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Sex differences in postprandial protein handling

# The muscle protein synthetic response to whey protein ingestion is greater in middle-aged women when compared with men

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**Rationale:** Muscle mass maintenance is largely regulated by the postprandial rise in muscle protein synthesis rates. It remains unclear whether postprandial protein handling differs between women and men.

**Methods:** Healthy men (43±3 y; BMI: 23.4±0.4 kg/m$^2$; *n*=12) and women (46±2 y; BMI: 21.3±0.5 kg/m$^2$; *n*=12) received primed continuous infusions of L-[ring-$^2H_5$]-phenylalanine and L-[ring-3,5-$^2H_2$]-tyrosine and ingested 25 g intrinsically L-[1-$^{13}$C]-phenylalanine-labeled whey protein. Blood samples and muscle biopsies were collected to assess dietary protein digestion and amino acid absorption kinetics as well as basal and postprandial myofibrillar protein synthesis rates.

**Results:** Plasma phenylalanine and leucine concentrations rapidly increased following protein ingestion (both P<0.001), with no differences between middle-aged women and men (Time×Sex *P*=0.307 and 0.529, respectively). The fraction of dietary protein-derived phenylalanine that appeared in the circulation over the 5 h postprandial period averaged 56±1 and 53±1% in the women and men, respectively (*P*=0.145). Myofibrillar protein synthesis rates increased (Time *P*=0.010) from 0.035±0.004 and 0.030±0.002 %/h in the post-absorptive state (t-test *P*=0.319) to 0.045±0.002 and 0.034±0.002 %/h in the 5 h postprandial phase in middle-aged women and men, respectively, with higher postprandial myofibrillar protein synthesis rates in women compared with men (t-test *P*=0.005). Middle-aged women showed a greater increase in myofibrillar protein synthesis rates during the early (0-2 h) postprandial period when compared with men (Time×Sex *P*=0.001).

**Conclusions:** There are no differences in post-absorptive myofibrillar protein synthesis rates between middle-aged women and men. The myofibrillar protein synthetic response to the ingestion of 25 g whey protein is greater in women when compared with men.

Contemporary stable isotope methodology was applied to show that women express higher postprandial muscle protein synthesis rates following ingestion of 25 g protein when compared to men.

## INTRODUCTION

Muscle mass maintenance is largely regulated by modulating post-absorptive and postprandial muscle protein synthesis rates. As there are obvious differences in body composition between men and women, with less muscle mass in women when compared with men (1,2), it has been speculated that this may be (partly) attributed to lower basal muscle protein synthesis rates in women when compared with men. However, the literature shows much discrepancy with studies showing similar (3-8) or even higher (7,9-11) post-absorptive muscle protein synthesis rates in women when compared with men. Since most studies indicate that there are no differences in post-absorptive muscle protein synthesis rates between women and men (3-8), we hypothesized that basal, post-absorptive muscle protein synthesis rates do not differ between women and men.

It has been well established that muscle protein synthesis rates are highly responsive to anabolic stimuli, such as physical activity and food intake (12). The skeletal muscle protein synthetic response to food ingestion is regulated on multiple levels, including dietary protein digestion and amino acid absorption (13), postprandial insulin release and subsequent microvascular perfusion (14,15), amino acid delivery to and uptake in skeletal muscle tissue



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(16), and intramyocellular anabolic signaling (17,18). Dietary protein digestion and amino acid absorption kinetics represent important processes modulating postprandial muscle protein accretion (19-21). Impairments in dietary protein digestion and amino acid absorption, or an increase in splanchnic amino acid retention, may attenuate the postprandial availability of amino acids for muscle protein synthesis (22). So far, no data are available on potential differences in dietary protein digestion and amino acid absorption kinetics between women and men, and only few studies have assessed potential sex differences in the postprandial muscle protein synthetic response to food intake (10,11). No differences in clamp-induced increases in muscle protein synthesis rates have been observed between women and men of various age groups under conditions of hyperinsulinemia and hyperaminoacidemia (6,7). In contrast, others have reported sex differences in the anabolic response to food intake in such a way that the meal-induced increase in muscle protein synthesis was greater in men than in women (10,11). Clearly, there is discrepancy in the literature on the proposed sex differences in postprandial protein handling. We hypothesize that postprandial muscle protein synthesis rates following the ingestion of a meal-sized amount of protein are lower in women when compared with men.

In the present study we compare post-absorptive myofibrillar protein synthesis rates, dietary protein digestion and amino acid absorption kinetics, as well as the postprandial muscle protein synthetic response to the ingestion of 25 g whey protein between middle-aged women and men. Primed continuous intravenous infusions of L-[ring-$^{2}H_5$]-phenylalanine and L-[ring-3,5-$^{2}H_2$]-tyrosine in combination with the ingestion of 25 g intrinsically L-[1-$^{13}$C]-phenylalanine-labeled whey protein were applied to allow assessment of basal myofibrillar protein synthesis rates as well as postprandial protein handling *in vivo* in healthy middle-aged women and men.

## METHODS

### Participants

Healthy, recreationally active women and men (age: 30-55 y; BMI: 19-25 kg/m$^2$) were recruited by advertisements in local newspapers. Participants' characteristics are presented in **Table 1**. Women were pre- or peri-menopausal; none of them had a post-menopausal status. None of the participants used medications known to affect protein digestion, amino acid absorption, or protein metabolism. Before written informed consent was obtained, all participants were informed about the purpose of the study, the experimental procedures, and possible risks involved with participation. This study was approved by the Medical Ethics Committee of Maastricht University Medical Centre+, and the procedures followed were in accordance with the latest version of the Declaration of Helsinki. The trial was conducted between May and October 2013 at Maastricht University Medical Centre+, the Netherlands and was part of a larger project on muscle protein metabolism in middle-aged individuals.

### Screening

At least 1 week before the experimental trial, participants underwent a medical screening. After an overnight fast, participants arrived at the laboratory at 08:30 h by car or public transportation. A fasting blood sample was obtained, after which a standard 75 g oral glucose tolerance test (OGTT) was performed to assess glucose tolerance. After the 2 h OGTT, blood pressure, body weight, body height, and body composition (by dual-energy X-ray absorptiometry, DXA; Hologic Inc., Discovery A, QDR series, Marlborough, USA) were assessed. Whole-body and regional lean mass and percent body fat was determined using the software package Apex version 2.3 (en-CORE 2005, version 9.15.00, Hologic, Marlborough, USA). Finally, a single slice CT scan (Philips Brilliance 64, Philips Medical Systems, Best, the Netherlands) was



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performed to assess *quadriceps* muscle cross-sectional area (CSA). The scanning characteristics were as follows: 120 kV, 300 mA, rotation time of 0.75 s, and a field of view of 500 mm. With subjects lying supine with their legs extended and feet secured, a 3 mm thick axial image was taken 15 cm proximal to the top of the patella. Muscle area was selected between -29 and +150 Hounsfield units (23), after which the *quadriceps* muscle was manually traced using ImageJ software (version 1.45d, National Institute of Health, Maryland, USA) (24-26). After the screening session, subjects were equipped with a triaxial physical activity monitor (Actigraph GTX3, Actigraph Inc., Pensacola, USA) worn on the hip over a 5 d period, providing information on the habitual physical activity level of the participants. Habitual physical activity was determined by the sum of accelerometer counts of a 24 h period. Analyses were carried out using Actilife software (version 6, Actigraph Inc., Pensacola, USA).

**Diet and physical activity prior to testing**

All volunteers were instructed to refrain from physical exercise and alcohol intake, and to keep their diet as constant as possible for 48 h prior to the experimental trial. All subjects received the same standardized meal on the evening prior to the experimental trial (2.3 MJ, composed of 19 energy percent protein, 40 energy percent carbohydrate, and 41 energy percent fat).

Participants' habitual energy intakes were calculated from 3-d dietary records and estimated with the use of the Harris-Benedict equation with a physical activity level of 1.5. The days of recording included 2 weekdays and 1 weekend day. Energy and macronutrient intakes were calculated with the use of the Dutch Nutrients Database (NEVO-online version 2013/4.0; http://nevo-online.rivm.nl/).

**Experimental trial**

A schematic overview of the experimental trial is provided in **Figure 1**. After an overnight fast, participants arrived at the laboratory by car or public transport at 08:00 h. A Teflon catheter was inserted into an antecubital vein for stable isotope infusion. A second Teflon catheter was inserted into a dorsal hand vein of the contralateral arm and placed in a hot box (60°C) for arterialized blood sampling (27). After taking a baseline blood sample (t=-210 min), the plasma phenylalanine and tyrosine pools were primed with a single dose of L-[ring-$^{2}H_{5}$]-phenylalanine (3.6 µmol/kg fat free mass (FFM)) and L-[ring-3,5-$^{2}H_{2}$]-tyrosine (1.1 µmol/kg FFM), after which continuous L-[ring-$^{2}H_{5}$]-phenylalanine (0.060 µmol/kg FFM/min) and L-[ring-3,5-$^{2}H_{2}$]-tyrosine (0.018 µmol/kg FFM/min) intravenous infusions were initiated (t=-210 min) using a calibrated IVAC 560 pump (San Diego, USA).

After resting in a supine position for 90 min, a second arterialized blood sample was drawn and the first muscle biopsy was collected from the *vastus lateralis* muscle (t=-120 min). Subsequent arterialized blood samples were collected at t=-90, -60, -30, and 0 min during the post-absorptive period with a second muscle biopsy taken from the same leg (new incision) at t=0 min, marking the end of the post-absorptive period. Immediately after the second biopsy, participants ingested a single bolus of 25 g intrinsically L-[1-$^{13}$C]-phenylalanine-labeled whey protein, signifying the beginning of a 5 h period for the determination of postprandial myofibrillar protein synthesis rates. Arterialized blood samples were then drawn at t=20, 40, 60, 90, 120, 150, 180, 240, and 300 min. A third and fourth muscle biopsy were collected from separate incisions in the other leg at t=120 and 300 min, respectively, to determine postprandial myofibrillar protein synthesis rates during the early (0-2 h) and entire (0-5 h) postprandial phase.

Blood samples were collected into EDTA-containing tubes and centrifuged at 1000*g* for 10 min at 4°C. Aliquots of plasma were frozen in liquid nitrogen and stored at -80°C. Muscle biopsy



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samples were obtained from the middle region of the *m. vastus lateralis*, ~15 cm above the patella and ~3 cm below entry through the fascia, using the percutaneous needle biopsy technique (28). Muscle samples were dissected carefully and freed from any visible non-muscle material and were immediately frozen in liquid nitrogen and stored at -80 °C until further analysis.

**Preparation of intrinsically labeled protein and beverage composition**

Intrinsically L-[1-$^{13}$C]-phenylalanine-labeled whey protein was prepared by infusing L-[1-$^{13}$C]-phenylalanine into a lactating Holstein cow, collecting milk, and purifying the whey fraction as described previously (29-31). The enrichment of L-[1-$^{13}$C]-phenylalanine in the whey protein was 7.6 mole percent excess (MPE). The whey protein met all chemical and bacteriologic specifications required for human consumption. Participants received a beverage containing 25 g whey protein (containing 2.3 g leucine) dissolved in 350 mL water flavored with 1.5 mL vanilla (IMCD Benelux N.V., Rotterdam, the Netherlands).

**Plasma analyses**

Plasma glucose concentrations were analyzed using enzymatic assays on an automated spectrophotometer (ABX Pentra 400 autoanalyzer, Horiba ANX, Montpellier, France). Plasma insulin concentrations were determined with radioimmunoassay (RIA) kits (Human Insulin specific RIA, Millipore Corporation, MA, USA). Plasma amino acid concentrations and enrichments were determined by gas chromatography-mass spectrometry (GC-MS; Agilent 7890A GC/5975C; MSD, Little Falls, USA). Specifically, the internal standards [U-$^{13}C_6$]-leucine, [U-$^{13}C_9$ $^{15}$N]-phenylalanine, and [U-$^{13}C_9$ $^{15}$N]-tyrosine were added to the plasma sample. The plasma was deproteinized with 5-sulfosalicylic acid. Free amino acids were purified using cation exchange AG 50W-X8 resin columns (mesh size: 100-200 µm, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA, USA). The purified amino acids were converted into *N-tert*-Butyldimethylsilyl derivatives using MTBSTFA before analysis by GC-MS. The amino acid concentrations were determined using electron impact ionization by monitoring ions at mass/charge (m/z) 302 and 308 for unlabeled and [U-$^{13}C_6$]-leucine respectively, 336 and 346 for unlabeled and [U-$^{13}C_9$ $^{15}$N]-phenylalanine, and 466 and 476 for unlabeled and [U-$^{13}C_9$ $^{15}$N]-tyrosine, respectively. The plasma phenylalanine and tyrosine $^{13}$C and $^{2}$H enrichments were determined using selective ion monitoring m/z 336, 337, and 341 for unlabeled and labeled (1-$^{13}$C and ring-$^{2}H_5$) phenylalanine, respectively, and m/z 466, 467, 468, and 470 for unlabeled and labeled (1-$^{13}$C, ring-3,5-$^{2}H_2$, and ring-$^{2}H_4$) tyrosine, respectively. Standard regression curves from a series of known standard enrichment values against the measured values were applied to assess linearity of the mass spectrometer and to account for isotopic fractionation that may have occurred during analysis. Phenylalanine and tyrosine enrichments were corrected for the presence of both the $^{13}$C and $^{2}$H isotopes.

**Muscle analyses**

Myofibrillar protein-enriched fractions were extracted from ~50 mg of wet muscle by hand-homogenizing in 7 µL/mg homogenization buffer using a Teflon pestle. Samples were centrifuged at 2000*g* for 5 min at 4ºC. The pellet was washed with 500 µL dd$H_2O$ and centrifuged at 200*g* for 10 min at 4ºC. The myofibrillar proteins in the pellet were solubilized by adding 1 mL of 0.3 M NaOH and heating at 50ºC for 30 min with vortex mixing every 10 min. Samples were centrifuged at 9500*g* for 5 min at 4ºC. The supernatant containing the myofibrillar proteins was collected and the collagen pellet was discarded. Myofibrillar proteins were precipitated by adding 1 mL of 1 M PCA and centrifuging at 400*g* for 10 min at 4ºC. The



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myofibrillar protein pellet was washed twice with 1 mL of 70% ethanol and hydrolyzed overnight in 2 mL of 6 M HCl at 110°C for 18 h. The free amino acids from the hydrolyzed myofibrillar protein pellet were dried under nitrogen stream while being heated to 120°C. The dried amino acids were then dissolved in 25% acetic acid solution, passed over cation exchange AG 50W-X8 resin columns (mesh size: 100-200 µm, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA, USA), eluted with 2 M $NH_4OH$, and dried under nitrogen stream. The L-[ring-$^2H_5$]-phenylalanine enrichments in the purified amino acids were determined by GC-MS analysis. To reduce the signal-to-noise ratio during GC-MS analysis at low tracer enrichments, phenylalanine was enzymatically decarboxylated to phenylethylamine prior to derivatization with MTBSTFA.

L-[ring-$^2H_5$]-phenylalanine enrichments of the myofibrillar protein-enriched fractions were determined by selected ion monitoring for phenylalanine mass to charge ratio at 183 ($m$ + 5) to 180 ($m$ + 2) and a single linear standard curve (to avoid slope influences on the measured TTR) from mixtures of known $m$ + 5 to $m$ + 0 ratios. To avoid saturation of the MS and eliminate bias due to any potential concentration dependencies (22), the split ratio was adjusted prior to the injection of each sample so that nearly equal amounts of phenylalanine were injected for all samples and standards. The derivatized amino acids were separated on a 30 m × 0.25 mm × 0.50 µm HP-5MS column (temperature program: 80˚C for 1 min; 30˚C·min$^{-1}$ ramp to 300˚C; hold for 10 min Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation that may have occurred during the analysis.

**Calculations**

Ingestion of L-[1-$^{13}$C]-phenylalanine-labeled protein, intravenous infusion of L-[ring-$^2H_5$]-phenylalanine and L-[ring-3,5-$^2H_2$]-tyrosine, and arterialized blood sampling were used to assess whole-body amino acid kinetics in non-steady state conditions. Total, exogenous, and endogenous rates of phenylalanine appearance ($R_a$, in µmol/kg/min) and plasma availability of dietary protein-derived phenylalanine (i.e., the fraction of dietary protein-derived phenylalanine that appeared in the systemic circulation, $Phe_{plasma}$) were calculated using modified Steele's equations (32,33). These parameters were calculated as follows:

$$Total\,R_a = \frac{F_{iv} - \left[ pV \times C(t) \times \frac{dE_{iv}}{dt} \right]}{E_{iv}(t)} \quad (1)$$

$$Exo\,R_a = \frac{Total\,R_a \times E_{po}(t) + \left[ pV \times C(t) \times \frac{dE_{po}}{dt} \right]}{E_{prot}} \quad (2)$$

$$Endo\,R_a = Total\,R_a - Exo\,R_a - F_{iv} \quad (3)$$

$$Phe_{plasma} = \left( \frac{AUC_{Exo\,Ra}}{Phe_{prot}} \right) \times BW \times 100\% \quad (4)$$

where $F_{iv}$ is the intravenous tracer infusion rate (µmol/kg/min); $pV$ (0.125 L/kg) is the distribution volume (32); $C(t)$ is the mean plasma phenylalanine concentration between two consecutive time points; $dE_{iv}/dt$ is the time-dependent variation of plasma phenylalanine



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enrichment derived from the intravenous tracer; and $E_{iv}(t)$ is the mean plasma phenylalanine enrichment derived from the intravenous tracer between two consecutive time points. *Exo* $R_a$ represents the rate at which dietary protein-derived phenylalanine enters the circulation. $E_{po}(t)$ is the mean plasma phenylalanine enrichment derived from the oral tracer between two consecutive time points; $dE_{po}/dt$ is the time-dependent variation of plasma phenylalanine enrichment derived from the oral tracer; and $E_{prot}$ is the L-[1-$^{13}$C]-phenylalanine enrichment of the dietary protein. $AUC_{Exo\ Ra}$ represents the area under the curve (AUC) of *Exo* $R_a$, which corresponds to the amount of dietary protein-derived phenylalanine that appeared in the circulation over the 5-h postprandial period; $Phe_{prot}$ is the amount of dietary phenylalanine ingested; and *BW* is the participant's body weight in kg.

Total phenylalanine rate of disappearance ($R_d$) represents the rate of phenylalanine hydroxylation (first step in phenylalanine oxidation) plus the rate of phenylalanine utilization for protein synthesis. This parameter is calculated as follows:

$$Total\ R_d = Total\ R_a - pV \times \frac{dC}{dt} \quad (5)$$

$$Phe\ hydroxylation = Tyr\ R_a \times \frac{E_{Tyr}(t)}{E_{Phe}(t)} \times \frac{Phe\ R_d}{\left(F_{Phe} + Phe\ R_d\right)} \quad (6)$$

$$Protein\ synthesis = Total\ R_d - Phe\ hydroxylation \quad (7)$$

$$Phe\ net\ balance = Protein\ synthesis - Endo\ R_a \quad (8)$$

where $dC/dt$ is the time-dependent variation of plasma phenylalanine concentrations. *Tyr* $R_a$ is the total rate of tyrosine appearance based on the L-[ring-3,5-$^2H_2$]-tyrosine infusion and plasma enrichments. $E_{Tyr}(t)$ and $E_{Phe}(t)$ represent the mean plasma L-[ring-$^2H_4$]-tyrosine and L-[ring-$^2H_5$]-phenylalanine enrichment between 2 consecutive time points, respectively, *Phe* $R_d$ is the total phenylalanine rate of disappearance, and $F_{Phe}$ is the intravenous infusion rate of L-[ring-$^2H_5$]-phenylalanine (µmol/kg/min).

Myofibrillar protein fractional synthetic rates (FSR) were calculated using the standard precursor-product equation:

$$FSR = \frac{\Delta E_p}{E_{precursor} \cdot t} \cdot 100\% \quad (9)$$

where $\Delta E_p$ is the increment in myofibrillar protein-bound L-[ring-$^2H_5$]-phenylalanine enrichment after an incorporation period (MPE), $E_{precursor}$ is the weighted mean plasma L-[ring-$^2H_5$]-phenylalanine enrichment during that incorporation period (in MPE), and *t* is the incorporation period (h). For basal FSR, muscle biopsies at t=-120 and 0 min were used, and for postprandial FSR, biopsies at t=0, 120, and 300 min were used.

**Statistics**

All data are expressed as mean ± SEM, except for the descriptive measures in Tables 1 and 2, where SDs are used. Student's t-tests were applied to identify differences between sex at baseline and for 0-5 h muscle protein synthesis rates. For plasma and muscle time curves, repeated-measures ANOVAs with sex (as between-group factor), time (as within-group factor), and their interaction were used to identify differences between sex over time. Statistical significance was set at $P<0.05$. All calculations were performed using IBM SPSS Statistics (version 25, IBM Corp., Armonk, USA).



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## RESULTS

### Body composition

Fat percentage was higher in middle-aged women when compared with men (28.2±1.2 and 21.3±1.0%, respectively; $P$<0.001). Lean body mass (42.1±1.1 and 57.7±1.6 kg; $P$<0.001) and leg lean mass (6.9±0.2 and 9.6±0.3 kg; $P$<0.001) were lower in women compared with men, respectively. *Quadriceps* muscle CSA was also significantly lower in women compared with men (5384±192 and 7912±301 $mm^2$, respectively; $P$<0.001; **Table 1**).

### Plasma metabolites

Plasma glucose concentrations declined over time from 5.2 to 4.6 mmol/L ($P$<0.001) during the experimental trial, with no differences between women and men (Time×Sex $P$=0.551; **Figure 2A**). Plasma insulin concentrations increased after protein intake in both groups (Time $P$<0.001), with no differences between women and men (Time× Sex $P$=0.770; Figure 2B). Peak plasma insulin concentrations averaged 182±19 and 155±18 pmol/L in women and men, respectively ($P$=0.312). Both plasma phenylalanine and leucine concentrations rapidly increased following protein ingestion (both $P$<0.001), and did not differ between women and men (Time× Sex $P$=0.307 and 0.529, respectively; **Figure 3**).

### Plasma tracers

Plasma L-[ring-$^2H_5$]-phenylalanine enrichments are shown in **Figure 4**. The plasma L-[ring-$^2H_5$]-phenylalanine enrichments were lower in women when compared with men over the course of the experiment (Sex $P$=0.001). Plasma L-[ring-$^2H_5$]-phenylalanine enrichments transiently declined following protein ingestion in both groups (Time $P$<0.001), with no differences between women and men over time (Time× Sex $P$=0.533; Figure 4A). Plasma L-[1-$^{13}C$]-phenylalanine enrichments rapidly increased following protein ingestion ($P$<0.001), and were higher in women when compared with men (Sex $P$<0.001; Figure 4B).

### Whole-body amino acid kinetics and metabolism

**Figure 5** shows whole-body amino acid kinetics. Exogenous phenylalanine appearance rates (i.e., the rate at which dietary protein-derived phenylalanine enters the circulation) rapidly increased following protein ingestion (Time $P$<0.001). Exogenous phenylalanine appearance rates did not differ significantly between sexes over time (Time× Sex $P$=0.090; Figure 5A), but overall appearance was greater in women compared with men (Sex $P$<0.001). During the first 2 hours after protein ingestion 42±1 vs 41±1% of the dietary protein-derived phenylalanine had appeared in the circulation in women and men, respectively ($P$=0.287). Over the entire 5 h postprandial period, this was 56±1 vs 53±1% in women and men, respectively (Sex $P$=0.145). This represented 14.0±0.3 vs 13.3±0.3 g of the ingested dietary protein-derived amino acids in women and men, respectively ($P$=0.145; **Figure 6**). Endogenous phenylalanine appearance rates (i.e., the rate at which phenylalanine derived from whole-body protein breakdown enters the circulation) declined following protein ingestion (Time $P$<0.001) to a similar extent in men and women (Time× Sex $P$=0.335; Figure 5B). Protein ingestion increased total phenylalanine appearance rates in both groups (Time $P$<0.001) with no significant differences between sexes over time (Time× Sex $P$=0.113; Figure 5C). Total phenylalanine disappearance rates increased to a greater extent in women when compared with men (Time× Sex $P$=0.044; Figure 5D). Protein ingestion also increased total tyrosine appearance rates in both groups (Time $P$<0.001) with no significant differences between sexes over time (Time×Sex $P$=0.095; Figure 5E).

Basal and postprandial whole-body protein breakdown, synthesis, oxidation, and net balance are shown in **Figure 7**. Regardless of sex, protein ingestion decreased whole-body protein



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breakdown rates and increased whole-body protein synthesis rates, resulting in a more positive whole-body net protein balance when compared with the basal, post-absorptive state (Time $P<0.001$). Whole-body protein breakdown rates (Time×Sex $P=0.122$) and protein synthesis rates (Time×Sex $P=0.202$) did not differ between sexes over time. Oxidation rates and whole-body net protein balance increased to a greater extent in women than in men over time (Time×Sex $P=0.007$ and $P<0.001$, respectively).

**Muscle protein synthesis**

Myofibrillar protein fractional synthetic rates (FSR) were calculated based on the incorporation of L-[ring-$^2H_5$]-phenylalanine into myofibrillar protein and the weighted mean plasma L-[ring-$^2H_5$]-phenylalanine enrichments as precursor pool (**Figure 8**). Basal myofibrillar protein synthesis rates did not differ between middle-aged women and men and averaged 0.035±0.004 and 0.030±0.002 %/h, respectively (t-test $P=0.319$). Ingesting 25 g whey protein significantly increased myofibrillar protein synthesis rates (Time $P=0.010$). Middle-aged women showed a greater increase in myofibrillar protein synthesis rate (to 0.061±0.004 %/h) during the early (0-2 h) postprandial period when compared with men (to 0.031±0.002 %/h) (Time×Sex $P=0.001$). Postprandial myofibrillar protein synthesis rates assessed over the entire 5 h postprandial period averaged 0.045±0.002 and 0.034±0.002 %/h in the women and men, respectively, which was 30% higher in the women compared with the men (t-test $P=0.005$).

**Dietary intake and physical activity**

Dietary intake and physical activity data are presented in **Table 2.** Energy intake averaged 7.4±0.6 and 10.8±0.8 MJ/d in women and men, respectively ($P=0.003$). Habitual protein intake was lower in women when compared with men (69±7 vs 92±8 g/day, respectively; $P=0.04$), with no differences when expressed per kg BW (1.1±0.1 vs 1.2±0.1 g/kg BW/d; $P<0.05$). Women tended to have a lower step count than men (8796±590 vs 12574±1783 steps per day; $P=0.066$). The absolute time spent in sedentary and moderate-to-vigorous activities per day was lower in women (521±27 and 45±8 min, respectively) when compared with men (544±28 and 58±9, respectively; $P<0.05$), while the time spent performing light activities per day was higher in women (309±16 vs 288±23 min; $P<0.05$). The relative time spent performing sedentary, light, and moderate-to-vigorous physical activity did not differ between sexes ($P>0.05$).

## DISCUSSION

In the present study, we observed that post-absorptive myofibrillar protein synthesis rates did not differ between middle-aged women and men. Upon the ingestion of 25 g whey protein, protein digestion and absorption kinetics did not differ between sexes, with ~55% of the protein-derived amino acids appearing in the circulation over a 5 h postprandial period. The subsequent postprandial myofibrillar protein synthetic response, however, was substantially greater in middle-aged women when compared with men.

Muscle mass maintenance is largely regulated by post-absorptive muscle protein synthesis rates and the ability to increase muscle protein synthesis after protein ingestion. Consequently, it could be suggested that lower muscle mass in women compared with men may be associated with lower post-absorptive muscle protein synthesis rates in women. Although higher (~30%) basal MPS rates have been observed in older (65-80 y) post-menopausal obese women compared with men (10,11), most studies indicate that there are no differences in post-absorptive muscle protein synthesis rates between men and pre- and peri-menopausal women (3-8). In the present study, we confirm these findings as post-absorptive myofibrillar protein synthesis rates averaged



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0.035±0.004 and 0.030±0.002 %/h in middle-aged pre- or peri-menopausal women and middle-aged men, respectively (*P*=0.319; Figure 8). Clearly, lower muscle mass in women compared with men cannot be attributed to sex differences in basal, post-absorptive myofibrillar protein synthesis rates.

Besides post-absorptive muscle protein synthesis, skeletal muscle mass is also modulated by the capacity to increase muscle protein synthesis rates following food intake (22). The stimulation of muscle protein synthesis rates after protein ingestion is dependent on a cascade of processes, including dietary protein digestion, amino acid absorption, and splanchnic amino acid retention, which all regulate the postprandial availability of dietary protein-derived amino acids for muscle protein synthesis (19,20,34). In the current study, we applied continuous infusion of L-[ring-$^{2}H_{5}$]-phenylalanine in combination with the ingestion of intrinsically L-[1-$^{13}$C]-phenylalanine-labeled whey protein to assess dietary protein digestion and amino acid absorption kinetics. The rate of appearance of dietary protein-derived phenylalanine increased rapidly in both sexes following the ingestion of 25 g whey protein (Figure 5A). From the exogenous phenylalanine appearance rates depicted in Figure 5A, it is evident that the amount of protein-derived phenylalanine appearing in the circulation is greater in the middle-aged women compared with the men. However, this is simply attributed to the expression of phenylalanine appearance rates per kg body weight. When calculated over the 5 h postprandial period, 56±1% (14.0±0.3 g) and 53±1% (13.3±0.3 g) of the ingested dietary protein-derived amino acids had become available in the circulation in the women and men, respectively (*P*=0.145; Figure 6). This is the first study to compare dietary protein digestion and amino acid absorption kinetics *in vivo* between sexes, showing no significant differences between middle-aged women and men.

Dietary protein-derived amino acids in the circulation are subsequently taken up by tissues, thereby stimulating protein synthesis and inhibiting protein breakdown. This allows whole-body net protein balance to become more positive. We indeed observed a positive whole-body net protein balance following protein ingestion in both women and men (Figure 7). However, postprandial whole-body amino acid kinetics do not necessarily reflect changes in protein metabolism at the muscle tissue level. Therefore, muscle biopsies were collected 2 and 5 h after protein ingestion to assess early and more prolonged postprandial changes in myofibrillar protein synthesis rates (Figure 8). We observed an increase in postprandial myofibrillar protein synthesis rates following the ingestion of 25 whey protein when compared with basal, post-absorptive values (*P*=0.010). Women showed a greater increase in myofibrillar protein synthesis rates during the early (0-2 h) postprandial period when compared with men (0.061±0.004 vs 0.031±0.002 %/h in women and men, respectively; Time×Sex *P*=0.001; Figure 8). In addition, postprandial muscle protein synthesis rates assessed over the entire 5 h postprandial period was greater in women compared with men (0.045±0.002 and 0.034±0.002 %/h, respectively; t-test *P*=0.005; Figure 8). These data are in line with one study using a mixed meal in older obese individuals (11), but not with studies using clamp techniques (6,7), which do not represent a normal physiological response to the ingestion of a single meal-like amount of dietary protein.

Our data clearly show that middle-aged women have a greater myofibrillar protein synthetic response than men when ingesting the same meal-sized amount of (whey) protein. We can only speculate on reasons for the greater postprandial muscle protein synthetic response in women compared with men. First, we provided the women and men with the same absolute amount of whey protein. Though the same amount of protein-derived amino acids were released in the circulation after splanchnic sequestration (with 14.0±0.3 and 13.3±0.3 g being released over the 5 h postprandial period in the women and men, respectively), this resulted in more amino acids



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provided per kg lean mass in the women when compared with the men. With a total lean body mass of 42±1 and 58±2 kg in the women and men, respectively, the women received ~40% more dietary protein-derived amino acids when expressed per kg lean mass (0.33±0.01 vs 0.23±0.01 g amino acids per kg lean mass, respectively; $P<0.001$). Consequently, the greater relative amino acid provision to skeletal muscle tissue in the women compared with the men could be responsible for the more rapid and overall greater muscle protein synthetic response observed in the women. Of course, other factors such as habitual physical activity levels and hormonal environment could be responsible for a greater anabolic sensitivity in middle-aged women compared with men (20,22). However, our measurements of habitual physical activity using accelerometers showed no significant differences in daily physical activity level between sexes ($P>0.05$). Though the impact of circulating androgen levels on basal or postprandial muscle protein synthesis rates remains highly debated (8,9,35-37), they do not seem to be responsible for the differences in postprandial protein handling between middle-aged men and women.

The present findings show that middle-aged women have a greater myofibrillar protein synthetic response compared with men when ingesting the same amount of protein. This implies that women require less dietary protein to induce a similar muscle protein synthetic response when compared with men. These findings are of relevance for a variety of topics, including sports nutrition, nutritional support during energy intake restriction, and protein requirements for the aging population or during illness or inactivity. Clearly, sexual dimorphism in relation to postprandial protein handling should be taken into account when defining nutritional guidelines to support muscle mass maintenance or muscle hypertrophy in women. Therefore, more research will be required to assess relevant differences in dietary protein requirements and anabolic responses to protein ingestion between women and men.

In conclusion, there are no differences in post-absorptive myofibrillar protein synthesis rates between middle-aged men and women. The myofibrillar protein synthetic response to the ingestion of 25 g whey protein is greater in women when compared with men. Therefore, women will generally require less dietary protein to induce a similar myofibrillar protein synthetic response when compared with men.

**Acknowledgements**

JWvD, HMH, and LJCvL designed the study. IWKK and JWvD conducted the clinical experiments. IWKK and JvK performed the plasma and muscle stable isotope analyses. BBLG performed the medical procedures. AMHH and SHMG performed the (statistical) data analysis. AMHH and LJCvL wrote the manuscript and had primary responsibility for the final content. None of the authors had any personal or financial conflict of interest. All authors read and approved the final manuscript.

**Clinical trial registration number:** NTR4060 (www.trialregister.nl)

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collection and analysis, decision to publish, and preparation of the manuscript. The industrial partners have contributed to the project through regular discussion.

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**Figure 1**: Schematic representation of the outline of the study protocol.

**Figure 2**: Plasma glucose (A) and insulin (B) concentrations (mmol/Land pmol/L, respectively) in healthy women and men (*n*=12/group) during the fasting period and after ingestion of 25 g whey protein. Values represent mean ± SEM. A: Time *P*<0.001, Time×Sex *P*=0.551. B: Time *P*<0.001, Time×Sex *P*=0.770.

**Figure 3**: Plasma phenylalanine (A) and leucine (B) concentrations (µmol/L) after the ingestion of 25 g whey protein in healthy women and men (*n*=12/group). Values represent mean ± SEM. A: Time *P*<0.001, Time×Sex *P*=0.307. B: Time *P*<0.001, Time×Sex *P*=0.529.

**Figure 4**: Plasma L-[ring-$^{2}H_{5}$]-phenylalanine (A) and L-[1-$^{13}$C]-phenylalanine (B) enrichments (MPE) during the post-absorptive state and after the ingestion of 25 g whey protein in healthy women and men (*n*=12/group). Values represent mean ± SEM. A: Time *P*<0.001, Sex *P*=0.001, Time×Sex *P*=0.533. B: Time *P*<0.001, Sex *P*<0.001, Time×Sex *P*=0.098.

**Figure 5**: Whole-body amino acid kinetics. Exogenous rate of appearance (Ra; A), endogenous Ra (B), total Ra (C) and total rate of phenylalanine disappearance (Rd; D) (µmol phenylalanine/kg/min), and total tyrosine Ra (µmol tyrosine/kg/min) in women and men (*n*=12/group) during the post-absorptive period and after ingesting 25 g whey protein is shown. Values represent mean ± SEM. A: Time *P*<0.001, Sex *P*<0.001, Time×Sex *P*=0.090. B: Time *P*<0.001, Sex *P*=0.531, Time×Sex *P*=0.335. C: Time *P*<0.001, Sex *P*=0.055, Time×Sex *P*=0.113. D: Time *P*<0.001, Sex *P*=0.063, Time×Sex *P*=0.044. E: Time *P*<0.001, Sex *P*=0.019, Time×Sex *P*=0.095.

**Figure 6**: The amount (g) of dietary protein-derived phenylalanine that appeared in the plasma over the early 2 h and entire 5 h postprandial period following ingestion of 25 g whey protein in



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healthy women and men (*n*=12/group). Values represent mean ± SEM. 0-2 h: *P*=0.287; 0-5 h: *P*=0.145.

**Figure 7**: Whole-body protein breakdown, synthesis, oxidation, and net balance during the post-absorptive period and after the ingestion of 25 g whey protein in healthy women and men (*n*=12/group). Values represent mean ± SEM. Breakdown: Time *P*<0.001, Sex *P*=0.465, Time×Sex *P*=0.122. Synthesis: Time *P*<0.001, Sex *P*=0.132, Time×Sex *P*=0.202. Oxidation: Time *P*<0.001, Sex *P*=0.874, Time×Sex *P*=0.007. Net Balance: Time *P*<0.001, Sex *P*=0.010, Time×Sex *P*<0.001.

**Figure 8**: Myofibrillar protein fractional synthetic rates (FSR) assessed using intravenous L-[ring-$^{2}H_5$]-phenylalanine infusions during the post-absorptive state (Basal) and over the early (0-2 h; Time *P*<0.001, Sex *P*<0.001, Time×Sex *P*=0.001) and entire (0-5 h; Time *P*=0.010, Sex *P*=0.029, Time×Sex *P*=0.260) postprandial period after the ingestion of 25 g whey protein in healthy women and men (*n*=12 per group). Values represent mean ± SEM. # significantly different between men and women.

**Table 1.** Subjects' characteristics

| | Women (*n*=12) | Men (*n*=12) |
|---|---|---|
| Age, y | 46 ± 7 | 43 ± 9 |
| Weight, kg | 61.4 ± 6.3 * | 76.5 ± 5.6 |
| BMI, kg/m$^2$ | 21.3 ± 1.6 * | 23.4 ± 1.5 |
| Diastolic blood pressure, mmHg | 68 ± 11 | 73 ± 9 |
| Systolic blood pressure, mmHg | 116 ± 12 | 125 ± 16 |
| Fat, % | 28.2 ± 4.0 * | 21.3 ± 3.4 |
| LBM, kg | 42.1 ± 3.8 * | 57.7 ± 5.5 |
| Leg lean mass, kg | 6.9 ± 0.8 * | 9.6 ± 1.2 |
| Quadriceps CSA, mm$^2$ | 5384 ± 665 * | 7912 ± 1042 |
| $HbA_{1c}$, % | 5.4 ± 0.4 | 5.2 ± 0.3 |
| OGIS, mL·min$^{-1}$·m$^{-2}$ | 472 ± 53 | 451 ± 42 |

Abbreviations: BMI, body mass index; CSA, cross-sectional area; $HbA_{1c}$, glycated hemoglobin; OGIS, oral glucose insulin sensitivity. Values are mean ± SD. Data were analyzed using unpaired Student's *t*-tests. * Significant difference between sex, *P*<0.005.

**Table 2.** Habitual dietary intake and physical activity

| | Women (*n*=12) | Men (*n*=12) |
|---|---|---|
| Energy intake (MJ·d$^{-1}$) | 7.4 ± 1.9 * | 10.8 ± 2.8 |
| Carbohydrate/Protein/Fat (energy percent) | 44/16/33 | 47/15/33 |
| Protein intake (g·kg$^{-1}$·d$^{-1}$) | 1.1 ± 0.3 | 1.2 ± 0.3 |
| Time accelerometer worn·d$^{-1}$ (min) | 875 ± 82 | 890 ± 74 |
| Total steps·d$^{-1}$ (steps) | 8796 ± 1956 ^ | 12574 ± 6178 |
| Sedentary time·d$^{-1}$ (min) | 521 ± 94 * | 544 ± 96 |
| Light intensity activity time·d$^{-1}$ (min) | 309 ± 55 * | 288 ± 79 |
| MVPA time·d$^{-1}$ (min) | 45 ± 28 * | 58 ± 32 |
| %·24 h$^{-1}$ sedentary activities | 59 ± 8 | 61 ± 9 |
| %·24 h$^{-1}$ light activities | 36 ± 5 | 32 ± 7 |
| %·24 h$^{-1}$ MVPA | 6 ± 4 | 7 ± 3 |

Values are mean ± SD. En%: energy %, MVPA: moderate-to-vigorous physical activity. Data were analyzed by using unpaired Student's *t*-tests, * Significant difference between sex, *P*<0.05. ^ Trend for difference between sex *P*=0.066.



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**Figure 2**

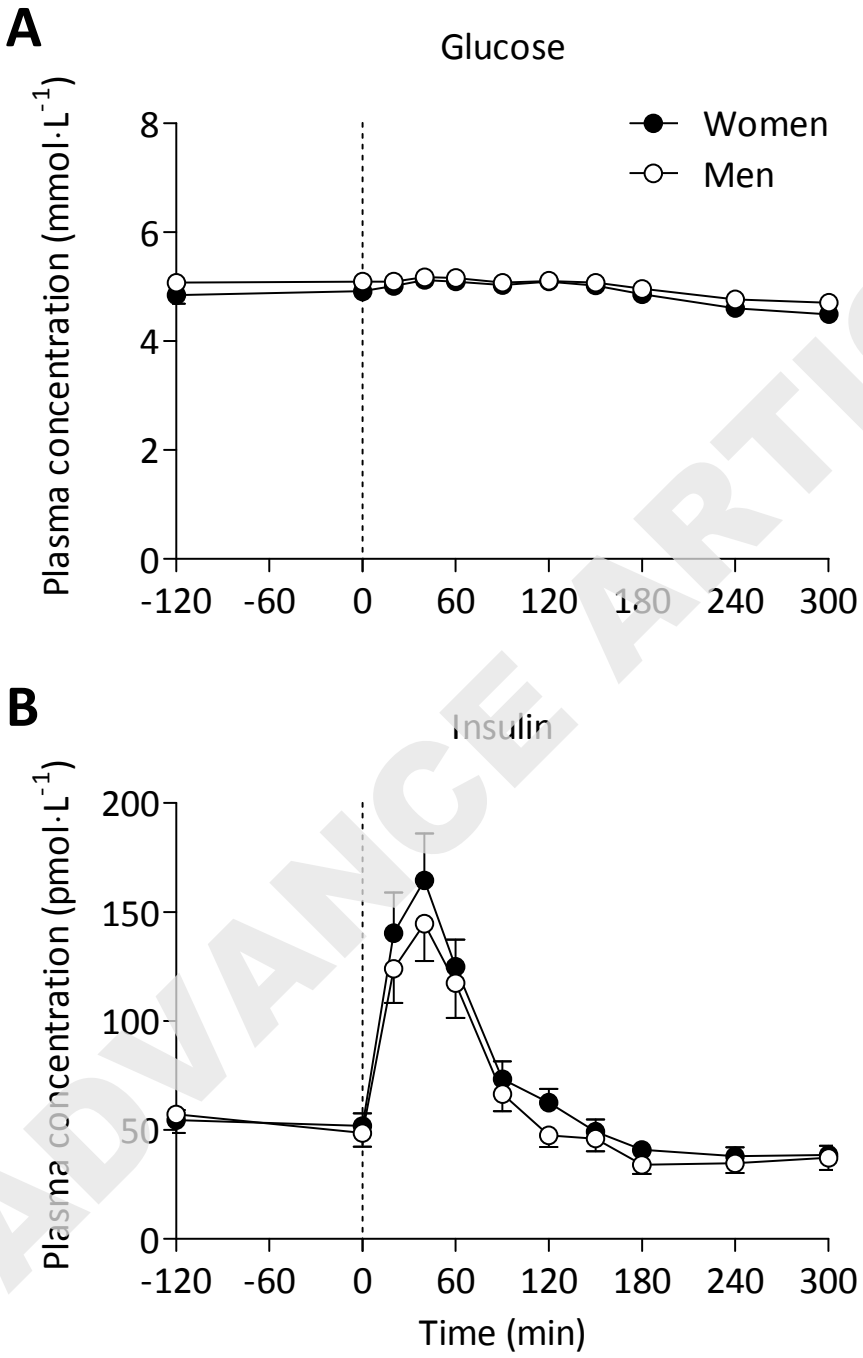


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**Figure 3**

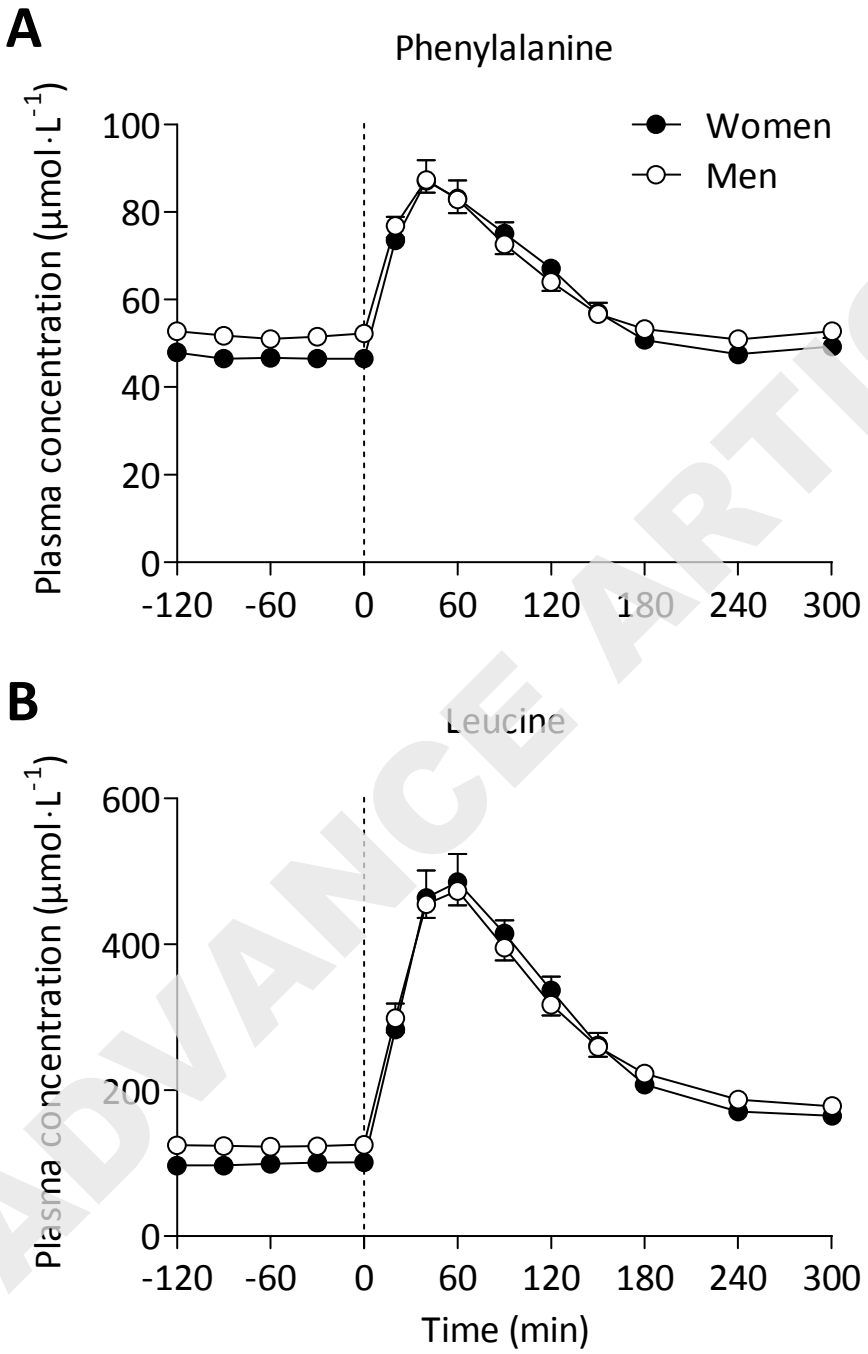


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**Figure 4**

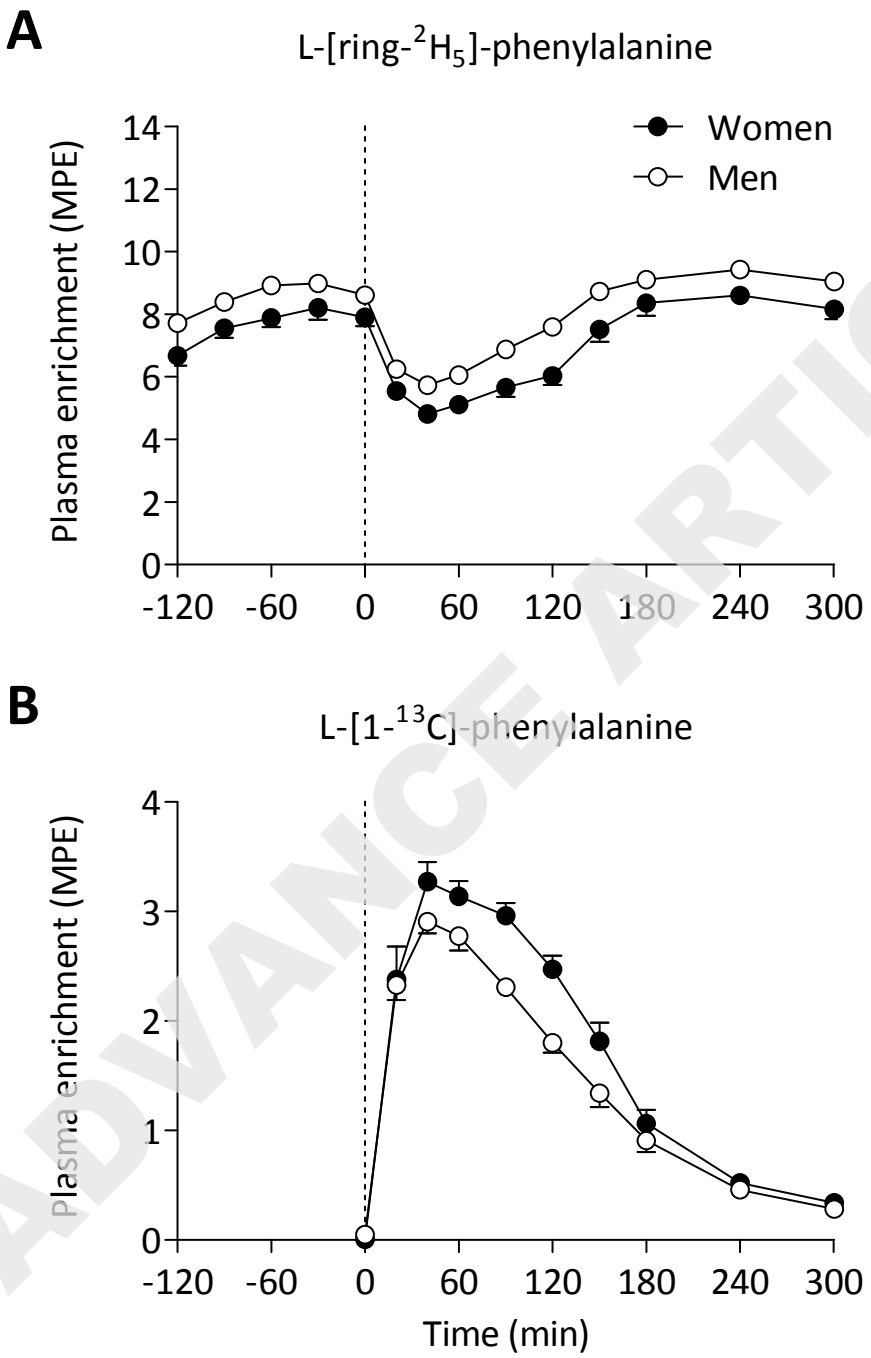


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**Figure 5**

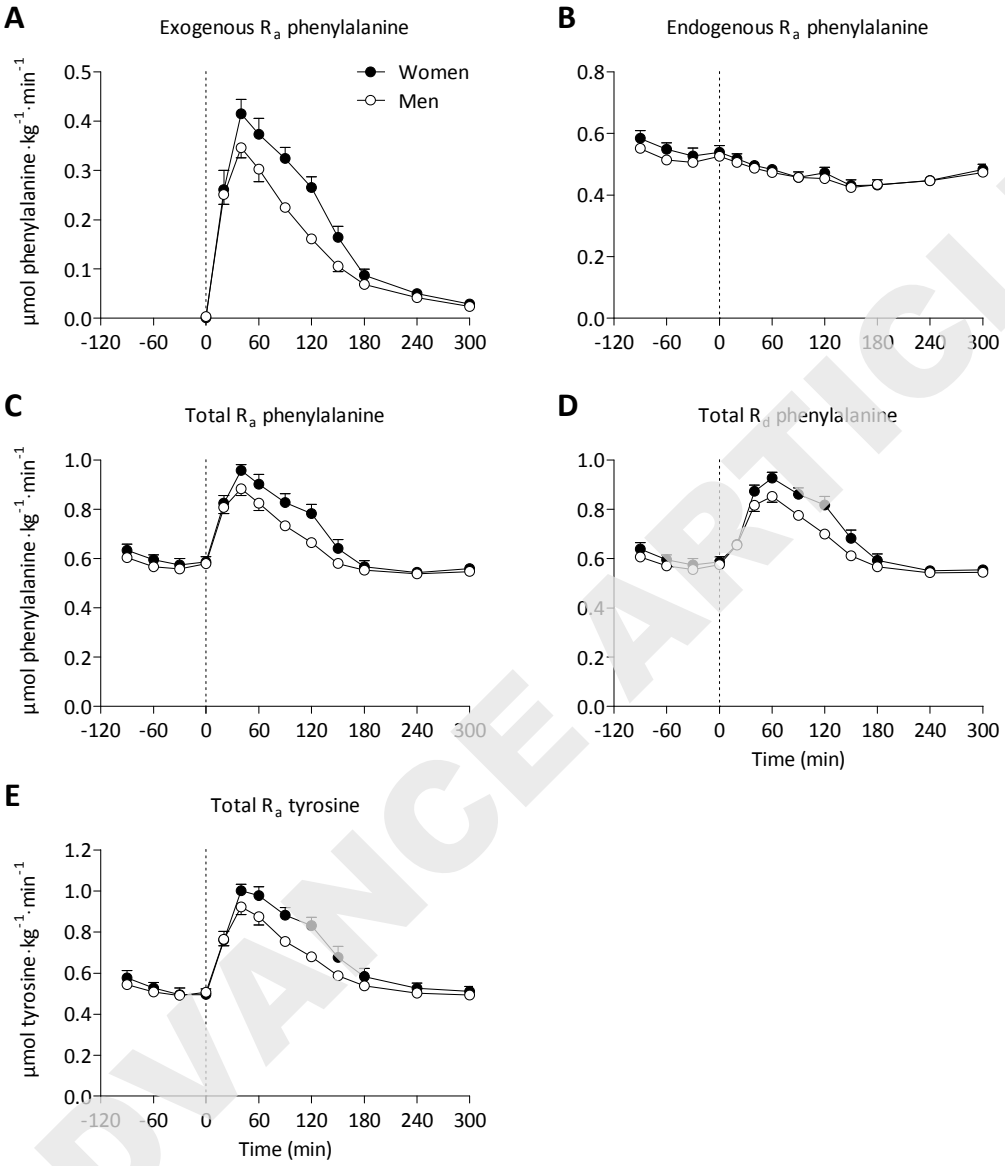


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**Figure 6**

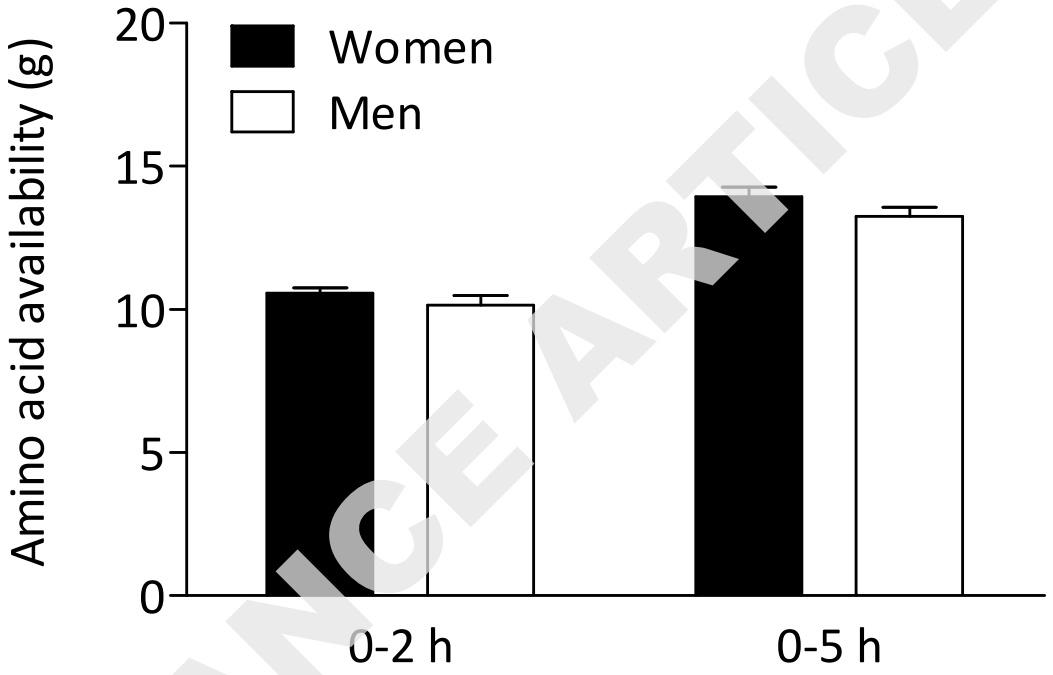


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**Figure 7**

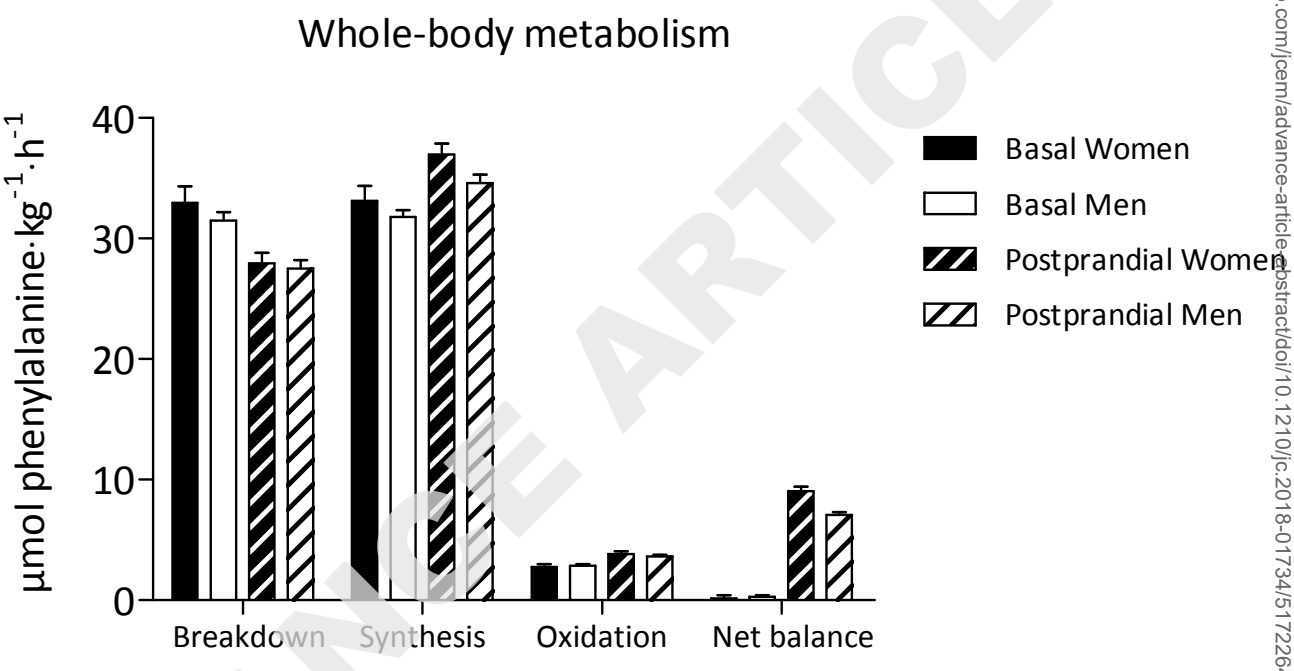


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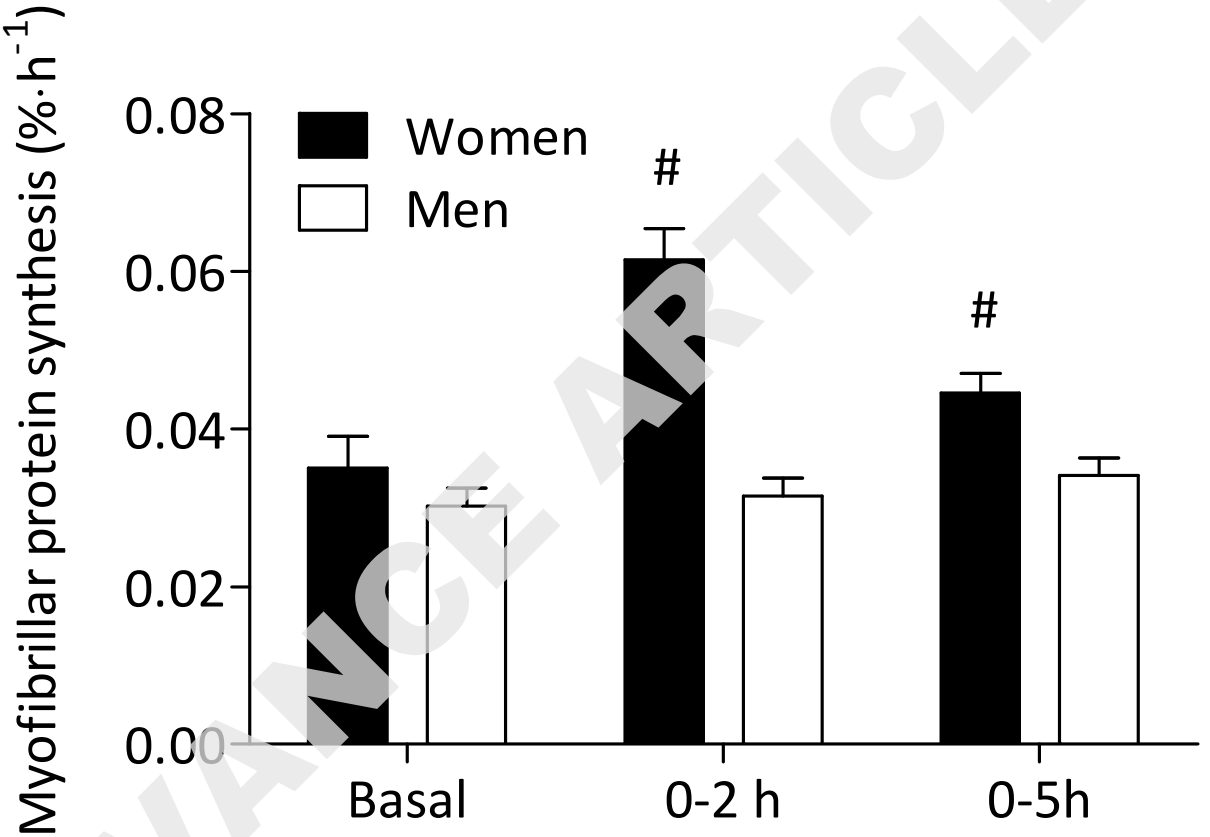
**Figure 8**

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