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

Abstract

Short periods of bed rest lead to the loss of muscle mass and quality. It has been speculated that dietary feeding pattern may impact upon muscle protein synthesis rates and, therefore, modulate the loss of muscle mass and quality. We subjected 20 healthy men (age: 25±1 y, BMI: 23.8±0.8 kg·m^{-2}) to one week of strict bed rest with intermittent (4 meals/day) or continuous (24 h/day) enteral tube feeding. Participants consumed deuterium oxide for 7 days prior to bed rest and throughout the 7-day bed rest period. Prior to and immediately after bed rest, lean body mass (DXA), quadriceps cross-sectional area (CSA; CT), maximal oxygen uptake capacity (VO_2peak), and whole-body insulin sensitivity (hyperinsulinaemic-euglycaemic clamp) were assessed. Muscle biopsies were collected 7 days prior to, 1 day prior to, and immediately after bed rest to assess muscle tracer incorporation. Bed rest resulted in 0.3±0.3 vs 0.7±0.4 kg lean tissue loss and a 1.1±0.6 vs 0.8±0.5% decline in quadriceps CSA in the intermittent vs continuous feeding group, respectively (both P<0.05), with no differences between groups (both P>0.05). Moreover, feeding pattern did not modulate the bed rest-induced decline in insulin sensitivity (-46±3% vs 39±3%; P<0.001) or VO_2peak (-2.5±2.2 vs -8.6±2.2%; P<0.010)(both P>0.05). Myofibrillar protein synthesis rates during bed rest did not differ between the intermittent and continuous feeding group (1.33±0.07 vs 1.50±0.13%·d^{-1}, respectively; P>0.05). In conclusion, dietary feeding pattern does not modulate the loss of muscle mass or the decline in metabolic health during one week of bed rest in healthy men.

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Introduction

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

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

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

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

To test this hypothesis, we subjected 20 young, healthy men to one week of bed rest while being tube-fed in energy balance using either a continuous (24 h) or an intermittent (4 boluses daily) enteral feeding protocol. Muscle mass (CT, DXA) and metabolic health (VO_2peak, whole-body insulin sensitivity via hyperinsulinaemic-euglycaemic clamp) were assessed prior to and after one week of bed rest. Muscle protein synthesis rates were assessed for one week prior to bed rest and during one week of bed rest using deuterated water administration and muscle biopsy sampling. This is the first study to compare the impact of continuous versus intermittent enteral feeding on changes in muscle mass and quality during one week of bed rest *in vivo* in humans.

Methods

Participants

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

Experimental outline

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

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

One week of bed rest

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

Dietary intake

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

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

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

Body composition

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

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

Metabolic health

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

Deuterium oxide loading and body water enrichments

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

one daily 50 mL oral dose every morning of the study period. Daily saliva samples were collected using a cotton swab at 18:00 on every study day, to determine body water enrichment. Samples were frozen in liquid nitrogen and stored at -80°C. Body water ^{2}H-alanine enrichments were measured as described elsewhere (32). In short, samples were centrifuged at 10,000 *g* to remove debris and subsequently diluted 70-fold with ddH_2O to achieve deuterium enrichments within the detection limits of the GC-C-IRMS. Samples were prepared for analysis using the protocol by Scrimgeour and colleagues (51). This involved placing small plastic cups holding 4 mg of catalyst (5% platinum on alumina, 325 mesh, Sigma-Aldrich, St. Louis, USA) inside 3 mL glass vials, after which 300 µL of diluted saliva sample was added and vials were sealed using rubber septums and a screw cap. Air in each vial was evacuated and replaced by hydrogen gas simultaneously, after which vials were left at 21 °C for 24 h for deuterium equilibration to occur between the hydrogen gas and the saliva samples. The deuterium enrichment of the hydrogen gas was then measured in duplicate on a GC-C-IRMS (Micromass Optima IRMS fitted with a Multiprep and Gilson autoinjector, Micromass UK Limited, Manchester, UK). Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for deuterium loss during equilibration.

Myofibrillar protein synthesis

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

hand-homogenizing on ice using a pestle in a standard extraction buffer (10 μL·mg^{-1}). The samples were spun at 2500 *g* and 4ºC for 5 min. The pellet was washed with 500 μL ddH_2O and centrifuged at 2500 *g* and 4ºC for 10 min. The myofibrillar protein was solubilized by adding 1 mL of 0.3 M NaOH and heating at 50ºC for 30 min with vortex mixing every 10 min. Samples were centrifuged at 9500 *g* and 4ºC for 5 min, the supernatant containing the myofibrillar proteins was collected and the collagen pellet was discarded. Myofibrillar proteins were precipitated by the addition of 1 mL of 1 M PCA and spinning at 700 *g* and 4ºC for 10 min. The myofibrillar protein was washed twice with 70% ethanol and hydrolyzed overnight in 2 mL of 6 M HCL at 110˚C. The free amino acids from the hydrolyzed myofibrillar protein pellet were dried under a continuous nitrogen stream while being heated at 120ºC. The free amino acids were then dissolved in 25% acetic acid solution, passed over cation exchange AG 50W-X8 resin columns (mesh size: 100-200, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA, USA), and eluted with 2 M NH_4OH. Thereafter, the eluate was dried and the purified amino acids were derivatized to their N(O,S)-ethoxycarbonyl ethyl esters (33). The derivatized samples were measured using a gas chromatography-isotope ratio mass spectrometer (GC-IRMS; Thermo Fisher Scientific, MAT 253; Bremen, Germany) equipped with a pyrolysis oven and a 60 m DB-17MS column (no. 122-4762; Agilent, Wilmington, DE, USA) and 5 m precolumn. Ion masses 2 and 3 were monitored to determine the $^2H/^1H$ ratios of muscle protein bound alanine. A series of known standards was applied to assess linearity of the mass spectrometer and to control for the loss of tracer.

Skeletal muscle gene expression

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

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

Nitrogen balance

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

Statistics

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

Results

Body composition

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

Maximal oxygen uptake capacity and whole-body insulin sensitivity

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

Cumulative muscle protein synthesis

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

Skeletal muscle gene expression

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

Nitrogen balance

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

Discussion

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

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

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

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

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

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

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

Consequently, the observed muscle atrophy (**Figure 1** and **2**) may be largely caused by an increase in muscle protein breakdown rates. Though data are quite limited, all available direct (57) and indirect (23) measurements of muscle protein breakdown rates suggest no changes in postabsorptive muscle protein breakdown rates following several weeks of muscle disuse. However, we (19, 61, 62) and others (1, 59) have demonstrated a rapid but transient increase in molecular proxies for muscle protein breakdown during the first few days following the onset of muscle disuse. In line, we observed an increase in MAFBx expression following bedrest in both treatment groups (**Figure 5**). Although it remains unclear whether muscle protein breakdown rates are increased following short-term disuse, and if so, whether this is attributed to increased postabsorptive and/or postprandial muscle protein breakdown rates, our data seem to support previous suggestions that muscle protein breakdown is increased following the onset of disuse (1, 19, 59, 61, 62). It has been suggested that continuous enteral feeding may have a greater impact on muscle protein breakdown due to the continuous insulin-mediated suppression of proteolysis (29), whereas intermittent feeding has a greater impact on protein synthesis due to the repeated hyperinsulinaemia and hyperaminoacidaemia (9). Although we did not assess muscle proteolysis, mRNA expression of key proteins involved in the regulation of muscle protein breakdown did not show differences between feeding strategies. Consequently, our data do not support that large differences in muscle protein breakdown rates exist between continuous versus intermittent enteral feeding (**Figure 5**).

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

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

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

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

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Conflict of interest

LJCvL has received research grants, consulting fees, speaking honoraria, or a combination of these, from Friesland Campina and Nutricia Research. LBV has received speaking honoraria from Nutricia Research. None of the other authors have disclosed any conflicts of interest.

Author contributions

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

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Tables

Table 1: Participants´ characteristics

	Intermittent (*n*=10)	**Continuous (*n*=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 ($kg \cdot m^{-2}$)	23.5 ± 1.3	24.0 ± 1.0
HbA_{1c} (%)	5.2 ± 0.1	5.2 ± 0.2
RMR ($MJ \cdot d^{-1}$)	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 (*n*=10)		Continuous (*n*=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 (*n*=10)		**Continuous (*n*=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 ($g \cdot cm^{-2}$)	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±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±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±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±SEM.