle Atrophy F-box
219 (MAFbx), Forkhead box protein O1 (FoxO1), mammalian target of rapamycin (mTOR),
220 Muscle RING-finger protein-1 (MuRF1), and ribosomal protein 70-kDa S6 kinase (P70S6K).
221 Ct values of the target genes were normalized to Ct values of 18S, and final results were
222 calculated as relative expression against the standard curve.

223

224 *Nitrogen balance*

225 On every day of the bed rest period, 24 h urine collection was performed starting from the
226 second voiding of the day until the first voiding on the day after. Urine was collected into
227 containers with 10 mL of 4 M HCl. After the total daily urine production was measured,
228 aliquots of urine were snap-frozen in liquid nitrogen and stored at -80°C. The Dumas
229 combustion method was used to determine nitrogen using the vario MAX cube CN (Elementar
230 Analysensysteme, Germany) as described before (60).

231

232 *Statistics*

233 The two-tailed sample size calculation ($\alpha=0.05$, power=0.8) was based on an expected $29\pm 5\%$
234 decline in insulin sensitivity following one week of bed rest with intermittent feeding (20), and
235 an expected 25% worsening thereof (i.e. $-36\pm 5\%$) in the continuous feeding group (54). This
236 resulted in a required sample size of $n=10$ participants per group. Baseline differences between
237 groups were assessed using an independent samples *t*-test. Changes over time were analyzed
238 using a Repeated Measures ANOVA with time (free-living vs bed rest or pre- vs post-bed rest)
239 as within-subjects factor and group (intermittent vs continuous) as between-subjects factor. In

240 case of a significant interaction, a Bonferroni post hoc test was applied to locate individual
241 differences. Statistical data analysis was performed using SPSS version 24.0 (IBM Corp,
242 Armonk, NY, USA). Statistical significance was set at $P < 0.05$. All data are expressed as
243 means \pm SEM.

244 **Results**

245

246 *Body composition*

247 The two experimental groups did not differ in any of the participants' characteristics (**Table 1**)
248 prior to the start of the study (all $P>0.05$). After one week of bed rest, quadriceps cross-
249 sectional area (CSA; **Figure 2A**) had declined by $1.1\pm 0.6\%$ (from 7513 ± 522 to 7430 ± 511
250 mm^2) and $0.8\pm 0.5\%$ (from 7544 ± 549 to 7469 ± 522 mm^2) in the intermittent and continuous
251 feeding groups, respectively ($P<0.05$). No differences were observed between groups
252 (interaction effect, $P>0.05$). Bed rest led to an average 0.62 ± 0.19 kg decline in total body mass
253 ($P<0.01$; **Table 3**), which was predominantly attributed to a loss of trunk lean mass (-0.52 ± 0.12
254 and -0.36 ± 0.19 kg in the intermittent and continuous feeding group, respectively; $P<0.01$),
255 which did not differ between groups ($P>0.05$). Due to the maintenance of energy balance
256 during bed rest, no changes in whole-body fat mass were observed (interaction effect, $P>0.05$).

257

258 *Maximal oxygen uptake capacity and whole-body insulin sensitivity*

259 VO_2peak (**Figure 1B**) declined from 40.3 ± 3.0 to 38.9 ± 2.5 $\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ following bed rest
260 with intermittent feeding and from 44.8 ± 3.1 to 40.7 ± 2.6 $\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ following bed rest with
261 continuous feeding (time effect $P<0.001$), with no differences between groups (interaction
262 effect, $P>0.05$). Glucose infusion rate (**Figure 1C**), representing whole-body insulin
263 sensitivity, declined by $46\pm 3\%$ following bed rest with intermittent and $39\pm 3\%$ following bed
264 rest with continuous feeding (time effect $P<0.001$), with no differences between groups
265 (interaction effect, $P>0.05$).

266

267 *Cumulative muscle protein synthesis*

268 Analyses of daily saliva samples revealed a gradual increase in body water enrichments
269 (**Figure 3**; time effect $P<0.001$), with no differences between groups. Cumulative myofibrillar
270 protein fractional synthesis rates (FSR; **Figure 4**) were not different between groups during the
271 free-living period. Moreover, no significant differences between free-living and bed rest (time
272 effect, $P>0.05$) or between groups during bed rest (interaction effect $P>0.05$, treatment effect
273 $P>0.05$) were found.

274

275 *Skeletal muscle gene expression*

276 Skeletal muscle mRNA expression of genes involved in muscle mass regulation, are depicted
277 in **Figure 5**. For mTOR and P706SK, both key players in the regulation of muscle protein
278 synthesis, no significant effects were found (interaction effect, all $P>0.05$). FoxO1 and MuRF1
279 mRNA expression also were not influenced by bed rest or dietary feeding pattern (interaction
280 effect, both $P>0.05$). MAFBx (**Figure 5D**) mRNA expression showed a time effect ($P<0.01$)
281 but no interaction effect ($P>0.05$), demonstrating increased expression following bed rest in
282 both feeding strategies. Skeletal muscle mRNA expression of the housekeeping gene 18S was
283 not affected by bed rest or dietary feeding pattern (interaction and time effect both $P>0.05$).

284

285 *Nitrogen balance*

286 Dietary nitrogen intake during bed rest, derived from dietary protein intake, was on average
287 15.0 ± 0.6 and 15.4 ± 0.5 $\text{g}\cdot\text{d}^{-1}$ in the intermittent and continuous feeding groups, respectively,
288 with no differences over time or between groups (both $P>0.05$). Urinary nitrogen loss showed
289 a time effect ($P<0.05$), such that urinary nitrogen loss was greater on day 7 than on day 1. From
290 these data, 24h nitrogen balance was calculated (**Figure 6**). We show that 7 days of bed rest,
291 irrespective of dietary feeding pattern (interaction effect, $P>0.05$), leads to a decline in whole-
292 body nitrogen balance (time effect, $P<0.05$). However, a significant treatment effect ($P<0.05$)

293 indicated that at all time points the continuous feeding group was in a more positive nitrogen
294 balance.

295 **Discussion**

296 In the current study, we observed that one week of strict bed rest reduced muscle mass, lowered
297 oxygen uptake capacity, and impaired insulin sensitivity in healthy volunteers fed in energy
298 balance. Dietary feeding pattern, i.e. enteral food administration in an intermittent versus
299 continuous manner, did not impact the bed rest-induced decline in muscle mass and metabolic
300 health. Moreover, measures of muscle protein synthesis rates and markers of muscle protein
301 breakdown were not influenced by the pattern of food administration.

302 In line with previous work in our laboratory (20) as well as others (7, 23, 24, 52, 56), we show
303 the impact of one week of bed rest on muscle mass and metabolic health. The average 525 ± 219
304 g loss of lean tissue and 0.9 ± 0.4 % decline in quadriceps CSA was less than what we had
305 expected based upon the 1.4 ± 0.2 kg lean tissue loss and 3.2 ± 0.9 % decline in quadriceps CSA
306 we recently observed following one week of bed rest in our laboratory (20). The apparent
307 discrepancy may be attributed to the enteral feeding regimens as opposed to normal food
308 consumption (13) and/or the composition of the standard enteral feeds (which are typically
309 higher in protein and/or branched chain amino acids content than normal foods). Daily protein
310 intake in the present study was $1.25 \text{ g} \cdot \text{kg body weight}^{-1} \cdot \text{d}^{-1}$ (**Table 2**) compared to $0.98 \text{ g} \cdot \text{kg}$
311 $\text{body weight}^{-1} \cdot \text{d}^{-1}$ in our previous study (20). Furthermore, the enteral feeding product had a
312 branched-chain amino acid content (22 g per 100 g protein) that is even higher than milk or
313 beef (11). The anabolic properties of the BCAAs (14, 34) may have contributed to the lesser
314 muscle loss (45, 52) in the present study when compared to our previous work. The observed
315 muscle atrophy was accompanied by a substantial $\sim 5\%$ decline in maximal oxygen uptake
316 capacity and a $\sim 40\%$ decrease in whole-body insulin sensitivity (**Figure 1**). To put this in
317 perspective, such a decline in muscle mass and metabolic health is similar to what is generally
318 observed over many years of aging (15, 42, 46). Clearly, it is of important clinical relevance to
319 gain more insight in the mechanisms underlying disuse-induced atrophy and insulin resistance,

320 to develop interventions that can attenuate a decline in muscle mass and health during short
321 episodes of muscle disuse.

322 We hypothesized that dietary feeding pattern would modulate the rate of muscle atrophy as
323 well as the bed rest-induced impairments in oxygen uptake capacity and insulin sensitivity.
324 Therefore, we provided 20 healthy subjects with nasogastric feeding tubes to allow continuous
325 and intermittent feeding with exactly the same clinical enteral feeding product. To mimic the
326 ingestion of various meals we administered the enteral feed in an intermittent pattern, providing
327 four daily boluses mimicking three main meals and a pre-bed snack, to half of the participants.
328 In contrast, the continuous enteral feeding group received the same amount of food
329 continuously (24/7). Previous work has suggested that dietary feeding pattern forms an
330 important factor driving postprandial muscle protein synthesis. Specifically, ingestion of a
331 single meal-like bolus of 20 g protein is required to significantly increase muscle protein
332 synthesis rates and inhibit protein breakdown, thereby resulting in net muscle protein accretion
333 (10, 30, 62, 67, 68). Based upon these findings it has been suggested that each main meal should
334 contain ample protein to allow such a postprandial anabolic response, and that a dietary intake
335 pattern containing less protein in each meal would be suboptimal in maintaining muscle mass.
336 In support, some studies (2, 4, 12, 21, 26, 65) but certainly not all (3, 36, 37, 39, 40) have
337 shown a more positive impact of bolus feeding on muscle protein synthesis and/or muscle
338 protein retention when compared to more frequent feeding of smaller quantities of food.
339 Subjects in the intermittent enteral feeding group were administered 4 daily boluses containing
340 28 ± 1 g protein, 83 ± 4 g carbohydrate and 27 ± 1 g fat. This amount of high quality protein would
341 provide sufficient amino acids to stimulate muscle protein synthesis, inhibit muscle protein
342 breakdown and, as such, stimulate postprandial muscle protein accretion. Although a minor
343 delay in protein digestion may occur when other macronutrients are co-ingested with protein
344 (27, 28), this does not modulate total plasma amino acid availability or postprandial muscle

345 protein synthesis rates (27, 28). As such, the repeated stimulation of muscle protein synthesis
346 with the intermittent mixed meal feeding pattern should theoretically lead to an attenuated
347 decline in skeletal muscle mass and metabolic health when compared to a situation where
348 participants are fed in a continuous manner. In contrast to our hypothesis, we observed no
349 differences in the decline in muscle mass, oxygen uptake capacity or insulin sensitivity
350 following one week of bed rest combined with continuous versus intermittent feeding (**Figure**
351 **2, Table 3**). Therefore, we conclude that feeding pattern does not modulate the decline in
352 muscle mass and health during short periods of bed rest in healthy volunteers when fed in
353 energy balance.

354 To assess whether potential differences in muscle mass loss during continuous versus
355 intermittent feeding could be (partly) explained by differences in daily muscle protein synthesis
356 rates, we applied the deuterated water method as a means to assess muscle protein synthesis
357 rates over a more extended time frame (32). In the present study, muscle protein synthesis rates
358 averaged $\sim 1.4 \pm 0.1\% \cdot d^{-1}$. These findings are in agreement with previous studies from our lab
359 (32) as well as others (38, 66) applying the deuterated water method. In line with the absence
360 of measurable differences in muscle mass loss between the intermittent and continuous feeding
361 regimen, no differences were observed in daily protein synthesis rates between groups
362 (1.33 ± 0.07 vs $1.50 \pm 0.13\% \cdot d^{-1}$ with intermittent and continuous feeding, respectively; **Figure**
363 **4**). To our surprise we also did not observe significant differences in daily protein synthesis
364 rates assessed in the week prior to bed rest and the week during bedrest, independent of the
365 feeding regimen applied during bed rest (1.33 ± 0.04 vs $1.41 \pm 0.07\% \cdot d^{-1}$ during free-living and
366 bed rest, respectively). This is surprising as lower postabsorptive (23, 25, 57) and postprandial
367 (8, 45) muscle protein synthesis rates have been reported in young individuals following 1-4
368 weeks of bed rest. In contrast, our data seem to be more in line with recent work showing that
369 a shorter period (i.e. 5 days) of bed rest does not affect muscle protein synthesis rates in healthy

370 young volunteers. Nonetheless, the amount of leg muscle mass lost in the present study (i.e.
371 less than 50 g) may have been insufficient to allow the detection of significant declines in daily
372 protein synthesis rates using the deuterated water method (58). More work is required applying
373 deuterated water as a means to assess the impact of changes in muscle protein synthesis rates
374 as a key factor in explaining net muscle loss during (short) periods of disuse.

375 Consequently, the observed muscle atrophy (**Figure 1** and **2**) may be largely caused by an
376 increase in muscle protein breakdown rates. Though data are quite limited, all available direct
377 (57) and indirect (23) measurements of muscle protein breakdown rates suggest no changes in
378 postabsorptive muscle protein breakdown rates following several weeks of muscle disuse.

379 However, we (19, 61, 62) and others (1, 59) have demonstrated a rapid but transient increase
380 in molecular proxies for muscle protein breakdown during the first few days following the
381 onset of muscle disuse. In line, we observed an increase in MAFBx expression following
382 bedrest in both treatment groups (**Figure 5**). Although it remains unclear whether muscle
383 protein breakdown rates are increased following short-term disuse, and if so, whether this is
384 attributed to increased postabsorptive and/or postprandial muscle protein breakdown rates, our
385 data seem to support previous suggestions that muscle protein breakdown is increased
386 following the onset of disuse (1, 19, 59, 61, 62). It has been suggested that continuous enteral
387 feeding may have a greater impact on muscle protein breakdown due to the continuous insulin-
388 mediated suppression of proteolysis (29), whereas intermittent feeding has a greater impact on
389 protein synthesis due to the repeated hyperinsulinaemia and hyperaminoacidaemia (9).

390 Although we did not assess muscle proteolysis, mRNA expression of key proteins involved in
391 the regulation of muscle protein breakdown did not show differences between feeding
392 strategies. Consequently, our data do not support that large differences in muscle protein
393 breakdown rates exist between continuous versus intermittent enteral feeding (**Figure 5**).

394 Though muscle protein synthesis rates (using deuterated water) and markers of muscle protein
395 breakdown do not seem to support this (**Figures 4 and 5**), our observations of nitrogen balance
396 seem to indicate that continuous feeding leads to greater whole-body nitrogen retention when
397 compared with intermittent feeding (**Figure 6**). This is in agreement with some (37) but not all
398 (12) work in patients, and could suggest that continuous feeding may lead to better preservation
399 of whole-body protein during more prolonged bed rest. Although a positive nitrogen balance
400 during bed rest has been shown before in some (23, 53) but not all (35, 49) studies, it seems to
401 be at odds with the decline in lean mass that was observed in the present study (**Figures 1 and**
402 **2**). Due to the nature of the whole-body nitrogen balance method, it is impossible to determine
403 the tissue(s) responsible for the greater nitrogen retention, which likely include splanchnic
404 tissues, other organs, as well as the impact on the microbiota. However, as we failed to see any
405 preservation of muscle mass or metabolic health with continuous versus intermittent feeding,
406 we assume that the observed greater nitrogen retention following continuous versus
407 intermittent feeding is not *per se* reflective of skeletal muscle tissue.

408 This is the first study to assess the impact of continuous versus intermittent enteral feeding
409 during bed rest in healthy men fed in energy balance. Under these conditions, the enteral
410 feeding pattern had no impact on the decline in muscle mass, oxygen uptake capacity, and
411 insulin sensitivity. These data are important for clinical practice where the proposed benefits
412 of intermittent over continuous enteral feeding strategies are currently a topic of intense debate
413 (17). Bed-rested individuals under conditions of reduced energy intake tend to lose more
414 muscle mass than those fed in energy balance (7). This seems to be in line with the observation
415 that muscle protein synthesis rates are lower during caloric restriction (31, 41, 44). It could be
416 speculated that dietary feeding pattern has a more potent effect under conditions of an energy
417 and/or protein deficit. Therefore, similar approaches should be applied to assess the impact of
418 different feeding strategies on muscle health. However, under conditions where appropriate

419 energy and protein is provided to support muscle mass maintenance, enteral feeding pattern
420 does not modulate the decline in muscle mass or metabolic health during a short period of
421 bedrest. Of course, besides appropriate nutrition some level of physical activity and/or muscle
422 contraction will always be required to allow preservation of skeletal muscle mass and metabolic
423 health during a period of disuse (18, 19, 43). As such, strategies need to be developed to define
424 the minimal amount of physical activity required to maintain muscle mass and metabolic
425 function under conditions where malnutrition is no longer evident.

426 In conclusion, dietary feeding pattern does not modulate the decline in skeletal muscle mass,
427 oxidative capacity, or insulin sensitivity during one week of bed rest in healthy men fed in
428 energy balance.

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438

439 **Conflict of interest**

440 LJCvL has received research grants, consulting fees, speaking honoraria, or a combination of
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443

444 **Author contributions**

445 MLD and LJCvL designed the study. MLD, JSJS, IWKK, GNM-N, and GPH organized and
446 performed the experiments. AMH and APG performed the sample analyses. MLD analyzed
447 the data. MLD, JSJS, IWKK, AMH, LBV, and LJCvL interpreted the data. MLD drafted the
448 manuscript. MLD and LJCvL edited and revised the manuscript, and all authors approved the
449 final version.

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Tables

Table 1: Participants' characteristics

	Intermittent (<i>n</i>=10)	Continuous (<i>n</i>=10)
Age (y)	27 ± 1	24 ± 1
Body mass (kg)	77.5 ± 5.1	77.3 ± 5.1
Height (m)	1.81 ± 0.03	1.79 ± 0.03
BMI (kg·m⁻²)	23.5 ± 1.3	24.0 ± 1.0
HbA_{1c} (%)	5.2 ± 0.1	5.2 ± 0.2
RMR (MJ·d⁻¹)	7.6 ± 0.4	7.6 ± 0.3

BMI, body mass index; HbA_{1c}, glycated hemoglobin; RMR, resting metabolic rate

Table 2: Dietary intake

	Intermittent (<i>n</i>=10)		Continuous (<i>n</i>=10)	
	Free-living	Bed rest	Free-living	Bed rest
Energy (MJ·d⁻¹)	11.3 ± 0.7	9.8 ± 0.4 *	10.8 ± 0.3	10.1 ± 0.4 *
Protein (g·kg BW⁻¹·d⁻¹)	1.4 ± 0.1	1.2 ± 0.1 *	1.4 ± 0.1	1.3 ± 0.1 *
Protein (g·d⁻¹)	108 ± 7	94 ± 4 *	107 ± 5	96 ± 3 *
Carbohydrates (g·d⁻¹)	323 ± 19	276 ± 12 *	302 ± 8	282 ± 10 *
Fat (g·d⁻¹)	100 ± 6	89 ± 4 *	95 ± 4	91 ± 3 *
Fibers (g·d⁻¹)	32 ± 2	35 ± 2 *	31 ± 1	36 ± 1 *
Protein (En%)	16 ± 0	16	17 ± 0	16
Carbohydrate (En%)	48 ± 1	47	47 ± 1	47
Fat (En%)	33 ± 1	34	33 ± 0	34
Fibers (En%)	2 ± 0	3 *	2 ± 0	3 *

Values (means±SEM) represent parameters of dietary intake from *n*=20 healthy, male volunteers during 7 days of free-living and 7 days of strict bed rest. During bed rest, participant were fed a standard enteral food product in an intermittent (4 meals per day) or continuous (24 h per day) manner. Abbreviations: BW, body weight; En%, energy percentage; MJ, Mega Joule. * Significantly different from corresponding free-living values.

Table 3: Body composition prior to and after 7 days of strict bed rest in participants fed either intermittently (4 boluses per day) or in a continuous manner.

	Intermittent (<i>n</i> =10)		Continuous (<i>n</i> =10)	
	Pre	Post	Pre	Post
Total mass (kg)	77.7 ± 4.9	77.3 ± 5.0 *	77.6 ± 5.3	76.8 ± 5.1 *
Fat mass (kg)	18.2 ± 2.1	18.3 ± 2.1	17.7 ± 2.3	17.6 ± 2.3
Fat percentage (%)	22.9 ± 1.9	23.2 ± 1.9	22.3 ± 1.2	22.4 ± 1.3
Lean mass (kg)	57.0 ± 3.4	56.6 ± 3.4 *	57.2 ± 3.1	56.5 ± 2.9 *
Trunk lean mass (kg)	28.6 ± 1.8	28.0 ± 1.7 *	28.0 ± 1.7	27.6 ± 1.6 *
Leg lean mass (kg)	9.5 ± 0.7	9.5 ± 0.6	9.5 ± 0.6	9.4 ± 0.5
Arm lean mass (kg)	3.5 ± 0.2	3.5 ± 0.2	3.5 ± 0.2	3.4 ± 0.2
BMD (g·cm⁻²)	1.16 ± 0.03	1.17 ± 0.03 *	1.16 ± 0.02	1.15 ± 0.02

Values (means±SEM) represent parameters of body composition from *n*=20 healthy, male volunteers before (pre) and after (post) 7 days of strict bed rest, as measured by DXA. BMD, bone mineral density. * Significantly different from corresponding pre-values.

Figure legends

Figure 1: Lean body mass (**A+B**), whole-body oxygen uptake capacity (**C+D**), and whole-body insulin sensitivity (**E+F**) at baseline and following 7 days of strict bed rest in healthy, young men, nasogastric tube fed in an intermittent ($n=10$) or continuous ($n=10$) feeding pattern. Panels **A**, **C**, and **E** represent individual data, whereas panels **B**, **D**, and **F** display group means. GIR, glucose infusion rate. * Significantly different from pre-bed rest values ($P<0.05$). Values are means \pm SEM.

Figure 2: Individual participants' quadriceps cross sectional area (CSA; **A**) and group mean changes in quadriceps CSA (**B**), following 7 days of strict bed rest in healthy, young men, nasogastric tube fed in an intermittent ($n=10$) or continuous ($n=10$) feeding pattern. * Significantly different from pre-bed rest values ($P<0.05$). Values are means \pm SEM. Panel **C** (pre bed rest) and **D** (post bed rest) display representative CT scans from a participant with an average decline in quadriceps CSA.

Figure 3: Body water deuterium enrichments, measured the day after ingestion of 8 x 50 mL of 70% deuterium oxide (Test 1) and every subsequent day, in healthy, young men under free-living (Test 1-BR1) and bed rested (BR1-Test 3) conditions. On all days, a 50 mL maintenance dose was provided. During bed rest, participants were nasogastric tube fed in an intermittent or continuous feeding pattern. Values are means \pm SEM.* Significantly different from Test 1 ($P<0.001$).

Figure 4: Myofibrillar protein synthesis, expressed as fractional synthetic rate (FSR) per day, during free-living and bed-rested conditions in healthy, young men. Data are displayed as

participants' individual FSR. During bed rest, food was administered via a nasogastric tube in either an intermittent ($n=10$; 4x bolus per day) or continuous ($n=10$, 24 h per day) pattern. A Repeated Measures ANOVA revealed no significant effects.

Figure 5: Skeletal muscle mRNA expression of genes involved in the regulation of muscle protein synthesis (i.e. mTOR (**A**) and P70S6K (**B**)) and muscle protein breakdown (i.e. FoxO1 (**C**), MAFBx (**D**), and MuRF1 (**E**)). Biopsies were taken between the free-living and the bed rested period (pre), and immediately following bed rest (post). * Significantly different from corresponding pre-bed rest values ($P<0.01$).

Figure 6: Daily nitrogen balance during 7 days of strict bed rest. Participants were fed a standard enteral food product via a nasogastric tube, in either an intermittent ($n=10$; 4x bolus per day) or continuous ($n=10$, 24 h per day) pattern. * Significant time effect ($P<0.001$). Values are means \pm SEM.