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Randomized Control Trials

Even or skewed dietary protein distribution is reflected in the whole-body protein net-balance in healthy older adults: A randomized controlled trial

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SUMMARY

Background & aim: For older adults, the dietary protein intake has shown to be skewed towards the evening meal. Resultingly, the vital source of essential amino acids could be insufficient after some meals, while after the evening meal the dietary protein could be suboptimally utilized for protein synthesis. The present study explored if an even distribution of the protein intake could improve the dietary amino acid absorption and whole-body protein net-balance.

Methods: Twenty-four healthy elderly males and females were included in a randomized controlled trial. Ten days of habituation to either an EVEN (n = 12) or SKEWED (n = 12) protein intake, was followed by a trial day. The total protein intake was controlled at 1.5 g/kg LBM, divided into 30% at each main meal in EVEN, and into 15% at breakfast and lunch and 60% at dinner in SKEWED. Snacks with 5% of the protein intake were served between meals. Energy intake in the meals and snacks were equal in both groups. Intrinsically labelled $^{2}H_{5}$-phenylalanine minced meat was served as the dietary protein to assess the amino acid absorption. On the trial day, infusion of $^{2}H_{8}$-phenylalanine and $^{2}H_{2}$-tyrosine, and blood samples taken over 11 h were used to measure whole-body protein turnover. Vastus lateralis muscle biopsies were taken to measure 9 h muscle protein FSR.

Results: Amino acid absorption rates and concentrations were greater in EVEN compared to SKEWED protein intake. Whole-body protein breakdown rates were lower with similar protein synthesis rates, and consequently the net-balance was greater in EVEN after breakfast and lunch compared to SKEWED and were the same in both groups after dinner. Muscle protein FSR were not different between EVEN and SKEWED.

Conclusions: The whole-body protein net-balance was more positive in EVEN compared to SKEWED for an extended time of the measured period, driven by a lower whole-body protein breakdown in EVEN.

Clinical Trials registration: NCT03870425, https://clinicaltrials.gov/ct2/show/NCT03870425.

1. Introduction

The age-related decline in muscle mass, strength, and function [1,2], termed sarcopenia [3–5], compromises metabolic health as well as the ability to perform everyday activities [4,6,7]. The total amount of daily dietary protein intake to maintain muscle mass with aging has been debated [8]. The recommendation from the WHO is to consume 0.8 g protein/kg body weight/day, which has been shown to maintain whole-body protein balance in adults [9].

Abbreviations: AA, amino acids; EAA, essential amino acids; MPE, Mole percent excess; MPS, muscle protein synthesis; PITC, phenylisothiocyanate; Ra, rate of appearance; Rd, rate of disappearance.

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However, to ensure a positive muscle protein balance in older adults a total amount of 1.2 g protein/kg body weight/day may be needed [10–12]. The importance of how the total dietary protein intake is distributed throughout the daily meals is less explored. It has been shown that for healthy older adults with a mean age of 71 years, a protein bolus of 0.4 g/kg body weight is needed to maximally stimulate myofibrillar FSR at rest, indicating that a greater protein amount will not be utilized for skeletal muscle protein biosynthesis [13]. Therefore, to obtain maximal stimulation of protein synthesis and to gain full utilization of especially the essential amino acids (EAA) of the dietary protein intake, it is hypothesized that each meal should contain 0.4 g protein per kg body weight emphasizing an even distribution of meal-protein throughout the day.

For older adults, is has been shown that the daily dietary protein intake is characterized by a skewness towards the evening meal [14,15]. In this context, an epidemiological study on elderly (75–96 years) has shown that a skewed protein intake is associated with frailty [16], a systematic review has indicated that evenness of dietary protein intake is associated with a greater muscle mass [17], and a cross-sectional study has found evenness of protein intake to be associated with greater lean mass and strength [18]. Furthermore, a study on adults (25–52 years of age) has shown that an even protein distribution at the main meals improves mixed muscle protein synthesis (MPS) more than a skewed distribution [19]. Conversely, a study on older adults found no difference between even and skewed dietary protein distribution in mixed MPS [20]. Also, intervention studies with a skewed protein intake (approx. 70% of the daily protein intake in one of four daily meals) compared to an equal intake have shown a superior effect of the skewed pattern in terms of nitrogen retention [21], lower amino acid oxidation [22], and an increase in fat free mass [23]. The current knowledge base is therefore modest and ambiguous.

From an aging perspective, the dietary protein intake must ensure a positive protein net-balance to maintain muscle mass, whether it is caused by an increase in whole-body protein synthesis or decrease in whole-body protein degradation. Notably, a decreased whole-body protein breakdown would indicate a state of amino acid sufficiency. Therefore, the aim of the present project is to create evidence of how the protein distribution pattern affects the dietary amino acid utilization as well as stimulate and influence whole-body protein turnover. Amino acid uptake, amino acid oxidation, as well as the whole-body protein synthesis and breakdown will be assessed through a day with even or skewed intake of the dietary proteins. The cornerstone of the present project is the use of an intrinsically amino acid tracer labeled meat product, which provides a unique opportunity to trace the dietary protein-derived amino acid uptake and utilization. It is hypothesized that an even daily protein distribution would result in a greater utilization and absorption of dietary amino acids, and that the magnitude of whole-body protein net-balance would be reflected by the intake pattern, but will not be greater after the evening meal with skewed compared to even protein distribution.

2. Methods

2.1. Subjects

Healthy elderly males and females between 65 and 80 years of age were included in the study between June 2019 and May 2020, and block randomized to either the EVEN (n = 12) or SKEWED (n = 12). Prior to inclusion, all subjects were given written and oral information about the purpose, study design, and possible risks of participating in the study. All subjects gave their written consent before enrollment to the study, in accordance with the Declaration of Helsinki. Before the project was commenced, the study approved by the Ethics Committee of Copenhagen and Frederiksberg (H-18026529) and was registered at ClinicalTrials.net (NCT03870425) with the primary and secondary outcomes specified.

Screening at inclusion ensured that all subjects adhered to the inclusion criteria of being between 65 and 80 years of age, having a BMI between 18.5 and 30, were non-smokers and non-vegetarians, were absent of diseases such as diabetes or any other metabolic diseases, gastrointestinal diseases, impaired kidney or liver function, inflammatory diseases, hypertension, signs of arteriosclerosis, did not take any medications affecting protein turnover, and physically active no more than 5 h per week except walking and activity associated with transportation. Height, weight, and blood pressure were measured for all subjects, and blood samples were taken for health screening. Whole body DXA-scans were carried out to determine LBM.

After screening and enrollment by the research staff, the randomization allocated the subjects into two intervention groups based on the dietary protein distribution that should be followed; an even protein distribution in the EVEN group (n = 12) and a protein distribution that was skewed towards the evening meal in the SKEWED group (n = 12). With the unpaired group design, we would be able to determine a 17% difference in the primary outcome protein net-balance with a power of 0.80, a significance level of 0.05 and a coefficient of variation of 15% when including 12 older adults to each group. The block-randomization was performed by lab technicians not involved in the project by drawing from sequential numbered sealed envelopes. An equal number of males and females were enrolled in each group (Table 1). After inclusion and before the trial day, one subject from the EVEN group dropped out due to stomach problems and one subject in the SKEWED group were excluded due to a previous pancreatectomy. Those subjects were replaced to ensure a total of 12 subjects in each group completing the study. The CONSORT diagram of the participant flow has previously been published [24] (Supplemental Fig. 1).

2.2. Diet design

The study was divided into a 10-day run-in period to ensure habituation to the EVEN and SKEWED dietary protein distribution, immediately followed by a one-day trial day on day 11 at Institute of Sports Medicine Copenhagen, Bispebjerg and Frederiksberg Hospital, Denmark. Importantly, the 10-day run-in period consisted of an initial 7-day habituation period with all food delivered at home followed by a 3-day trial period with all foods prepared and eaten at the research ward. The specific dietary design of the 7-day habituation period, and the dietary design and data of the 3-day trial period have previously been published [24]. In general, the meals were designed with an equal number of calories at breakfast, lunch, and dinner for both groups, but with the dietary protein distributed with 1/3 of the total daily intake at breakfast, lunch, and dinner in the EVEN group, and with 1/6 at breakfast and lunch, and 4/6 of the total daily protein intake at dinner in the SKEWED group.

2.3. Trial day design

After an overnight fast from 9:00 PM the night before, the subjects were transported by taxi and arrived at the hospital at 7:00 AM. Initially, a catheter was placed in an antecubital vein of the forearm/elbow of each arm; one catheter for tracer infusion, and the other for continuous blood sampling.

Infusion of stable isotope labeled amino acids was commenced after a background blood sample had been drawn. At 8:00 AM the primed continuous infusion of $^{2}H_{8}$-phenylalanine (prime 2.2 μmol/kg LBM, infusion 3.3 μmol/kg LBM/hour) and $^{2}H_{2}$-tyrosine (prime

0.7 μmol/kg LBM, infusion 0.9 μmol/kg LBM/hour) was initiated to enable assessment of whole-body protein turnover and measurement of muscle protein synthesis.

Three main meals (breakfast, lunch, and dinner) were served at 8:00 AM, 12:30 AM, and 17:00 AM, corresponding to time points 0 min, 270 min, and 540 min (Fig. 1). In addition, two snacks were served at 10:15 AM and 14:45 AM, corresponding to time points 135 min and 405 min. The calorie intake was divided with 30% of the total daily intake at each of the three main meals, and 5% at each of the two snacks in both the EVEN and SKEWED group. The total calorie intake was calculated on basis of LBM with the adjusted Harris Benedict equation with a correction factor of 1.53 for light activity [25]. The total protein intake was set to 1.5 g/kg LBM, corresponding to approximately 1.0–1.2 g/kg body weight. This protein intake was in EVEN divided with 30% (corresponding to approximately 22 g) at the three main meals, and 5% at each of the two snacks (Table 1). In the SKEWED group, the total protein intake was divided with 15% (corresponding to approximately 11 g) at breakfast and lunch, 60% (corresponding to approximately 44 g) at dinner, and 5% at each of the two snacks.

The food was based on whole foods and was produced by the research kitchen at the Department of Human Nutrition, University of Copenhagen. The fat content was identical in all meals and snacks, and in both groups (31 E%), whereas the amount of carbohydrates was altered depending on the amount of protein in the meal, to achieve the same number of calories in all meals. Importantly, to be able to measure the dietary protein uptake the protein source at all meals and snacks was primarily $^{2}H_{5}$-phenylalanine intrinsically labeled minced meat [26]. To minimize dilution of the intrinsically labeled amino acid tracer, all other whole foods chosen for the meals were low on protein. All meals and snacks were weighted and prepared by the research staff, and the food intake was closely observed. All subjects finished their meals and snacks. Besides the meals and snacks, only intake of water was allowed.

Throughout the entire day, the subjects were lying or sitting in a bed except from when visiting the restroom and from two bouts of 15 min of cycling on a cycle ergometer (Monark Exercise AB, Sweden). Each of these bouts of cycling was completed with a cadence of 60–80 rpm and with a heart rate at 60–80% of their estimated maximal heart rate. The cycling was not designed to be physical training but was included to ensure that the subjects were not completely immobilized for the entire day and was feasible despite being connected to the tracer infusion pump.

2.4. Blood sampling

Venous blood samples were drawn at baseline in the morning before tracer infusion and meal intake, right before lunch and dinner, and every half hour during the first 2 h after each main meal (Fig. 1). Also, a blood sample was drawn 1 h after each of the two snacks. The blood samples were collected in k3-EDTA and Z serum clot activator vacutainer tubes from the vein catheter that was not used for tracer infusion. Upon collection the k3-EDTA tubes were placed on ice for 10–30 min and the Z serum clot activator tubes were placed at room temperature between 30 and 45 min. Hereafter, the samples were centrifuged at 3200 g for 10 min at 4 °C to isolate the plasma and serum, respectively, which subsequently were aliquoted into 500 μL tubes and stored at −80 °C until further analysis.

2.5. Muscle tissue sampling

By randomization on each subject either the dominant or non-dominant leg was chosen as the site for all biopsies. The muscle biopsies were taken from the lateral portion of the vastus lateralis muscles from a proximal, medial, and distal site with 3 cm in-between. The order of the biopsy; proximal, medial, and distal as either the first, second, and third biopsy, was randomized as well. The first biopsy was taken at 10:00 AM three days prior to the trial day from which data has been published [24]. The second and third biopsy were used in the present study and was taken at 10:00 AM and 19:00 AM; 120 and 660 min, respectively, on the trial day (Fig. 1).

The muscle biopsies were taken under local anesthetic treatment (lidocaine, 1%). The biopsies were taken with 4-mm Bergström biopsy needles (Stille, Stockholm, Sweden) with manual suction. From the muscle specimen, blood, visible fat, and connective tissue were quickly removed, and the muscle specimen was rinsed with saline water before snap frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.6. Insulin

Serum insulin concentration was analyzed by ELISA (80-INSHU-E01.1, ALPCO, NH, USA) according to the instruction of the manufacturer. Serum samples and insulin standards of 50 μL were loaded in replicates on a pre-coated 96-well plate, and 200 μL of insulin primary anti-body was added to each well before incubation on an ELISA plate shaker at room temperature for 60 min. Hereafter, the samples were discarded, and the plate was washed 6 times. Next, 200 μL of HRP streptavidin solution was added to each well followed by 15 min incubated on an ELISA plate shaker at room temperature, whereafter 200 μL of stop solution was added to each well. Immediately hereafter, the intensity in each well was measured at 450 nm on a 96-well plate reader (Multiscan FC, Thermo-Fisher Scientific). The insulin concentrations were calculated on basis of the standard curve.

2.7. Mass spectrometry

Mass spectrometry analysis on the muscle biopsies was performed on 20 mg of wet weight muscle tissue per sample. The muscle tissue was homogenized in homogenization buffer (0.02 M Tris [pH 7.4], 0.15 M NaCl, 2 mM EDTA, 2 mM EGTA, 0.5% TritonX-100 and 0.25 M sucrose). The proteins were hydrolyzed by addition of 1 mL 1 M HCl and 1 mL resin slurry, and left overnight at 110 °C.

For mass spectrometry analysis of plasma amino acid concentration and tracer precursor enrichment, 200 μL of plasma per sample was used to extract the free amino acids. Isotopically labeled internal standards (uniformly labeled $^{13}C/^{15}N$) of each amino acid, was added to the plasma in a 50% acetic acid solution.

The solution from either the muscle- or plasma samples was poured over cation exchange columns with resin (AG 50 W-X8 resin, Bio-Rad Laboratories), which had been prepared by adding 3 × 2 mL 1 M HCl. The resin columns were washed 5 times with 3 mL deionized water before the amino acids were eluted into collection vials by adding 2 × 2 mL 4 M NH_4OH.

Subsequently, to measure the plasma amino acid tracer enrichment and muscle protein tracer incorporation, the solvents eluted from the resin columns were evaporated under a stream of N_2 flow at 70 °C and samples were derivatized into their PITC-derivative with phenylisothiocyanate (PITC). Ten microliters of the derivatized samples were loaded and analyzed by LC–MS/MS (TSQ Quantiva; Thermo Fisher Scientific, San Jose, CA) as described elsewhere [27].

2.8. Equations for calculating protein turnover

Calculations of whole-body protein turnover was performed over an 11-h postprandial period. With the fluctuating amino acids

levels in such a period, the pool size is changing over a certain time interval, whereby the tracers are in a non-steady state. Therefore, calculations were based on the Steele equation to take the non-steady state into account [28]. The following equations in the non-steady state are based on the delta values between two adjacent time points.

Total rate of appearance (R_a) of phenylalanine was calculated by:

$$Total\ R_a = \frac{IR}{\hat{E}_t} - \frac{pV \times \left[\frac{\hat{C}_t}{1+\hat{E}_t}\right] \times \left[\frac{\Delta E_t}{\Delta t}\right]}{\hat{E}_t} \quad (1)$$

where IR denotes the tracer ($^2H_8$-phenylalanine) infusion rate in μmol x kg LBM^{-1} x min^{-1}, $\hat{E}_t$ is the mean tracer enrichment of values surrounding time t, pV denotes the volume of distribution, which is set to be 0.125, $\hat{C}_t$ is the mean tracee (phenylalanine) concentration of values surrounding time t, ΔE_t is the change in tracer enrichment between values surrounding time t, and Δt is the change in time between two adjacent time points in minutes.

The R_a of exogenous dietary protein derived phenylalanine was calculated by:

$$Exogenous\ R_a = \frac{Total\ R_a \times \hat{E}_{OT(t)} + pV \times \hat{C}_t \times \left[\frac{\Delta E_{OT(t)}}{\Delta t}\right]}{E_{protein}} \quad (2)$$

where $\hat{E}_{OT(t)}$ denotes the mean and $\Delta E_{OT(t)}$ denotes the delta plasma enrichment of the oral tracer ($^2H_5$-phenylalanine) of values surrounding time t, and $E_{protein}$ is the intrinsically labeled tracer ($^2H_5$-phenylalanine) enrichment of the ingested dietary protein.

The hydroxylation of phenylalanine to tyrosine, the rate limiting step in phenylalanine oxidation, representing the amino acid oxidation was given by:

$$Phe_{hydroxylation} = Tyr\ R_a \times \frac{\hat{E}_{Tyr}}{\hat{E}_{Phe}} \quad (3)$$

where Tyr R_a denotes the rate of appearance of tyrosine calculated using equation (1), knowing the tracer ($^2H_2$ tyrosine) IR, tracer enrichment, and tracee (tyrosine) concentration. $\hat{E}_{Tyr}$ and $\hat{E}_{Phe}$ is the mean enrichment of values surrounding time t of tyrosine and phenylalanine, respectively.

The total rate of disappearance (R_d) of phenylalanine was calculated by:

$$Total\ R_d = pV \times \left[\frac{\Delta C_t}{\Delta t}\right] + Total\ R_a \quad (4)$$

Table 1
Subject characteristics, total calorie, total protein intake, and protein intake at main meals.

	EVEN	SKEWED	*p-value*
N	12	12	–
Sex Ratio (w/m)	6/6	6/6	–
Age (years)	69 ± 4	70 ± 3	*0.784*
BMI (kg/m^2)	25.2 ± 3.4	27.0 ± 2.7	*0.163*
LBM (kg)	47.1 ± 8.4	49.5 ± 8.6	*0.502*
Calorie intake (kcal)	1961 ± 349	2062 ± 360	–
Total protein (g/kg LBM)	1.51 ± 0.002	1.50 ± 0.002	–
Protein morning (g/kg LBM)	0.45 ± 0.001	0.23 ± 0.001	–
Protein lunch (g/kg LBM)	0.45 ± 0.001	0.23 ± 0.001	–
Protein dinner (g/kg LBM)	0.45 ± 0.001	0.90 ± 0.001	–

Data are shown as mean ± SD. Age, BMI, and LBM was compared by two-tailed unpaired t-test, which has previously been published [24].

where ΔCt denotes the change in concentration of the tracee (phenylalanine) between values surrounding time t.

The endogenous R_d representing the whole-body protein synthesis was calculated by

$$Endogenous\ R_d = Total\ R_d - Phe_{hydroxylation} \quad (5)$$

The endogenous R_a representing the whole-body protein breakdown was calculated by

$$Endogenous\ R_a = Total\ R_a - Exogenous\ R_a - IR \quad (6)$$

The net-balance in whole-body protein turnover was calculated by:

$$Net\ balance = Endogenous\ R_d - Endogenous\ R_a \quad (7)$$

2.9. Muscle protein synthesis

The fractional synthesis rate (FSR) of mixed muscle proteins was calculated based on direct incorporation of the infused $^2H_8$-phenylalanine tracer:

$$FSR = \left[\frac{\Delta E_{protein}}{\hat{E}_{precusor} \times \Delta t}\right] \times 100 \quad (8)$$

where FSR denotes the fractional synthetic rate in % x hour^{-1}. $\Delta E_{protein}$ is the change in muscle protein enrichment of $^2H_8$-phenylalanine from the first biopsy at 120 min to the last biopsy at 660 min, the time interval which defines Δt, and also the time period over which the mean precursor enrichment ($\hat{E}_{precursor}$) of $^2H_8$-phenylalanine was measured in plasma.

2.10. Statistics

Plasma levels of amino acids (AA), plasma stable isotope tracer enrichment, plasma phenylalanine rate of appearance, as well as whole-body turnover data were analyzed by two-way ANOVA with repeated measures for time. Whenever main effect of time or group, or interaction was found subsequent Student-Newman-Keuls posthoc analysis was performed. Serum insulin levels were log transformed to ensure homoscedasity of the data before analyzed by two-way ANOVA with repeated measures for time, and Dunett's posthoc test was performed after a main effect of time was found. The scope of the study was to explore the difference between the EVEN and SKEWED protein distribution. Therefore, only significant group differences are denoted in the figures of plasma levels of AA, serum insulin, plasma stable isotope tracer enrichment, plasma phenylalanine rate of appearance, and whole-body protein turnover. Mixed muscle FSR was analyzed by two-tailed unpaired t-test. Unless otherwise stated data is shown as mean ± SEM, with the significant level set to $p < 0.05$. All subjects were included in all analyses, and data was analyzed by Sigma Plot version 13.0 (Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Plasma amino acids and serum insulin

The plasma levels of BCAA were greater in EVEN compared to SKEWED from 90 min after breakfast until time point 570 min, corresponding to 30 min after dinner (Fig. 2A). At time point 630 and 660 min, corresponding to 1.5 and 2 h after dinner, the plasma BCAA levels were greater in SKEWED compared to EVEN.

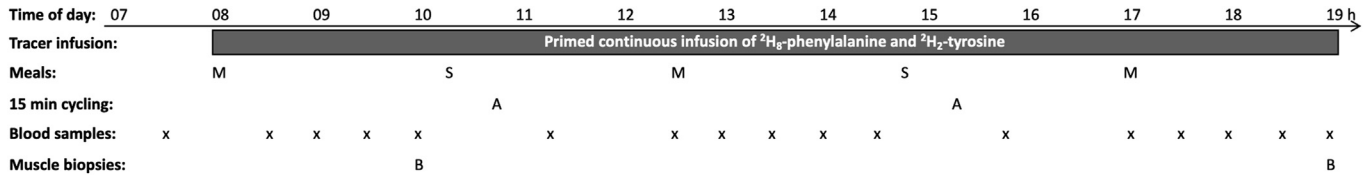

Fig. 1. Design of the trial day where whole-body protein turnover rates were assessed in response to an EVEN or SKEWED dietary protein distribution over an 11-h postprandial period with primed continuous infusion of 2H8-phenylalanine and 2H2-tyrosine. Main meals are denoted with M and snacks with S. Two times during the infusion trial the subjects where physical active (A) completing 15 min of ergometer cycling. Blood samples (x) were taken in an overnight fasting condition and throughout the postprandial period. Muscle tissue sampling (B) was performed 2 and 11 h into the postprandial period.

For EAA the plasma levels were higher in EVEN compared to SKEWED from 90 min after breakfast until time point 300 min, and from time point 360 min until time point 540 min, corresponding to the time of dinner (Fig. 2B). At time point 630 and 660 min, 1.5 and 2 h after dinner, the plasma EAA levels were greater in SKEWED compared to EVEN.

The plasma levels of total amino acids (AA) were higher in EVEN compared to SKEWED from 90 min after breakfast until time point 570 min, corresponding to 30 min after dinner (Fig. 2C). At no time point was the plasma level of total AA greater in SKEWED compared to EVEN.

For all time points from 30 to 660 min the serum insulin levels were higher compared to baseline (Fig. 2D). Insulin levels fluctuated with an increase after each meal, but at no time points were the insulin levels different between EVEN and SKEWED.

3.2. Plasma phenylalanine and tyrosine

Plasma phenylalanine levels were greater in EVEN compared to SKEWED at time point 120 min, and higher in SKEWED compared to EVEN at time point 630 and 660 min (Fig. 3A). Tyrosine plasma levels were higher in EVEN compared to SKEWED at time point 30 min, and from 90 min until 570 min (Fig. 3B). At time point 660 min the plasma level of tyrosine was higher in SKEWED compared to EVEN.

The continuously infused $^{2}H_{8}$-phenylalanine tracer was only different between SKEWED and EVEN at time point 390 min, being higher in SKEWED (Fig. 3C), whereas the continuously infused $^{2}H_{2}$-tyrosine tracer was higher in SKEWED compared to EVEN at time point 30 min, 120–270 min, and 330–540 min (Fig. 3D).

3.3. Phenylalanine rate of appearance

Total R_a of phenylalanine was only higher in EVEN compared to skewed at time point 90 min (Fig. 4A). The exogenous meal derived phenylalanine R_a, calculated from the intrinsically $^{2}H_{5}$-phenylalanine label, was greater after breakfast and lunch in EVEN compared to SKEWED; from 60 min to 540 min, but was not different between EVEN and SKEWED after the dinner (Fig. 4B).

3.4. Whole-body protein turnover

Hydroxylation of phenylalanine to tyrosine, the rate limiting step in phenylalanine oxidation, increased after the morning meal to a greater degree in EVEN compared to SKEWED, being higher in EVEN at time points 90 and 120 min (Fig. 5A). For the remaining of the trial day, the phenylalanine hydroxylation was not different between EVEN and SKEWED.

There was an overall interaction effect on the endogenous R_d of phenylalanine, representing whole-body protein synthesis (Fig. 5B). However, the posthoc test could not identify specific timepoints where the R_d was different between EVEN or SKEWED (Fig. 5B). The AUC for the entire measured period of the endogenous R_d of phenylalanine was not different between EVEN or SKEWED (Supplemental Fig. 2A).

Endogenous R_a of phenylalanine, representing whole-body protein breakdown, was higher in SKEWED compared to EVEN at time 285–345 min and 430–555 min (Fig. 5C). At no time point was the endogenous R_a higher in EVEN compared to SKEWED. The AUC of the endogenous R_a of phenylalanine was higher in SKEWED compared to EVEN (Supplemental Fig. 2B).

The difference between endogenous R_a and R_d of phenylalanine, which represents the protein net-balance between whole-body protein synthesis and breakdown, was positive throughout the entire post-prandial period, but higher in EVEN compared to SKEWED at the time interval from 15 to 555 min (Fig. 5D). At no time point was the net-balance greater in SKEWED compared to EVEN. The AUC of the protein net-balance was higher in EVEN compared to SKEWED (Supplemental Fig. 2C).

3.5. Muscle protein synthesis

Mixed muscle protein FSR measured between time point 120 min and time point 660 min was not different between EVEN and SKEWED (Fig. 6).

4. Discussion

In the present study, it was found that an EVEN meal-distribution of dietary protein intake over the day resulted in a more positive whole-body protein net-balance compared to a SKEWED meal-distribution pattern, whereas muscle protein FSR was the same in EVEN and SKEWED. Therefore, the protein distribution pattern was reflected in a whole-body protein net synthesis. The difference in the net-balance was primarily driven by a lower whole-body protein breakdown rate in EVEN compared to SKEWED. To our knowledge, this is the first study to show the time-course of whole-body protein turnover in response to EVEN and SKEWED dietary protein distribution. Importantly, the subjects were habituated to the distribution pattern prior to the trial, and total calorie and total protein intake were matched between the groups.

Moore and colleagues have previously shown that 0.4 g protein per kg body weight was needed to maximally stimulate muscle protein synthesis in older adults [13]. This amount was used in the present study design as a guideline for the optimal protein amount in all main meals in the EVEN group. In the SKEWED group we chose half of the optimal protein intake at breakfast and lunch, and double the amount at dinner, thus enabling identical total protein intake in the EVEN and SKEWED groups for the entire day. The higher protein intake at dinner in SKEWED was not expected to yield additional benefit in stimulating protein synthesis compared to the dinner in the EVEN group. Measuring the mixed MPS over 9 h from 120 to 660 min, we did not see a difference between EVEN and

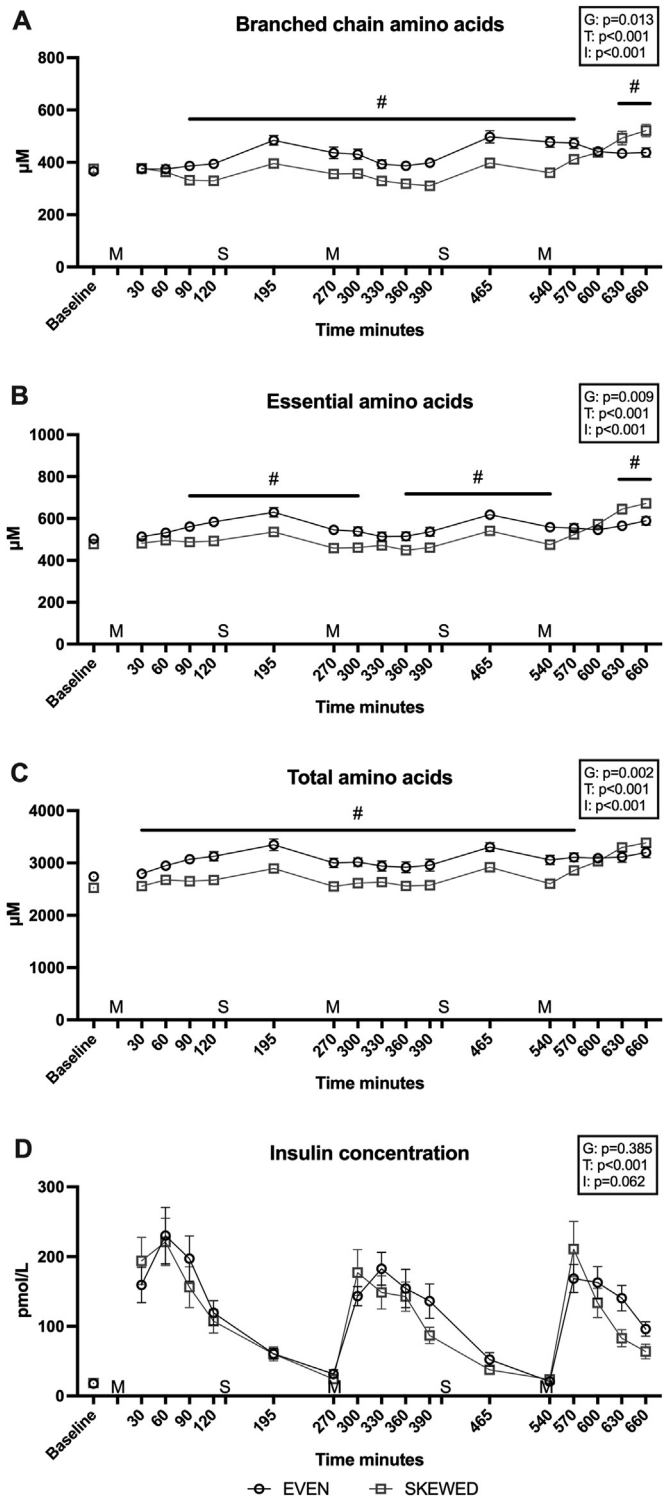

Fig. 2. Plasma (A) branched chain amino acids, (B) essential amino acids, (C) total amino acids, and (D) serum insulin levels shown as mean ± SEM in EVEN and SKEWED groups at selected time points over an 11-h postprandial period. M denote main meal, S denote snack. Data were analyzed by two-way ANOVA with repeated measures for time, with subsequent Student-Newman-Keuls posthoc-test or Dunnett's test for serum insulin. Top right box showing ANOVA results for group (G), time (T), and interaction (I) effect. Only significant group differences are denoted. # denote difference between EVEN and SKEWED, $p < 0.05$. Serum insulin data was log transformed before statistical testing but is depicted on a linear scale.

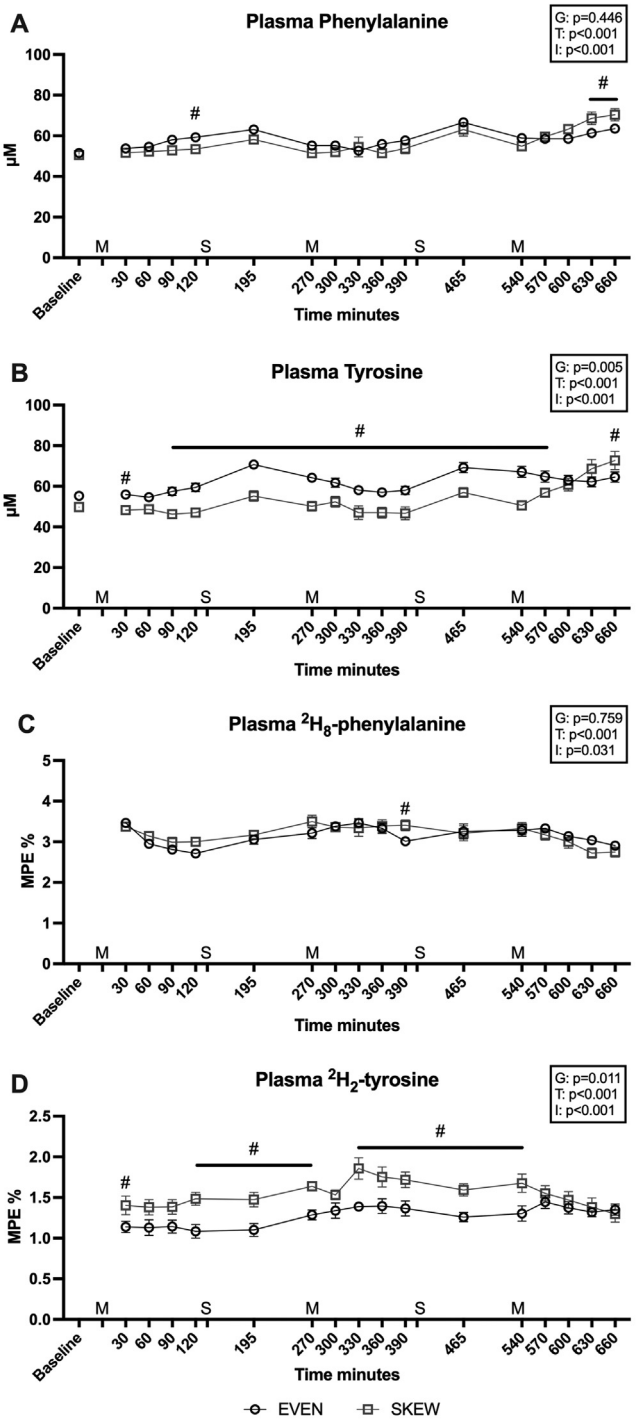

Fig. 3. Plasma (A) phenylalanine and (B) tyrosine concentrations, and plasma (C) 2H8-phenylalanine, (D) 2H2-tyrosine mole percent excess (MPE) enrichment shown as mean ± SEM in EVEN and SKEWED groups at selected time points over an 11-h postprandial period. M denote main meal, S denote snack. Data was analyzed by two-way ANOVA with repeated measures for time, with subsequent Student-Newman-Keuls posthoc-test. Top right box showing ANOVA results for group (G), time (T), and interaction (I) effect. Only significant group differences are denoted. # denote difference between EVEN and SKEWED, $p < 0.05$.

SKEWED. It is important to note that the 9 h MPS did not span the entire 11 h as the muscle biopsy at 120 min was designed for the preceding 3 days integrated MPS measurement. Yet, the 9 h MPS

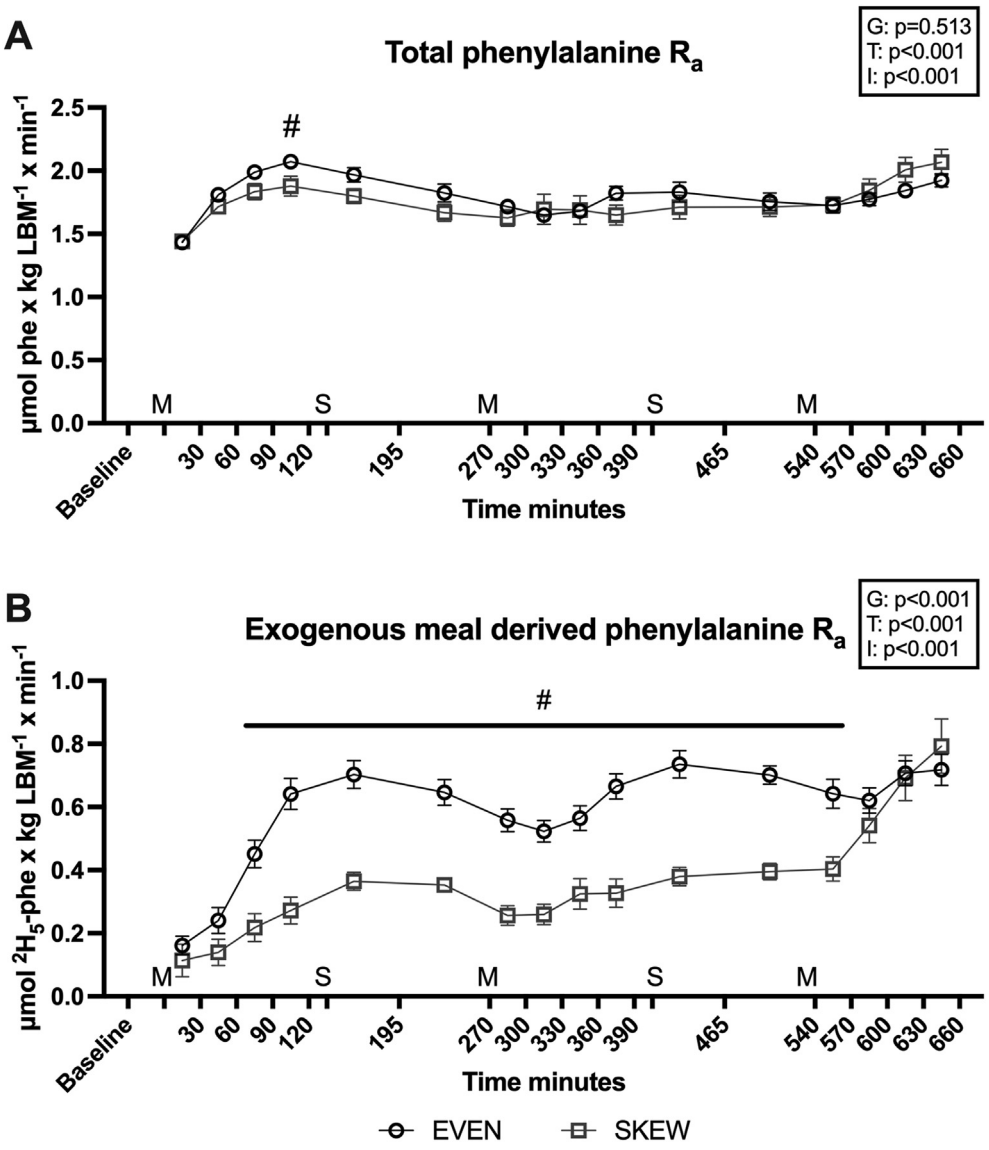

Fig. 4. Plasma (A) total phenylalanine rate of appearance (Ra) and (B) exogenous meal derived phenylalanine Ra shown as mean ± SEM in EVEN and SKEWED groups at selected time points over an 11-h postprandial period. M denote main meal, S denote snack. Data was analyzed by two-way ANOVA with repeated measures for time, with subsequent Student-Newman-Keuls posthoc-test. Top right box showing ANOVA results for group (G), time (T), and interaction (I) effect. Only significant group differences are denoted. # denote difference between EVEN and SKEWED, $p < 0.05$.

with no difference between EVEN and SKEWED is in line with the integrated measurement of mixed MPS [24]. Notably, in contrast to the studies by Moore and colleagues, where subjects were investigated in response to a rapidly digested protein bolus in the overnight fasted state in the resting condition, the present study applied multiple mixed meals with whole foods ensuring intake of all macronutrients as well as physical activity to refrain the subjects from being inactive. Previously, it has been shown that the food matrix affects the amino acid absorption rate and peak levels [26,29]. It could be speculated that when a protein is ingested as part of a mixed meal, the peak MPS response could be lower and more prolonged with less impact of an EVEN or SKEWED distribution pattern. Therefore, an intake of a stand-alone protein bolus may indicate the anabolic potential of such a protein intake, whereas the translation ability of the results could be higher when studying e.g. a given protein dose within a mixed food matrix. The MPS rate levels from the present study are similar to the study by Kim and colleagues [20]. In a comparable group of older adults investigating the impact of even and skewed protein distributions patterns, they also found that the mixed MPS was not influenced by the distribution pattern. In contrast, in young subjects, Mamerow and colleagues found a greater MPS when having an EVEN compared to a SKEWED dietary protein distribution [19]. An age difference in the effect of protein distribution in relation to stimulation of muscle protein synthetic processes therefore could exist.

The whole-body protein net-balance is more positive in EVEN compared to SKEWED for most of the post-prandial period; from 9:30 AM to 5:30 PM. Although the overall interaction effect was significant for R_d, the posthoc tests revealed no time-point where the whole-body protein synthesis rates were different between EVEN and SKEWED (Fig. 5B). Furthermore, the AUC for R_d of the entire trial was not different between EVEN and SKEWED (Supplemental Fig. 2A). The difference seen in whole-body protein breakdown thus, seems to drive the difference in net-balance between EVEN and SKEWED distribution. This finding indicates that when meals contain sufficient amounts of dietary proteins, the

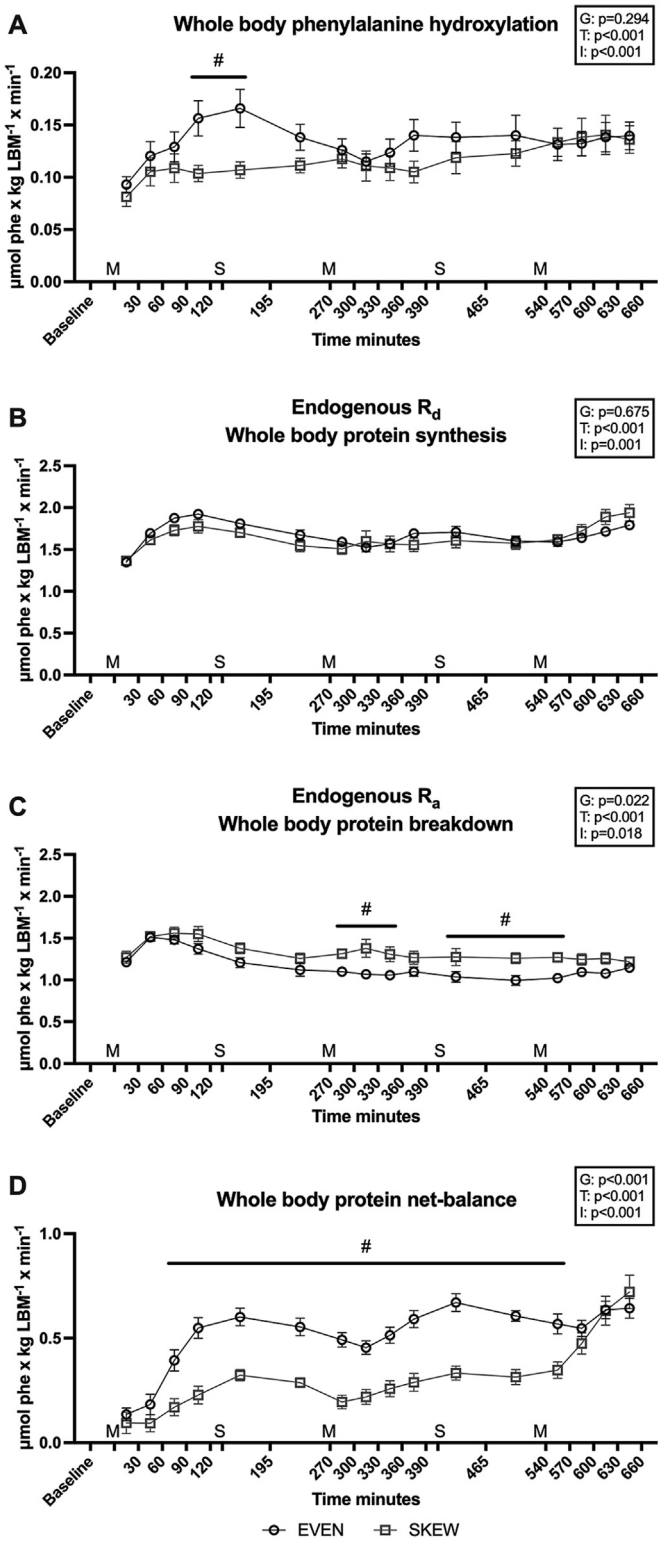

Fig. 5. Whole-body phenylalanine (A) hydroxylation, (B) endogenous rate of disappearance (Rd), (C) endogenous rate of appearance (Ra), and (D) net-balance shown as mean ± SEM in EVEN and SKEWED groups at selected time points over an 11-h postprandial period. M denote main meal, S denote snack. Data was analyzed by two-way ANOVA with repeated measures for time, with subsequent Student-Newman-Keuls posthoc-test. Top right box showing ANOVA results for group (G), time (T), and interaction (I) effect. Only significant group differences are denoted. # denote difference between EVEN and SKEWED, $p < 0.05$.

release of amino acids from endogenous body proteins due to protein breakdown are dampened and thus tissue proteins are spared. Comparable effect was seen by Kim and colleagues, where a greater whole-body net-protein balance in adult subjects (age 18–40 years) was observed when consuming a greater protein amount (70 g) of what is known to maximally stimulate muscle protein synthesis (40 g) [30]. Importantly, the net-balance was mainly affected by lower whole-body protein breakdown when ingesting 70 g of protein. Muscle proteins constitutes 40% of the whole-body protein pool and it is believed that muscle protein turnover constitutes 35–50% of the whole-body protein turnover rate [31]. Therefore, it cannot directly be stated to what degree the decreased whole-body protein breakdown rate is influenced by a lower breakdown rate of muscle proteins. Yet, the muscle protein synthesis in the present study was not different between EVEN and SKEWED, neither when measured acutely nor when measured over multiple days [24]. This was the same response seen in whole-body protein synthesis. Therefore, it would have been highly interesting to elucidate if the muscle protein breakdown was spared to a greater degree in EVEN, as seen with whole-body protein breakdown. Such impaired muscle protein breakdown could ensure maintenance of muscle mass with an even intake of dietary protein, which has previously been indicated [17]. Although muscle protein synthesis is known to be an important and indicative measurement of the anabolic potential of exercise interventions [32], it could be speculated that acute or integrated muscle protein breakdown is a more significant measurement when exploring the muscle mass sparing potential of a dietary protein intervention alone. Such, integrated muscle protein breakdown measurement is applicable [33]. The determining factor for the lower protein breakdown in EVEN compared to SKEWED could be the greater exogenous amino acid appearance and greater concentrations of amino acids, which is seen for an extended period in the EVEN group. To ensure a sufficient level of precursor amino acids to maintain important processes such as immune function, energy production, and liver and blood protein synthesis the dependence on breakdown of whole-body protein could be lowered with the EVEN distribution of protein intake. The greater amino acid appearance and concentration for an extended period of the day with the EVEN distribution

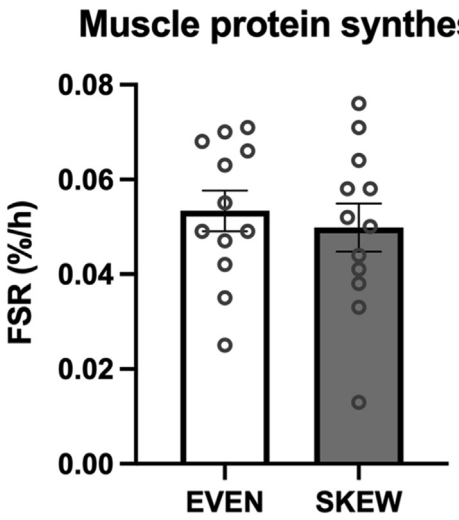

Fig. 6. Mixed muscle FSR measured over 9 h. Individual data points with mean ± SEM are shown for the EVEN and SKEWED groups. Data were analyzed by two-tailed unpaired t-test with significant level set to $p < 0.05$.

would spare the efflux of amino acids from the muscle protein pool, which is one of the easily accessible amino acid pools for protein breakdown.

The phenylalanine hydroxylation was different between the EVEN and SKEWED group after the breakfast meal. This contrasted with our hypothesis, where we had expected that the dinner bolus of 0.9 g protein/kg LBM in SKEWED would have resulted in the greatest amino acid oxidation. It has previously been shown that the habitual protein intake affects the phenylalanine hydroxylation after a meal [34]. Therefore importantly, the subjects were habituated through 10 days of exposure to the even or skewed distribution pattern with the same level of total protein intake. Thus, we do not believe that the phenylalanine oxidation was an artefact from not being accustomed to the protein distribution pattern. The protein content of the breakfast in SKEWED is only half compared to EVEN, thus the increase in the phenylalanine hydroxylation after breakfast reflects the phenylalanine content of the meal. Interestingly, the biggest change in phenylalanine hydroxylation seems to occur after breakfast in EVEN, whereas after lunch, the subsequent meal, the meal derived protein is better utilized resulting in less of a change in phenylalanine hydroxylation. Therefore, it could be speculated that the greatest change in amino acid oxidation occurs when going from the overnight postabsorptive state to the postprandial state. The perspective on this diurnal observation is that most acute (few hours) measurements of postprandial responses are conducted in overnight fasted participants at times before midday. Whether extrapolation of such data to whole day are feasible could be questioned by these findings.

It is important to note, with the time-window that was chosen after dinner peak levels in the SKEWED group could have been missed. Importantly however, when the measurements were stopped at 660 min not much of a change in phenylalanine hydroxylation, whole-body protein synthesis, or whole-body protein breakdown were seen (Fig. 5). It has been shown that 2 h and onwards after intake of 15, 30, or 45 g of protein the exogenous rate of appearance decreases for alle three doses of protein [35]. Therefore, although we are not sure if we have seen the peak level in e.g., exogenous R_a and protein net-balance in the SKEWED group when the measurements were stopped at 660 min, we would expect those to decrease in both the SKEWED and EVEN group. Furthermore, in the period after the measurements were stopped it is not expected that the exogenous R_a or the protein net-balance would stay elevated in the SKEWED group to a degree that would counteract the inferior exogenous R_a and net-balance that was seen compared to the EVEN group during the preceding 11 h.

The study setup with intravenous infusion of amino acid tracers required the subjects to be bedridden for most of the trial hours, only interrupted by two times 15 min of cycling. Therefore, the activity level during the trial was low. It is highly likely that the circulating EAA after either the EVEN or SKEWED intake saturated the need for MPS, within the state of low activity as seen for the older adults in the present study. Exercise is known to improve the amino acid sensitivity and MPS response of skeletal muscle [36–38]. In this respect, it would be highly relevant to elucidate if a higher level of physical activity or exercise for older adults determines if a beneficial effect of an even or skewed protein intake exists.

It is important to note that at all meals intrinsically labelled minced beef was served to being able to measure the endogenous uptake of meal-protein derived amino acids. Minced beef is known to be a high-quality protein with a digestibility that is comparable to milk and egg proteins [39,40]. Furthermore, minced meat has shown to be more rapidly digested and absorbed compared to beef steak [41]. Therefore, we do not believe that the restriction of the study to serve beef at all meals affects the interpretation or translation ability of the results.

5. Conclusion

In healthy older adults, fluctuations in whole-body protein net-balance were seen throughout a trial day in response to the amount of protein given at each of three main meals of an EVEN and SKEWED dietary protein distribution. A greater net-balance was seen with the EVEN distribution after breakfast and lunch compared to SKEWED and was the same in both groups after dinner. The difference in net-balance was driven by a difference in whole-body protein breakdown, which for an extended period was lower in EVEN. The muscle protein fractional synthesis rate over 9 h were not different when following the EVEN or SKEWED dietary protein distribution.

Authors' contributions

Jakob Agergaard: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing original draft, **Thomas Ehlig Hjermind Justesen:** Investigation, Project administration, Writing - review & editing, **Simon Elmer Jespersen:** Investigation, Project administration, Writing - review & editing, **Thomas Tagmose Thomsen:** Investigation, Project administration, Writing - review & editing, **Lars Holm:** Conceptualization, Methodology, Writing - review & editing, **Gerrit van Hall:** Formal analysis, Methodology, Supervision, Writing - review & editing.

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Conflict of interest

None of the authors have any conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2023.04.004.

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