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Supplementing Breakfast with a Vitamin D and Leucine–Enriched Whey Protein Medical Nutrition Drink Enhances Postprandial Muscle Protein Synthesis and Muscle Mass in Healthy Older Men

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Abstract

Background: A promising strategy to help older adults preserve or build muscle mass is to optimize muscle anabolism through providing an adequate amount of high-quality protein at each meal.

Objective: This ''proof of principle'' study investigated the acute effect of supplementing breakfast with a vitamin D and leucine–enriched whey protein medical nutrition drink on postprandial muscle protein synthesis and longer-term effect on muscle mass in healthy older adults.

Methods: A randomized, placebo-controlled, double-blind study was conducted in 24 healthy older men [mean ± SD: age 71 ± 4 y; body mass index (in kg/m^2) 24.7 ± 2.8] between September 2012 and October 2013 at the Unit of Human Nutrition, University of Auvergne, Clermont-Ferrand, France. Participants received a medical nutrition drink [test group; 21 g leucine-enriched whey protein, 9 g carbohydrates, 3 g fat, 800 IU cholecalciferol (vitamin D_3), and 628 kJ] or a noncaloric placebo (control group) before breakfast for 6 wk. Mixed muscle protein fractional synthesis rate (FSR) was measured at week 0 in the basal and postprandial state, after study product intake with a standardized breakfast with the use of L-[$^2H_5$]-phenylalanine tracer methodology. The longer-term effect of the medical nutrition drink was evaluated by measurement of appendicular lean mass, representing skeletal muscle mass at weeks 0 and 6, by dual-energy X-ray absorptiometry.

Results: Postprandial FSR (0–240 min) was higher in the test group than in the control group [estimate of difference (ED): 0.022%/h; 95% CI: 0.010%/h, 0.035%/h; ANCOVA, $P = 0.001$]. The test group gained more appendicular lean mass than the control group after 6 wk (ED: 0.37 kg; 95% CI: 0.03, 0.72 kg; ANCOVA, $P = 0.035$), predominantly as leg lean mass (ED: 0.30 kg; 95% CI: 0.03, 0.57 kg; ANCOVA, $P = 0.034$).

Conclusions: Supplementing breakfast with a vitamin D and leucine-enriched whey protein medical nutrition drink stimulated postprandial muscle protein synthesis and increased muscle mass after 6 wk of intervention in healthy older adults and may therefore be a way to support muscle preservation in older people. This trial was registered at www.trialregister.nl as NTR3471. *J Nutr* doi: https://doi.org/10.3945/jn.117.252510

Keywords: protein intake, leucine, vitamin D, muscle protein synthesis, muscle mass, sarcopenia

Introduction

Sarcopenia, the age-related loss of skeletal muscle mass and function (1), is partly the result of an impaired activation of postprandial muscle protein synthesis by anabolic stimuli [i.e., amino acids (AAs) and insulin] (2–5). In addition to increasing daily protein intake (6), nutritional strategies to overcome this anabolic resistance have focused on providing an adequate amount of protein per meal and using high-quality protein with readily available essential AAs (EAAs) and high

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leucine content. Supplementation with soluble milk protein (7), whey protein (8), or leucine (9, 10) was shown to effectively stimulate muscle protein synthesis in older adults. Consumption of an adequate amount of high-quality protein divided evenly over 3 meals seems to stimulate 24-h muscle protein synthesis more effectively than a skewed protein distribution (11). According to Paddon-Jones and Rasmussen (12), an intake of 25–30 g high-quality protein at each meal (breakfast, lunch, and dinner) is necessary to adequately stimulate muscle protein synthesis. However, the amount of protein traditionally ingested at breakfast is between 5 and 10 g (13–16), thus does not enable maximal muscle protein synthesis. A recent cross-sectional study stated that consumption of multiple meals with adequate protein content is positively associated with lean mass and muscle performance (17). In addition, for other nutrients, such as vitamin D, the importance in muscle protein metabolism is slowly emerging (18). Insufficient vitamin D status is common in older people and associated with functional outcomes such as less physical activity, increased risk of falls, and nursing home admissions (19–21).

Finally, few studies have investigated the effect of nutritional intervention without exercise on longer-term muscle outcomes related to sarcopenia (22). Tieland et al. (23) showed an improved physical performance with no effect on skeletal muscle mass after 24 wk of protein supplementation in frail elderly people. Bauer et al. (24) showed an improvement in muscle mass and lower-extremity function after 13 wk of a medical nutrition drink in sarcopenic older adults. Recently, Norton et al. (25) demonstrated an improved lean body mass in healthy adults aged 50–70 y after a 24-wk intervention with a supplement that contained protein, calcium, and vitamin D.

The goal of this randomized, placebo-controlled, double-blind "proof of principle" study was to investigate the acute and longer-term effect of a medical nutrition drink, consisting of a specific combination of whey protein, leucine, and vitamin D, integrated in the habitual diet of healthy older men. We hypothesized that supplementing breakfast with this medical nutrition drink would enhance muscle protein synthesis and thus enhance muscle mass over a longer period of time. Longer-term changes in physical performance, dietary intake, and postprandial glucose, insulin, and AA response were also explored.

Methods

Study design

The study was a randomized, placebo-controlled, double-blind, parallel-group, single-center trial to assess the acute effect of the consumption of a vitamin D and leucine-enriched whey protein medical nutrition drink before breakfast in healthy older adults on mixed muscle protein fractional synthesis rate (FSR) as the primary outcome. Longer-term effects were assessed on body composition, appendicular and leg lean mass, physical performance, dietary intake, nutritional biomarkers, and postprandial metabolic response.

Participants were recruited by the study team of the muscle metabolic adaptation group at the Unit of Human Nutrition, University of Auvergne, Clermont-Ferrand, France, through advertisements in local newspapers, on the radio or television, with flyers at public places, general practioners and health care professionals, and from existing databases. The local human ethics committee (CPP Sud-Est VI, France) and the French National Security Agency for Medicines and Health Products approved the study protocol. The study was conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonization Guidelines for Good Clinical Practice as appropriate for nutritional products, and local legislation. The Dutch Trial Registration number for this study is NTR3471 (www.trialregister.nl).

Participants

Healthy adult men aged ≥65 y with a BMI (in kg/m^2) between 20 and 30 were eligible for inclusion. Only men were included to minimize variation in FSR and changes in muscle mass. Exclusion criteria included comorbidities and medication use affecting gastrointestinal function or muscle metabolism and contraindications related to the muscle biopsy procedure or interfering with the purpose of the study (all exclusion criteria are described in **Supplemental Methods**: "Participants").

Study products and experimental breakfast

Upon entry into the study, participants were randomly assigned 1:1 to receive the vitamin D and leucine-enriched whey protein medical nutrition drink, containing 20 g whey protein, 3 g total leucine, including protein-bound and free L-leucine, 9 g carbohydrates, 3 g fat, and 800 IU cholecalciferol (vitamin D_3) with an energy value of 628 kJ (Nutricia Advanced Medical Nutrition; **Supplemental Table 1**), or a flavored watery placebo product before breakfast. Because the goal of the study was to investigate the acute and longer-term effect of breakfast supplementation with a medical nutrition drink in a double-blind setting, a noncaloric placebo drink was chosen to minimally affect the habitual diet. A computer random number generator was used to randomly assign participants to the test group (test product plus breakfast) or the control group (placebo product plus breakfast). The investigators, study staff, Nutricia Research staff, and participants were all blinded to study product allocation.

The test and placebo drink were provided in liquid form (200 mL) with similar taste and appearance and taken once daily before breakfast for a 6-wk period. The standardized breakfast used on the kinetic study visits at week 0 and week 6 consisted of 1 glass (250 mL) orange juice, 1 cup (150 mL) tea or coffee without sugar, bread (60 g), butter (12.5 g), and jam (30 g), providing 1567 kJ, 6 g proteins, 11 g fat, and 60 g carbohydrates.

Experimental protocol

For feasibility reasons, study assessments at baseline (week 0) and after 6 wk were each split into 2 separate visits (i.e., visits 1 and 2 for week 0 and visits 3 and 4 for week 6 measurements) (**Figure 1**). Visit 2 and visit 4 were 8-h kinetic visits. Study visits at each time point were maximal 10 d apart.

Week 0 (baseline). Nutritional status was measured using the Mini Nutritional Assessment Short-Form (26). The Physical Activity Scale for the Elderly (PASE) questionnaire (27) was used to assess physical activity, and cognitive performance was assessed by the Mini-Mental State Examination (28). Body composition was measured using DXA scanning (Discovery A; Hologic), handgrip strength was measured using a hydraulic hand dynamometer (Jamar), and physical performance was assessed using the Short Physical Performance Battery (SPPB) (29). Details regarding the performance of the handgrip measurement and the SPPB are described in the Supplemental Methods: "Muscle strength and physical performance."

Insulin sensitivity was measured with the oral glucose tolerance test and by calculating the HOMA-IR. Also measured were baseline

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Supplemental Methods and Results, Supplemental Tables 1–3, and Supplemental Figures 1–4 are 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.

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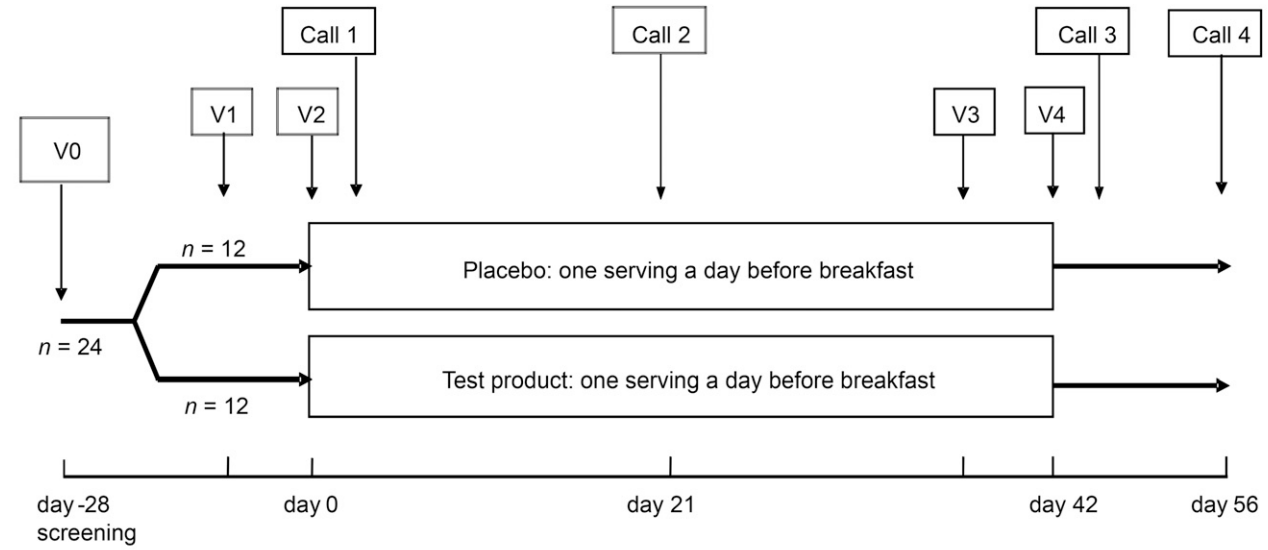
Abbreviations used: AA, amino acid; ALM, appendicular lean mass; C_{max}, peak concentration; EAA, essential amino acid; ED, estimate of difference; FSR, fractional synthesis rate; hs-CRP, high-sensitivity C-reactive protein; iAUC, incremental AUC; IGF-1, insulin-like growth factor; MMRM, mixed model for repeated measures; NEFA, nonesterified FA; PASE, Physical Activity Scale for the Elderly; PTH, parathyroid hormone; SPPB, Short Physical Performance Battery.



V0 = Screening
V1 = Handgrip strength, SPPB, DXA
V2 = First acute study visit, questionnaires
V3 = Handgrip strength, SPPB, DXA
V4 = Second acute study visit, questionnaires

Call 1 = Check-up, AE identification and compliance check after V2
Call 2 = AE identification and compliance check after V2
Call 3 = AE identification after V4
Call 4 = AE identification after V4

FIGURE 1 Schematic diagram of the study design, including measurements at each visit for healthy older men receiving placebo (n = 12) or the test product (n = 12). AE, adverse event; PASE, Physical Activity Scale for the Elderly; SPPB, Short Physical Performance Battery; V0, screening visit; V1 and V2, baseline visit (week 0); V3 and V4, week 6.

25-hydroxyvitamin D, parathyroid hormone (PTH), insulin-like growth factor (IGF-1), TGs, nonesterified FAs (NEFAs), and the inflammatory markers fibrinogen, high-sensitivity C-reactive protein (hs-CRP), TNF-α, and IL-6. Details regarding the biochemical analysis of plasma 25-hydroxyvitamin D, PTH, IGF-1, TGs, NEFAs, and markers of inflammation are described in Supplemental Methods: "Nutritional biomarkers and markers of inflammation in plasma."

During visit 2 the acute effect of the medical nutrition drink (test product) in addition to breakfast on muscle protein synthesis and on the glucose, insulin, and AA postprandial response was assessed. Participants were prepared to start the tracer protocol under comparable conditions (Supplemental Methods: "Preparation participants for study visits 2 and 4"). Dietary intake was assessed before the kinetic visit by using a 3-d dietary record. Participants were asked to fill out this record for 3 consecutive days, including 1 weekend day. A dietitian calculated the mean macronutrients intake, per day and per meal, by using Nutrilog software.

On the morning of the baseline kinetic visit, participants arrived at the study site at 0630 after an overnight fast (no food after 2200 on the preceding evening, water until 0700 on the study day). An 18-gauge polyethylene catheter was placed in a dorsal hand vein, and the hand was placed in a hot box (60°C) for arterialized blood sampling. Another catheter was placed in the contralateral arm for infusion of L-[$^2H_5$]-phenylalanine (Eurisotop). L-[$^2H_5$]-phenylalanine infusates were prepared by the local pharmacy. After a basal blood sampling, a primed (4.2 μmol/kg body weight bolus), continuous (0.07 μmol · kg body weight^{-1} · min^{-1}) infusion of L-[$^2H_5$]-phenylalanine was started at t = −240 min (T_{-240}). A first muscle biopsy specimen was taken after 240 min. Participants then consumed the study product within 5 min (T_0), immediately followed by consumption of the breakfast. A second muscle biopsy specimen was taken 240 min after intake of the study product (T_{240}). Throughout the 8 h, volunteers laid in bed in an upright position, and regular blood sampling was performed (at t = −120, −60, −40, −30, 0, 20, 40, 60, 80, 100, 120, 150, 180, 210, and 240 ± 5 min) to measure venous concentrations of glucose, insulin, and AAs (biochemical analysis described in Supplemental Methods: "Nutritional biomarkers and markers of inflammation in plasma") as well as arterialized L-[$^2H_5$]-phenylalanine enrichments.

Muscle biopsy specimens (~100 mg) were taken from vastus lateralis muscle ~10–15 cm above the patella and 3 cm below entry through the fascia by using locally anesthetized percutaneous needle biopsy technique. The specimens were cleaned from surrounding fat or connective tissues before being frozen in liquid nitrogen and stored at −80°C until analysis. Blood samples were collected with EDTA-coated or heparinized tubes, centrifuged at 1300 × g for 10 min at 4°C to isolate plasma, and stored at −80°C until analysis.

Week 6 (intervention). After 6 wk, the acute effect of the medical nutrition drink (test product) in addition to breakfast on glucose, insulin, and AA postprandial response was reevaluated as described for visit 2. Muscle protein synthesis was not repeated because the human ethics committee allowed only one study day with muscle biopsies. Body composition, handgrip strength, physical performance, dietary intake, HOMA-IR, vitamin 25-hydroxyvitamin D, PTH, IGF-1, TGs, NEFAs, and markers of inflammation (fibrinogen, hs-CRP, TNF-α, and IL-6) were reassessed.

Safety and compliance

Safety assessments and adverse events were recorded every 3 wk. This included changes in concomitant medication and nutritional supplements and also gastrointestinal tolerance. Product intake compliance was evaluated based on a self-reported diary completed daily by the study participants.

Biochemical determination of L-[$^2H_5$]-phenylalanine enrichment in plasma and muscle

Measurement of L-[$^2H_5$]-phenylalanine enrichment in free AAs from plasma was performed according to previously described methods (30) (Supplemental Methods: "Biochemical determination of L-[$^2H_5$]-phenylalanine enrichment in plasma").

To measure L-[$^2H_5$]-phenylalanine enrichment in muscle biopsy specimens, a 30-mg piece was used to precipitate proteins with 4% sulfosalicylic acid. After centrifugation, the pellet was dried, and protein was hydrolyzed in glass tubes by adding 6 mol/L HCl and heating the tubes at 110°C for 24 h. The residue was resuspended in 0.5 mol/L

sodium citrate buffer and filtered through a 0.20-μm filter (Interchim). Phenylalanine was then converted to phenylethylamine as previously described (31). Samples were derivatized with *N*-methyl-*N*-(tert-butyldimethylsilyl)-trifluoroacetamide/ethyl acetate (VWR International), and enrichment was measured using GC-MS. To avoid needing an extra biopsy specimen, the initial enrichment of muscle protein was assimilated to the basal (T_{-240}) enrichment in mixed plasma proteins as previously described (32). The same conversion method was applied for the measurements. *m/z* Ratios of 180 and 183 were measured by GC-MS.

Calculations

The mixed muscle protein FSR (expressed as %/h) was calculated by dividing incremental change in protein-bound L-[$^2H_5$]-phenylalanine enrichment by the enrichment in the plasma precursor pool for L-[$^2H_5$]-phenylalanine, as described by Kramer et al. (33) (Supplemental Methods: "Calculation of mixed muscle FSR").

Plasma AAs, glucose, and insulin parameters were calculated as (time to) peak concentrations (C_{max}) and the incremental AUC (iAUC). Baseline concentrations of AAs, glucose, and insulin were calculated as the mean of the concentrations in the 2 samples taken before intake of the study product with breakfast (i.e., at $t = -120$ and -30 min). Insulin sensitivity was assessed at baseline and before acute experiments (visit 2 and 4) by using the original calculation of the HOMA-IR: [fasting plasma insulin (mU/L) · fasting plasma glucose (mmol/L)] · 22.5^{-1} (34).

Statistics

The study was powered to detect a 50% increase (corresponding to an increase from 0.055% to 0.0825%/h) from basal FSR in the test group, and a 0% increase was expected in the control group. Assuming a pooled SD of 0.0191%/h, a significance level (α) of 0.05, and a power of 80%, a sample size of 9 participants/group was assumed to be sufficient to detect a statistically significant difference in FSR between the test product and the placebo product. With an anticipated dropout rate of 25%, 12 participants were needed per group.

Baseline characteristics and descriptive results are expressed as means ± SDs. Data in figures are expressed as means ± SEs. For the between-group comparison of the primary outcome (i.e., the postprandial FSR at 0–240 min), an ANCOVA with basal values as the covariate and "study group" as the factor was used, and expressed as estimates of differences (EDs) and 95% CIs (35). Similar ANCOVA models were used to analyze the effect of the 6-wk intervention on dietary intake, nutritional and body composition parameters, functional parameters, and insulin sensitivity. To determine whether postprandial muscle FSR was significantly different from basal values within groups, ANCOVA with basal values as the covariate was applied. The Wilcoxon-Mann-Whitney test was used to test differences in SPPB and balance test after the 6-wk intervention. The 2-sample *t* test was used to compare basal values between groups for FSR (visit 2), basal plasma concentrations of AAs, inflammatory markers, glucose and insulin, and the C_{max} and the iAUC of glucose, insulin, and AAs (visit 2 and 4). Furthermore, the plasma concentrations of AAs, glucose, and insulin at the individual time points were compared between groups using a mixed model for repeated measures (MMRM) per visit with the baseline and postprandial time point values as outcome, and "study group," "time point," and "study group × time point interaction" as fixed effects. To assess changes in amino acid, glucose and insulin concentrations between week 0 and week 6 for the test group compared with the control group, an MMRM was used with week 0 and week 6 values as the outcome, "week," "study group," and "interaction between week and study group" as fixed effects, and "participant" as a random effect. Statistical significance was set at $P < 0.05$, and all tests were 2-sided on the intention-to-treat data set. All analyses were performed in SAS Enterprise Guide 4.3 for Windows software (SAS Institute Inc.).

Results

Participants

The study included 24 healthy older men (71 ± 4 y; BMI: 24.7 ± 2.8) who were randomly assigned to the test or control group between September 2012 and July 2013. All participants completed the trial before October 2013, and no participants discontinued the study. Participants' baseline characteristics were similar between the groups (**Table 1**). Participants were cognitively healthy, had glucose tolerance and inflammation markers within normal reference ranges, and were not malnourished according to the Mini Nutritional Assessment Short-Form. The mean SPPB score was 11.1 ± 0.9, and the mean skeletal muscle mass index was 7.9 ± 1.0 kg/m^2. Participant-reported compliance to the nutritional intervention during the 6-wk study was 99% and similar between the 2 groups. Because all 24 participants completed the study without any major protocol deviations, the intention-to-treat population was identical to the per-protocol data group.

Mixed muscle protein synthesis

The primary outcome measure was the mixed muscle protein synthesis rate, which was calculated using protein-bound (**Table 2**) and arterialized plasma L-[$^2H_5$]-phenylalanine enrichments (**Figure 2**), and expressed as the FSR (**Figure 3**). Basal FSR (−240 to 0 min) did not differ between the 2 study groups (*t* test, $P = 0.62$). Postprandial FSR (0–240 min) was higher in the test group than in the control group (ED in FSR between test and control: 0.022%/h; 95% CI: 0.010%/h, 0.035%/h; ANCOVA, $P = 0.001$). In the test group, the increase in postprandial FSR was 94% ± 56% of the basal value (ANCOVA, $P < 0.001$). The control group also demonstrated a significant increase of 42% ± 36% from basal values (ANCOVA, $P < 0.001$). Additional information regarding individual FSR increments is provided in **Supplemental Figure 1**.

TABLE 1 Baseline demographic and clinical characteristics of healthy older men in the test group, who received a medical nutrition drink, and the control group, who received a placebo drink, in addition to breakfast for 6 wk[1]

Variable	Test group ($n = 12$)	Control group ($n = 12$)
Age, y	70.3 ± 4.3	70.8 ± 3.5
Body weight, kg	72.2 ± 9.5	72.8 ± 6.8
BMI, kg/m^2	24.4 ± 3.3	25.1 ± 2.5
Lean body mass, kg	55.2 ± 5.6	56.5 ± 3.9
Fat mass, %	20.5 ± 5.3	20.0 ± 4.9
Appendicular lean mass, kg	23.1 ± 2.8	23.3 ± 2.4
SMI, kg/m^2	7.8 ± 0.9	8.1 ± 1.1
MNA-SF score	13.4 ± 1.0	13.8 ± 0.4
Protein intake, g · kg body weight^{-1} · d^{-1}	1.3 ± 0.3	1.3 ± 0.2
Fasting plasma glucose,[2] mmol/L	5.62 ± 0.27	5.53 ± 0.64
2-h plasma glucose concentration,[2] mmol/L	5.32 ± 1.15	5.88 ± 0.79
Fasting plasma insulin,[2] mU/L	6.39 ± 2.72	5.94 ± 2.02
HOMA-IR index	2.09 ± 0.94	1.98 ± 1.40
HbA1c, %	5.73 ± 0.33	5.72 ± 0.32
Plasma 25-hydroxyvitamin D, nmol/L	35.3 ± 21.54	45.5 ± 23.56
Plasma hs-CRP, mg/L	3.14 ± 0.69	3.95 ± 1.85
Plasma TNF-α, pg/mL	1.32 ± 0.27	1.51 ± 0.38
Plasma IL-6, pg/mL	2.21 ± 2.08	2.14 ± 0.98
MMSE score	29.8 ± 0.4	29.0 ± 1.3
Handgrip strength, kg	34.7 ± 6.6	36.8 ± 5.0
SPPB score	11.2 ± 0.8	11.0 ± 1.0
PASE score	99.6 ± 53.9	142.4 ± 54.6

[1] Data are means ± SDs. HbA1c, glycated hemoglobin; hs-CRP, high-sensitive C-reactive protein; MMSE, Mini-Mental State Examination; MNA-SF, Mini-Nutritional Assessment Short-Form; PASE, Physical Activity Scale for Elderly; SMI, skeletal muscle mass index; SPPB, Short Physical Performance Battery.
[2] Collected from oral-glucose-tolerance test.

TABLE 2 Muscle protein-bound enrichment of L-[$^2H_5$]-phenylalanine (MPE) in healthy older men at baseline and after intake (t = 240 min) of a medical nutrition drink (test group) or placebo (control group), before a standardized breakfast, during the acute experiment at visit 2[1]

Time	Test group (n = 12)	Control group (n = 11)	P value[2]
0 min (basal)	0.0154 ± 0.0044	0.0143 ± 0.0028	
240 min	0.0429 ± 0.0078	0.0358 ± 0.0060	0.02*

[1] Data are means ± SDs. MPE, mole percent excess.
[2] Tested with ANCOVA with basal values as the covariate and ''study group'' as the fixed factor. *Statistically significant, $P < 0.05$.

Body composition, muscle mass, strength, and physical performance

After 6 wk of intervention, there was a significant gain in appendicular lean mass (ALM) in the test group compared with the control group (ED: 0.37 kg; 95% CI: 0.03, 0.72 kg; ANCOVA, P = 0.035) (individual results in **Supplemental Figure 2**), with a predominant gain in leg lean mass (ED: 0.30 kg; 95% CI: 0.03, 0.57 kg; ANCOVA, P = 0.034) (**Figure 4**). No increase in activity was observed with the PASE questionnaire. Results of this questionnaire even demonstrated a reduction in activity after 6 wk of intervention, which was most prominent in the test group (**Supplemental Table 2**). No important between-group differences were observed in body weight, lean body mass, arm lean mass, fat mass, handgrip strength, and SPPB outcomes (Supplemental Table 2).

Dietary intake and nutritional biomarkers

Dietary intake. Habitual dietary intake of energy, proteins, carbohydrates, and fat was similar in both study groups at baseline (week 0) (**Supplemental Results:** "Dietary intake" and **Supplemental Table 3**). Habitual protein intake was significantly lower in the test group after 6 wk compared with the control group, but no difference was observed when the medical nutrition drink was included. The test product affected the protein distribution over the 3 daily main meals (**Figure 5**). Protein intake after the 6-wk study was higher with breakfast in the test group than in the control group (ANCOVA, P < 0.01), was similar with lunch, and was decreased with dinner in the test group but increased in the control group (ANCOVA, P = 0.02).

Nutritional biomarkers. Plasma 25-hydroxyvitamin D concentrations were significantly higher after 6 wk in the test group than in the control group (ED: 19.7 nmol/L; 95% CI: 7.1, 32.3 nmol/L; ANCOVA, P = 0.004), whereas the concentration of PTH was significantly higher in the control group than in the test group after 6 wk of intervention (ED: 4.7 pg/mL; 95% CI: 0.1, 9.4 pg/mL; ANCOVA, P = 0.048) (Supplemental Table 3).

Plasma glucose and insulin, insulin sensitivity, AA concentrations, and inflammation

Baseline plasma glucose and insulin concentrations (**Figure 6**) were not significantly different between the study groups. Despite a lower glucose intake with breakfast in the control group (60 g) compared with the test group (69 g), C_{max} was significantly higher in the control group at week 0 (t test, P = 0.018) and week 6 (t test, P = 0.007) (Figure 6A, B). In addition, the time to reach C_{max} was significantly longer in the test group than in the control group (week 0: 124 compared with 60 min; t test, P = 0.002; week 6: 133 compared with 56 min; t test, P = 0.001). The iAUC for glucose was not different between groups at both visits. Both at week 0 and week 6, there were no differences in peak and iAUC insulin. The C_{max} and iAUC of glucose and insulin at week 6 were not significantly different from week 0 in the test group compared with the control. No difference was observed in insulin sensitivity at baseline (Table 1) and after 6 wk of intervention (Supplemental Results: "Insulin sensitivity").

A significantly higher C_{max} and iAUC of total AAs (**Supplemental Figure 3**), EAAs (**Figure 7**A, B), leucine (Figure 7C, D), and phenylalanine (**Supplemental Figure 4**), were observed in the test group than in the control group, both at wk 0 and wk 6 (t test, P < 0.05). In addition, C_{max} and iAUC of AAs, EAAs, and leucine in the test group at week 6 was significantly lower than at week 0 compared with the control group (MMRM; P < 0.05). The contribution of branched chain AAs to the total AA response for the iAUC was similar at both visits (46% compared with 4% at week 0 and 42% compared with 9% at week 6 for the test and control group, respectively). Finally, no statistically significant difference between study groups was observed for the inflammatory plasma parameters fibrinogen, hs-CRP, TNF-α, and IL-6 (data not shown).

Safety and tolerance

All participants completed the 6-wk study without major protocol deviations. No serious adverse events were reported. There were no relevant differences between groups in the occurrence of adverse events or any remarkable events. Study products were well tolerated, and none of the adverse events resulted in discontinuation of product consumption or study participation.

Discussion

This "proof of principle" study showed that the acute postprandial muscle protein synthesis response in healthy older men almost doubled when breakfast was supplemented with a vitamin D and leucine-enriched whey protein medical nutrition drink. Moreover, the test group gained appendicular muscle mass after 6-wk supplementation, with a predominant gain in

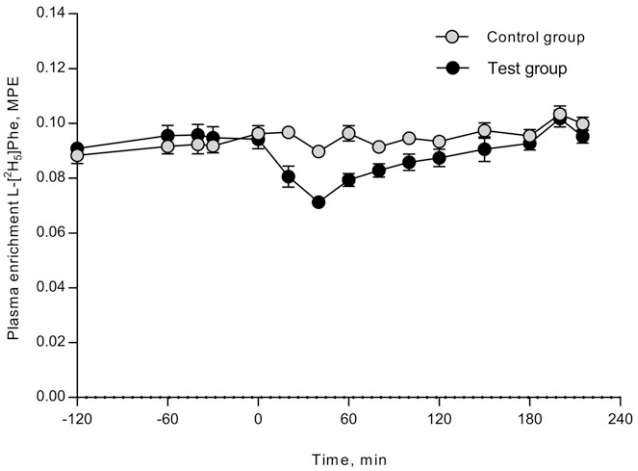


FIGURE 2 Arterialized plasma-corrected enrichments of L-[$^2H_5$]Phe in healthy older men in the control group, who received a placebo drink, and the test group, who received a medical nutrition drink, before a standardized breakfast during the acute experiment at visit 2. Data are means ± SEs; n = 12 in the test group and n = 11 in the control group. Because of a tracer infusion issue, no enrichment data were available from 1 participant in the control group. Data are means ± SEs. MPE, mole percent excess.

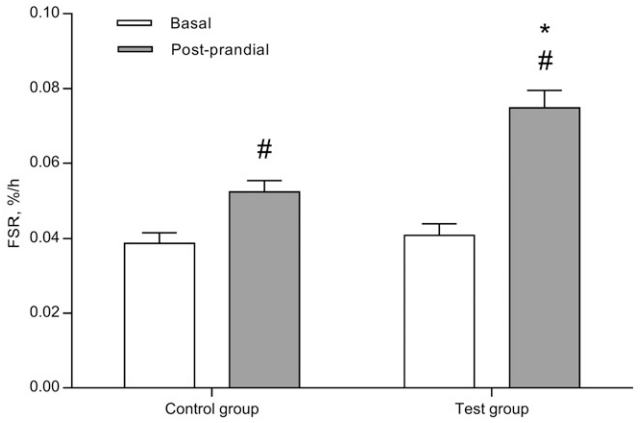


FIGURE 3 Mixed muscle protein FSR response in healthy older men, at baseline and after intake (postprandial) of placebo (control group) or a medical nutrition drink (test group), before a standardized breakfast during the acute experiment at visit 2. Data are means ± SEs; $n = 12$ in the test group and $n = 11$ in the control group. Because of a tracer infusion issue, no FSR data were available from 1 participant in the control group. *Different from control and #different from basal, $P < 0.05$. FSR, fractional synthesis rate.

leg lean mass. This study is unique in showing both an acute, mechanistic effect on muscle protein synthesis and a long-term, clinically relevant effect on ALM, of supplementing a medical nutrition drink to breakfast within a habitual diet.

To overcome the higher anabolic threshold in older adults (3–5), an adequate protein quantity per meal (25–30 g or ~0.40 g/kg body weight) (6, 12, 36), and high protein quality (2.5–2.8 g leucine and 10–15 g EAAs) (6, 12) have been proposed. Because breakfast is usually the lowest protein meal (13–16), we specifically aimed to supplement breakfast with a test product providing 21 g high-quality protein, including 3 g leucine. After 6 wk of intervention, dietary records showed a remarkable redistribution of protein intake over the meals in the test group but no important increase in total daily protein intake. Each of the meals in the test group exceeded 25 g protein, achieving multiple anabolic meal moments during the day (11, 12). However, Kim et al. (37, 38) recently showed no benefit of an even protein distribution in healthy older adults on acute and long-term muscle protein synthesis. This may suggest that other factors, such as protein quality, have played a more important role.

The test product, providing a leucine-enriched whey protein mixture, without a concomitant meal, has previously been shown effective in stimulating muscle protein synthesis in healthy and sarcopenic older adults (33, 39, 40). Whey protein is a quickly digestible protein source contributing to a rapid and high postprandial increase in EAAs, including leucine (41, 42). The additional stimulation of FSR by the test product compared with breakfast alone in the present study coincided with an increase in plasma leucine to a mean peak concentration >500 μmol/L and EAAs increasing to a mean peak concentration >1000 μmol/L. Leucine and EAAs are known to be key anabolic stimuli (5, 9, 43), and peak plasma levels show a positive correlation with postprandial FSR (8). We were surprised that the standardized breakfast (6 g protein, 60 g carbohydrate, and 11 g fat) resulted in a significant FSR increase. We can only speculate about the reason for this, taking into account that the FSR increase is a result of increased incorporation in muscle proteins of available AAs from the precursor pool. In our study, the protein-bound enrichment in muscle noticeably increased in both groups, but reached higher levels in the test group. An increase in protein-bound enrichment was observed in other studies after a bolus of protein (33, 44), but to a lower extent also after carbohydrates with fat (i.e., no protein, with an insulin peak of 51 mU/L) (33). This suggests that insulin and AAs are both contributing factors. In our study, the plasma precursor pool may have been slightly influenced by the larger protein amount in the test product. From these analyses, we can speculate that the muscle protein synthetic response in the control group was a result of a combination of the insulin response—related to the provision of carbohydrate—and the small amount of AAs from the breakfast. Because our subjects were not sarcopenic and insulin resistant, it is possible that the sensitivity of muscle protein synthesis to even a low amount of protein, such as in the breakfast in combination with a high insulin response, was able to increase muscle protein synthesis.

The 0.37 kg muscle mass in the test group over the 6-wk period is a relevant gain, considering that older adults aged >70 y lose an average of 5–10% of their muscle mass per decade. The gain of ~1.6% total appendicular muscle mass that we observed after the 6-wk intervention would translate, therefore, into saving of ~2 y of muscle mass decline.

The stimulation of FSR likely contributed to the observed gain. When the equation described by Horstman et al. (45) to calculate the hypothetical reduction in FSR (expressed as %/h) was reversed, taking into account that the FSR response was based on a 4-h observation, an increase in 0.022%/h would result in a muscle mass increase of 3.7%. This translates to an increase of $23.1 \times 0.037 = 0.85$ kg of appendicular muscle mass and approaches the observed uncorrected increase of 0.5 kg (Supplemental Table 2). Our result agrees with 2 recent studies investigating timed protein supplementation in older adults (24, 25). Bauer et al. (24) and Norton et al. (25) observed increases in ALM of 0.17 kg (95% CI: 0.004, 0.338 kg) and 0.27 kg (95% CI: 0.05, 0.48 kg), respectively, after longer periods of intervention. Although the result of our study was within the CIs of both studies, the question is why the ALM gain in our study was attained in one-half or even one-quarter of the time. It may be speculated that a 1–2% increase in ALM is the maximum achievable with an intake of ~1.5 g protein/d, and perhaps this gain is reached within 6 wk and subsequently sustained or redistributed to other tissues than muscle. To our knowledge, no studies reported repeated measurements of ALM at 6, 12, and

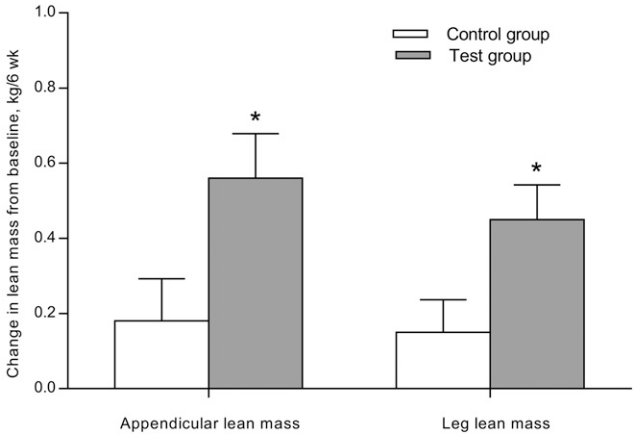


FIGURE 4 Increase in appendicular and leg lean mass of healthy older men in the control group, who received a placebo drink, and in the test group, who received a medical nutrition drink, before breakfast for 6 wk. Data are means ± SEs; $n = 12$ in both groups. *Different from control, $P < 0.05$.

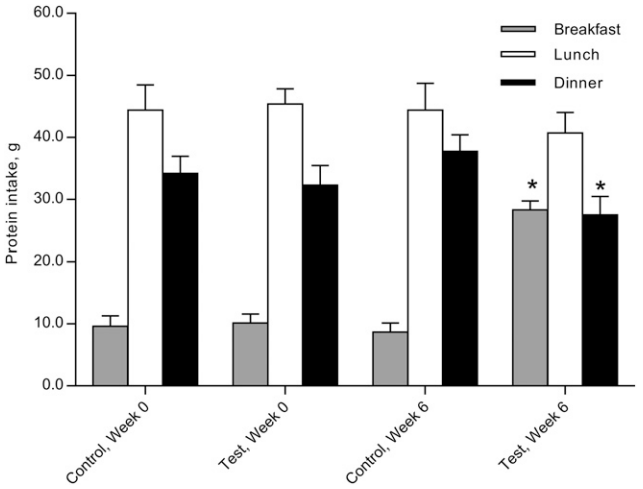


FIGURE 5 Protein intake distribution over the 3 daily main meals (breakfast, lunch, and dinner) at baseline and after 6 wk among healthy older men in the control group, who received a placebo drink, and in the test group, who received a medical nutrition drink before breakfast for 6 wk. Data are means ± SEs; n = 12 in both groups. *Different from control at this meal after 6 wk of intervention, $P < 0.05$.

24 wk. Therefore, we cannot substantiate this hypothesis at this moment.

The mean 25-hydroxyvitamin D concentration was low in the study population at baseline. Similar to other studies supplementing with vitamin D (46), we observed a noticeable improvement in the plasma 25-hydroxyvitamin D concentration. After supplementation for 6 wk, the mean exceeded the recommendation for older adults of >50 nmol/L (47) in the test group. Regarding the relevance of vitamin D for muscle (48), such an improvement in plasma 25-hydroxyvitamin D concentration may also have contributed to the observed increase in muscle mass in our study, although this study was not designed to determine such effect.

Surprisingly, the postprandial glucose peak was lower and delayed when breakfast was supplemented with the test product, despite the larger total carbohydrate load. The glucose peak lowering effect of whey protein, provided as a preload or added to a breakfast meal or carbohydrate drink, has been described in healthy young (49–52) and prediabetic adults (53, 54). In almost all of these studies, this coincided with higher peak insulin levels when whey protein was supplemented (50, 51, 53, 54). However, the postprandial insulin response did not differ between our study groups, similar to the study by Gunnerud et al. (52) in healthy young adults. This may be clinically relevant for older adults with impaired glucose control or type 2 diabetes. Another unexpected observation was the reduction in postprandial AA increments after 6 wk of intervention in the test group, which could not be attributed to the lowering concentrations of branched chain AAs. We speculate that this reduction may point toward better cellular utilization, uptake, or use of AAs, increased splanchnic AA extraction, or larger meal-induced inhibition of protein breakdown.

This study is rather unique in its combined measurement of acute muscle protein synthesis and longer-term intervention effect on muscle mass. The observed longer-term increase in muscle mass with the test product is in line with the acute stimulation of postprandial muscle protein synthesis. In addition, the study follows the recommendations for optimal quantity, quality, and timing of protein intake in older adults published in 2013 (6). Moreover, the improved glycemic control

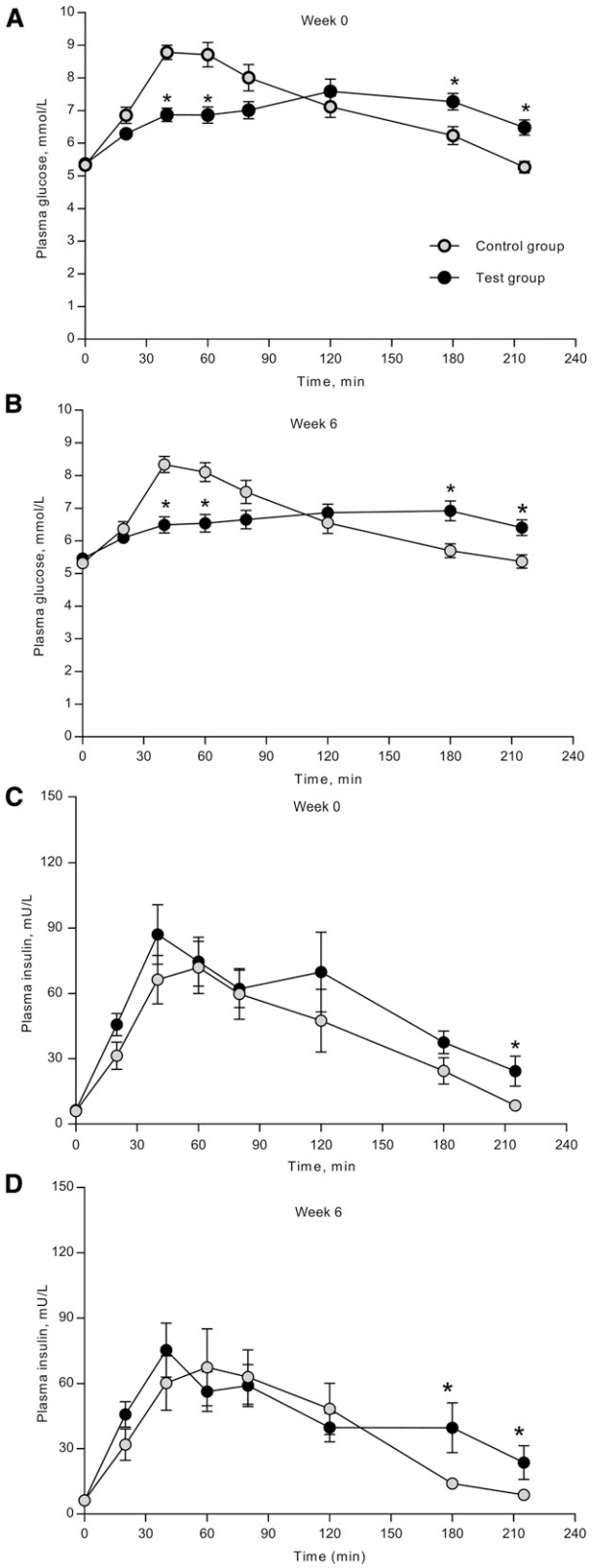


FIGURE 6 Plasma response of glucose (A and B) and insulin (C and D) of healthy older men in the control group, who received a placebo drink, and in the test group, who received a medical nutrition drink, before a standardized breakfast, during acute visits 2 (week 0; A and C) and 4 (week 6; B and D). Data are means ± SEs; n = 12 in both groups. *Different from control at the indicated time points, $P < 0.05$.

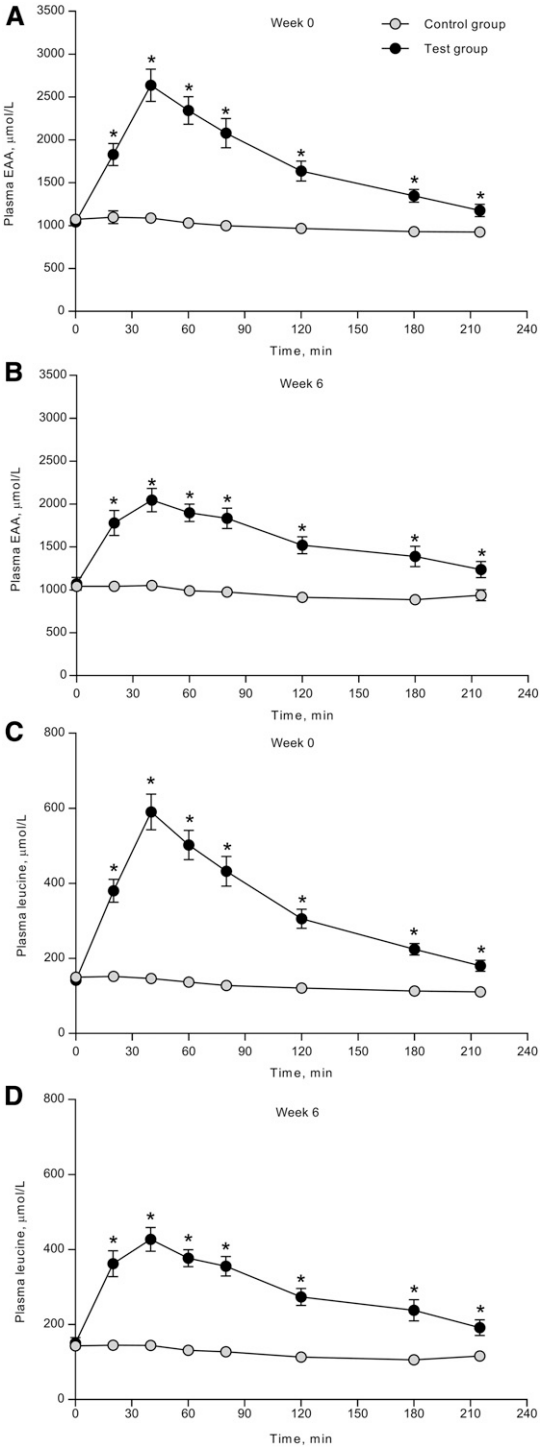


FIGURE 7 Plasma response of EAAs (A and B) and leucine (C and D) of healthy older men in the test group, who received a medical nutrition drink, and in the control group, who received a placebo drink, before a standardized breakfast during acute visits 2 (week 0; A and C) and 4 (week 6; B and D). Data are means ± SEs; n = 12 in both groups. *Different from control at the indicated time points, $P < 0.05$. EAA, essential amino acid.

after breakfast and unchanged long-term insulin sensitivity strengthens a positive rather than a negative glucose response to the test product despite the larger carbohydrate load.

The study is not without limitations, however. The muscle protein synthesis response was measured at baseline only and was not repeated after 6 wk of intervention as a result of local ethical restrictions regarding the collection of muscle biopsy specimens. Moreover, the study was limited to the measurement of muscle protein synthesis, whereas muscle protein breakdown and oxidation measurements would have been useful to understand the full metabolic picture. Despite an increase in muscle mass, we did not find an intervention effect of the 6-wk nutritional intervention on muscle strength, including handgrip strength and physical performance (i.e., SPPB). Also, after 6 wk, we observed a decrease in the PASE score in both groups, and most in the test group. Therefore, PASE-reported physical activity could not explain the larger increase in muscle mass in the test group. The applied measures of physical performance may not have been sensitive enough to detect effects caused by in mainly leg muscles in this relatively short intervention period.

Furthermore, the study population was healthy, with relatively good scores on these measurements at baseline. Exercise combined with adequate nutritional interventions could optimize effects on muscle strength and physical performance. Nutritional interventions alone might be more clinically relevant in compromised patient groups, especially for those who are unable to exercise. The same medical nutrition drink as in our study induced a similar muscle protein synthesis response in sarcopenic and healthy older adults (40), with an increase in muscle mass after a 12-wk intervention in sarcopenic older adults (24).

In conclusion, supplementing breakfast with a vitamin D and leucine-enriched whey protein medical nutrition drink increased muscle protein synthesis after breakfast, together with ensuring adequate postprandial EAA, leucine, and 25-hydroxyvitamin D plasma availability. This nutritional intervention also resulted in a more evenly distributed protein intake over the day and increased skeletal muscle mass after 6 wk of intervention in healthy older adults. Our study shows that supplementing breakfast with a vitamin D and leucine-enriched whey protein medical nutrition drink may be a way to support muscle preservation in older people.

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References

1. Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, Martin FC, Michel JP, Rolland Y, Schneider SM, et al. Sarcopenia: European consensus on definition and diagnosis: report of the European Working Group on Sarcopenia in older people. Age Ageing 2010; 39:412–23.
2. Mosoni L, Valluy MC, Serrurier B, Prugnaud J, Obled C, Guezennec CY, Mirand PP. Altered response of protein synthesis to nutritional state and endurance training in old rats. Am J Physiol 1995;268:E328–35.
3. Katsanos CS, Kobayashi H, Sheffield-Moore M, Aarsland A, Wolfe RR. Aging is associated with diminished accretion of muscle proteins after the ingestion of a small bolus of essential amino acids. Am J Clin Nutr 2005;82:1065–73.

4. Guillet C, Prod'homme M, Balage M, Gachon P, Giraudet C, Morin L, Grizard J, Boirie Y. Impaired anabolic response of muscle protein synthesis is associated with S6K1 dysregulation in elderly humans. FASEB J 2004;18:1586–7.
5. Cuthbertson D, Smith K, Babraj J, Leese G, Waddell T, Atherton P, Wackerhage H, Taylor PM, Rennie MJ. Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle. FASEB J 2005; 19:422–4.
6. Bauer J, Biolo G, Cederholm T, Cesari M, Cruz-Jentoft AJ, Morley JE, Phillips S, Sieber C, Stehle P, Teta D, et al. Evidence-based recommendations for optimal dietary protein intake in older people: a position paper from the PROT-AGE Study Group. J Am Med Dir Assoc 2013;14:542–59.
7. Walrand S, Gryson C, Salles J, Giraudet C, Migne C, Bonhomme C, Le Ruyet P, Boirie Y. Fast-digestive protein supplement for ten days overcomes muscle anabolic resistance in healthy elderly men. Clin Nutr 2016;35:660–8.
8. Pennings B, Boirie Y, Senden JM, Gijsen AP, Kuipers H, van Loon LJ. Whey protein stimulates postprandial muscle protein accretion more effectively than do casein and casein hydrolysate in older men. Am J Clin Nutr 2011;93:997–1005.
9. Katsanos CS, Kobayashi H, Sheffield-Moore M, Aarsland A, Wolfe RR. A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly. Am J Physiol Endocrinol Metab 2006;291:E381–7.
10. Rieu I, Balage M, Sornet C, Giraudet C, Pujos E, Grizard J, Mosoni L, Dardevet D. Leucine supplementation improves muscle protein synthesis in elderly men independently of hyperaminoacidaemia. J Physiol 2006;575:305–15.
11. Mamerow MM, Mettler JA, English KL, Casperson SL, Arentson-Lantz E, Sheffield-Moore M, Layman DK, Paddon-Jones D. Dietary protein distribution positively influences 24-h muscle protein synthesis in healthy adults. J Nutr 2014;144:876–80.
12. Paddon-Jones D, Rasmussen BB. Dietary protein recommendations and the prevention of sarcopenia. Curr Opin Clin Nutr Metab Care 2009; 12:86–90.
13. Paddon-Jones D, Campbell WW, Jacques PF, Kritchevsky SB, Moore LL, Rodriguez NR, van Loon LJ. Protein and healthy aging. Am J Clin Nutr 2015;101: 1339S–45S.
14. Rousset S, Patureau Mirand P, Brandolini M, Martin JF, Boirie Y. Daily protein intakes and eating patterns in young and elderly French. Br J Nutr 2003;90:1107–15.
15. Tieland M, Borgonjen-Van den Berg KJ, Van Loon LJ, de Groot LC. Dietary protein intake in Dutch elderly people: a focus on protein sources. Nutrients 2015;7:9697–706.
16. Tieland M, Borgonjen-Van den Berg KJ, van Loon LJ, de Groot LC. Dietary protein intake in community-dwelling, frail, and institutionalized elderly people: scope for improvement. Eur J Nutr 2012;51:173–9.
17. Loenneke JP, Loprinzi PD, Murphy CH, Phillips SM. Per meal dose and frequency of protein consumption is associated with lean mass and muscle performance. Clin Nutr 2016;35:1506–11.
18. Salles J, Chanet A, Giraudet C, Patrac V, Pierre P, Jourdan M, Luiking YC, Verlaan S, Migne C, Boirie Y, et al. 1,25(OH)2-vitamin D3 enhances the stimulating effect of leucine and insulin on protein synthesis rate through Akt/PKB and mTOR mediated pathways in murine C2C12 skeletal myotubes. Mol Nutr Food Res 2013;57:2137–46.
19. Bosaeus I, Rothenberg E. Nutrition and physical activity for the prevention and treatment of age-related sarcopenia. Proc Nutr Soc 2016;75:174–80.
20. Visser M, Deeg DJ, Puts MT, Seidell JC, Lips P. Low serum concentrations of 25-hydroxyvitamin D in older persons and the risk of nursing home admission. Am J Clin Nutr 2006;84:616–22; quiz 71–2.
21. Visser M, Deeg DJ, Lips P; Longitudinal Aging Study Amsterdam. Low vitamin D and high parathyroid hormone levels as determinants of loss of muscle strength and muscle mass (sarcopenia): the Longitudinal Aging Study Amsterdam. J Clin Endocrinol Metab 2003;88:5766–72.
22. Cruz-Jentoft AJ, Landi F, Schneider SM, Zuniga C, Arai H, Boirie Y, Chen LK, Fielding RA, Martin FC, Michel JP, et al. Prevalence of and interventions for sarcopenia in ageing adults: a systematic review. Report of the International Sarcopenia Initiative (EWGSOP and IWGS). Age Ageing 2014;43:748–59.
23. Tieland M, van de Rest O, Dirks ML, van der Zwaluw N, Mensink M, van Loon LJ, de Groot LC. Protein supplementation improves physical performance in frail elderly people: a randomized, double-blind, placebo-controlled trial. J Am Med Dir Assoc 2012;13:720–6.
24. Bauer JM, Verlaan S, Bautmans I, Brandt K, Donini LM, Maggio M, McMurdo ME, Mets T, Seal C, Wijers SL, et al. Effects of a vitamin D and leucine-enriched whey protein nutritional supplement on measures of sarcopenia in older adults, the PROVIDE study: a randomized, double-blind, placebo-controlled trial. J Am Med Dir Assoc 2015;16:740–7.
25. Norton C, Toomey C, McCormack WG, Francis P, Saunders J, Kerin E, Jakeman P. Protein supplementation at breakfast and lunch for 24 weeks beyond habitual intakes increases whole-body lean tissue mass in healthy older adults. J Nutr 2016;146:65–9.
26. Rubenstein LZ, Harker JO, Salva A, Guigoz Y, Vellas B. Screening for undernutrition in geriatric practice: developing the short-form mini-nutritional assessment (MNA-SF). J Gerontol A Biol Sci Med Sci 2001;56: M366–72.
27. Schuit AJ, Schouten EG, Westerterp KR, Saris WH. Validity of the Physical Activity Scale for the Elderly (PASE): according to energy expenditure assessed by the doubly labeled water method. J Clin Epidemiol 1997;50:541–6.
28. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state": a practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res 1975;12:189–98.
29. Guralnik JM, Simonsick EM, Ferrucci L, Glynn RJ, Berkman LF, Blazer DG, Scherr PA, Wallace RB. A short physical performance battery assessing lower extremity function: association with self-reported disability and prediction of mortality and nursing home admission. J Gerontol 1994;49:M85–94.
30. Vesali RF, Klaude M, Thunblad L, Rooyackers OE, Wernerman J. Contractile protein breakdown in human leg skeletal muscle as estimated by [2H3]-3-methylhistidine: a new method. Metabolism 2004;53:1076–80.
31. Calder AG, Anderson SE, Grant I, McNurlan MA, Garlick PJ. The determination of low d5-phenylalanine enrichment (0.002-0.09 atom percent excess), after conversion to phenylethylamine, in relation to protein turnover studies by gas chromatography/electron ionization mass spectrometry. Rapid Commun Mass Spectrom 1992;6:421–4.
32. Burd NA, West DW, Rerecich T, Prior T, Baker SK, Phillips SM. Validation of a single biopsy approach and bolus protein feeding to determine myofibrillar protein synthesis in stable isotope tracer studies in humans. Nutr Metab (Lond) 2011;8:15.
33. Kramer IF, Verdijk LB, Hamer HM, Verlaan S, Luiking Y, Kouw IW, Senden JM, van Kranenburg J, Gijsen AP, Poeze M, et al. Impact of the macronutrient composition of a nutritional supplement on muscle protein synthesis rates in older men: a randomized, double blind, controlled trial. J Clin Endocrinol Metab 2015;100:4124–32.
34. Wallace TM, Levy JC, Matthews DR. Use and abuse of HOMA modeling. Diabetes Care 2004;27:1487–95.
35. Vickers AJ, Altman DG. Statistics notes: analysing controlled trials with baseline and follow up measurements. BMJ 2001;323:1123–4.
36. Moore DR, Churchward-Venne TA, Witard O, Breen L, Burd NA, Tipton KD, Phillips SM. Protein ingestion to stimulate myofibrillar protein synthesis requires greater relative protein intakes in healthy older versus younger men. J Gerontol A Biol Sci Med Sci 2015;70:57–62.
37. Kim IY, Schutzler S, Schrader A, Spencer H, Kortebein P, Deutz NE, Wolfe RR, Ferrando AA. Quantity of dietary protein intake, but not pattern of intake, affects net protein balance primarily through differences in protein synthesis in older adults. Am J Physiol Endocrinol Metab 2015;308:E21–8.
38. Kim IY, Schutzler S, Schrader AM, Spencer HJ, Azhar G, Wolfe RR, Ferrando AA. Protein intake distribution pattern does not affect anabolic response, lean body mass, muscle strength or function over 8 weeks in older adults: a randomized-controlled trial. Clin Nutr 2017 Mar 3 (Epub ahead of print; DOI: 10.1016/j.clnu.2017.02.020).
39. Luiking YC, Deutz NE, Memelink RG, Verlaan S, Wolfe RR. Postprandial muscle protein synthesis is higher after a high whey protein, leucine-enriched supplement than after a dairy-like product in healthy older people: a randomized controlled trial. Nutr J 2014;13:9.
40. Kramer IF, Verdijk LB, Hamer HM, Verlaan S, Luiking YC, Kouw IW, Senden JM, van Kranenburg J, Gijsen AP, Bierau J, et al. Both basal and post-prandial muscle protein synthesis rates, following the ingestion of a leucine-enriched whey protein supplement, are not impaired in sarcopenic older males. Clin Nutr 2016 Sep 29 (Epub ahead of print; DOI: 10.1016/j.clnu.2016.09.023).
41. Dangin M, Guillet C, Garcia-Rodenas C, Gachon P, Bouteloup-Demange C, Reiffers-Magnani K, Fauquant J, Ballevre O, Beaufrere B. The rate of protein digestion affects protein gain differently during aging in humans. J Physiol 2003;549:635–44.

42. Luiking YC, Abrahamse E, Ludwig T, Boirie Y, Verlaan S. Protein type and caloric density of protein supplements modulate postprandial amino acid profile through changes in gastrointestinal behaviour: a randomized trial. Clin Nutr 2016;35:48–58.
43. Volpi E, Kobayashi H, Sheffield-Moore M, Mittendorfer B, Wolfe RR. Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults. Am J Clin Nutr 2003;78:250–8.
44. Gorissen SH, Burd NA, Hamer HM, Gijsen AP, Groen BB, van Loon LJ. Carbohydrate coingestion delays dietary protein digestion and absorption but does not modulate postprandial muscle protein accretion. J Clin Endocrinol Metab 2014;99:2250–8.
45. Horstman AM, Olde Damink SW, Schols AM, van Loon LJ. Is cancer cachexia attributed to impairments in basal or postprandial muscle protein metabolism? Nutrients 2016;8:499.
46. Bischoff-Ferrari HA, Dawson-Hughes B, Staehelin HB, Orav JE, Stuck AE, Theiler R, Wong JB, Egli A, Kiel DP, Henschkowski J. Fall prevention with supplemental and active forms of vitamin D: a meta-analysis of randomised controlled trials. BMJ 2009;339:b3692.
47. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, Durazo-Arvizu RA, Gallagher JC, Gallo RL, Jones G, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. J Clin Endocrinol Metab 2011;96:53–8.
48. Ceglia L, Harris SS. Vitamin D and its role in skeletal muscle. Calcif Tissue Int 2013;92:151–62.
49. Hoefle AS, Bangert AM, Stamfort A, Gedrich K, Rist MJ, Lee YM, Skurk T, Daniel H. Metabolic responses of healthy or prediabetic adults to bovine whey protein and sodium caseinate do not differ. J Nutr 2015;145:467–75.
50. Nilsson M, Holst JJ, Bjorck IM. Metabolic effects of amino acid mixtures and whey protein in healthy subjects: studies using glucose-equivalent drinks. Am J Clin Nutr 2007;85:996–1004.
51. Gunnerud UJ, Heinzle C, Holst JJ, Ostman EM, Bjorck IM. Effects of pre-meal drinks with protein and amino acids on glycemic and metabolic responses at a subsequent composite meal. PLoS One 2012; 7:e44731.
52. Gunnerud UJ, Ostman EM, Bjorck IM. Effects of whey proteins on glycaemia and insulinaemia to an oral glucose load in healthy adults; a dose-response study. Eur J Clin Nutr 2013;67:749–53.
53. Frid AH, Nilsson M, Holst JJ, Bjorck IM. Effect of whey on blood glucose and insulin responses to composite breakfast and lunch meals in type 2 diabetic subjects. Am J Clin Nutr 2005;82:69–75.
54. Jakubowicz D, Froy O, Ahren B, Boaz M, Landau Z, Bar-Dayan Y, Ganz T, Barnea M, Wainstein J. Incretin, insulinotropic and glucose-lowering effects of whey protein pre-load in type 2 diabetes: a randomised clinical trial. Diabetologia 2014;57:1807–11.