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Original article

Preserved anabolic threshold and capacity as estimated by a novel stable tracer approach suggests no anabolic resistance or increased requirements in weight stable COPD patients

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SUMMARY

Background & aims: Assessing the ability to respond anabolic to dietary protein intake during illness provides important insight in the capacity of lean body mass maintenance. We applied a newly developed stable tracer approach to assess in one session in patients with chronic obstructive pulmonary disease (COPD) and healthy older adults both the minimal amount of protein intake to obtain protein anabolism (anabolic threshold) and the efficiency of dietary protein to promote protein anabolism (anabolic capacity).

Methods: We studied 12 clinically and weight stable patients with moderate to very severe COPD (mean ± SE forced expiratory volume in 1 s: 36 ± 3% of predicted) and 10 healthy age-matched older adults. At 2-h intervals and in consecutive order, all participants consumed a mixture of 0.0, 0.04, 0.10 and 0.30 g hydrolyzed casein protein × kg ffm^{-1} × 2 h^{-1} and carbohydrates (2:1). We assessed whole body protein synthesis (PS), breakdown (PB), net PS (PS−PB) and net protein balance (phenylalanine (PHE) intake - PHE to tyrosine (TYR) hydroxylation) by IV primed and continuous infusion of L-[ring-$^{2}H_5$]−PHE and L-[$^{13}C_9$,^{15}N]-TYR. Anabolic threshold (net protein balance = 0) and capacity (slope) were determined on an individual basis from the assumed linear relationship between protein intake and net protein balance.

Results: We confirmed a linear relationship between protein intake and net protein balance for all participants (R^2 range: 0.9988−1.0, $p \leq 0.0006$). On average, the anabolic threshold and anabolic capacity were comparable between the groups (anabolic threshold COPD vs. healthy: 3.82 ± 0.31 vs. 4.20 ± 0.36 μmol PHE × kg ffm^{-1} × hr^{-1}; anabolic capacity COPD vs. healthy: 0.952 ± 0.007 and 0.954 ± 0.004). At protein intake around the anabolic threshold (0.04 and 0.10 g protein × kg ffm^{-1} × 2 h^{-1}), the increase in net PS resulted mainly from PB reduction ($p < 0.0001$) whereas at a higher protein intake (0.30 g protein × kg ffm^{-1} × 2 h^{-1}) PS was also stimulated ($p < 0.0001$).

Conclusions: The preserved anabolic threshold and capacity in clinically and weight stable COPD patients suggests no disease related anabolic resistance and/or increased protein requirements.

Trial registry: ClinicalTrials.gov; No. NCT01734473; URL: www.clinicaltrials.gov.

1. Introduction

During chronic or acute illness, metabolic changes occur that may alter the individual's ability to respond anabolic to dietary proteins. A response is considered anabolic when the rate of protein synthesis exceeds the rate of protein breakdown. Factors that may affect this anabolic response during illness include nutritional status, habitual protein intake, activity level, endocrine function, and the acute stress response [1,2]. Skeletal muscle mass will be lost

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List of abbreviations

COPD	chronic obstructive pulmonary disease
cTTR	tracer to tracee ratio corrected for natural abundance
DXA	dual-energy X-ray absorptiometry
EAR	estimated average requirement
FFM	fat-free mass
HADS	hospital anxiety and depression scale
IAAO	indicator amino acid oxidation
mMRC	modified medical research council dyspnea scale
PASE	physical activity scale for the elderly
PB	protein breakdown
PHE	phenylalanine
PS	protein synthesis
RDA	recommended dietary allowance
SGRQ-C	St. George's respiratory questionnaire for COPD patients
TYR	tyrosine
WbRa	whole body rate of appearance

during illness when the individual's ability to respond anabolic to dietary proteins is compromised and/or when the level of protein consumption that is needed to reach protein anabolism is elevated. Age, protein digestibility and absorption capacity, first pass splanchnic amino acid sequestration, changes in amino acid oxidation and other disease specific alterations in intermediary amino acid metabolism may play a role in this [3–5].

Protein requirements are currently being assessed by nitrogen balance and indicator amino acid oxidation (IAAO) techniques [6] although data are lacking in diseased populations. The nitrogen balance technique tends to overestimate nitrogen intake and protein retention, partly due to underestimation of miscellaneous nitrogen losses [3,7,8], and also requires habituation to varying protein intake levels [9]. The IAAO technique [10] assumes that the minimal requirement is met when oxidation of the indicator amino acid plateaus and ignores the contribution of protein breakdown to net protein balance [11,12]. The required CO_2 production measurements are sensitive to underestimation due to existing non-CO_2 routes [9], and may be difficult to perform in diseases such as COPD. In addition, both techniques require several study days to assess the individual's anabolic response.

Based on the consistent linear relationship between dietary essential amino acid intake and whole body net protein synthesis in several disease populations [13–18], we developed a novel stable tracer approach that is less time consuming because it can be completed in one session and has the potential to simultaneously assess the anabolic threshold (minimal amount of protein intake needed to obtain protein anabolism) and anabolic capacity (efficiency of dietary protein to stimulate protein anabolism) in clinical conditions, without changing the individual's habitual protein intake. The purpose of this initial study was to apply this novel stable tracer approach in a clinically and weight stable COPD group, as this group is characterized by disturbances in postabsorptive and prandial protein metabolism [19], but has the same linear anabolic response to intake of essential amino acids as healthy subjects [13].

2. Materials and methods

2.1. Subject inclusion

The study population comprised of 12 older adults with a clinical diagnosis of moderate to very severe airflow obstruction (grades II–IV), as defined by the established Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [20], and 10 healthy participants of similar age. We recruited participants in College Station, TX and surrounding areas from 2012 to 2015 through pulmonologist referral and local advertising. We assessed medical history and medication use as part of the screening process. None of the participants reported any recent weight loss ($\geq$5% in preceding 3 months or $\geq$10% in preceding 6 months). COPD patients were clinically stable and did not suffer from an exacerbation or any infection of the respiratory tract $\leq$4 weeks before the study. As maintenance therapy, 11 patients received bronchodilator treatment, 10 patients received inhalation corticosteroids, and 10 patients were on continuing oxygen therapy. The use of systemic corticosteroids $\leq$1 month before the study was an exclusion criterion as these medications are known to have systemic effects on metabolism and induce muscle dysfunction in COPD [21,22]. Other exclusion criteria were malignancy, recent myocardial infarction, recent surgery and severe endocrine, hepatic or renal disorders. Inclusion criteria for the healthy control group were healthy according to the investigator's judgment and not suffering from any acute or chronic disease or condition that may influence protein and amino acid metabolism (including inflammatory diseases). The exclusion criteria were history of untreated metabolic diseases including endocrine, hepatic or renal disorders, presence of fever within the last 3 days, use of protein or amino acid containing nutritional supplements within 1 month of study day, body mass index $\geq$35 kg/m^2 and <18.5 kg/m^2. We obtained written informed consent from all participants, and the Texas A&M University Institutional Review Board endorsed the study.

2.2. Study design

All participants were studied in a semi-recumbent position on a hospital bed during an 8-h experimental test day (Fig. 1). The test day started in the early morning after an overnight fast. First, a catheter was placed into an antecubital vein, then we collected the first blood sample to measure natural isotope enrichments. Consecutively, we started a primed, constant intravenous infusion of L-[ring-$^2H_5$]-phenylalanine (L-[$^2H_5$]–PHE: prime = 270 μmol; infusion = 270 μmol/h) and L-[U-$^{13}C_9$, ^{15}N]-tyrosine (L-[U-$^{13}C_9^{15}N$]-TYR: prime = 8.5 μmol; infusion = 8.5 μmol/h) to assess whole body protein metabolism. Additionally, we provided an oral bolus dose of L-[ring-$^2H_4$]-TYR to prime the plasma pool (L-[$^2H_4$]-TYR: prime = 25.5 μmol). Cambridge Isotopic Laboratories (Woburn, MA) supplied all the isotopes. We also placed a second catheter for arterialized venous blood sampling in a superficial dorsal vein of the contralateral hand or lower arm. The hand was placed in a thermostatically controlled heated box, a technique to mimic direct arterial sampling [23]. We obtained arterialized-venous blood samples at 80, 100, and 120 min after the start of infusion for postabsorptive assessments of plasma isotope enrichments, and amino acid concentrations. At 120 min, we started with the oral administration of a protein-carbohydrate mixture via sip feeding. Sips were provided every 20 min (as a surrogate for continuous feeding), and the dosage was increased every 2 h. We added L-[^{15}N]–PHE at 8% enrichment of the PHE content to the protein-carbohydrate mixture for the measurement of splanchnic PHE extraction. For postprandial assessments of plasma isotope enrichments, and amino acid concentrations we collected arterialized-venous blood samples at 200, 220, 240, 320, 340, 360, 440, 460, and 480 min. For the assessment of plasma insulin concentrations, we used blood samples collected at t = 120, 240, 360 and 480 min. Further details regarding sample processing and biochemical analysis can be found elsewhere [17].

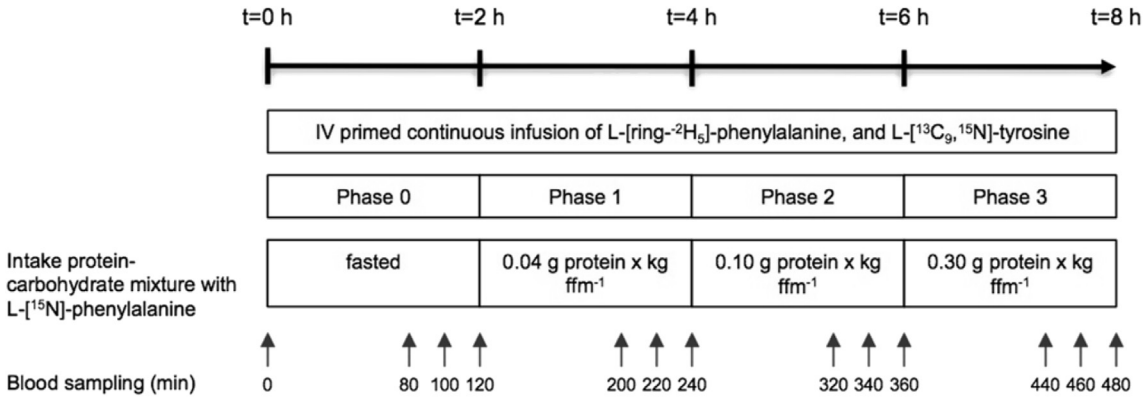

Fig. 1. Study design. ffm: fat-free mass.

2.3. Dietary intake

During the first two hours of feeding, participants received 0.04 g protein × kg fat-free mass (ffm)$^{-1}$ and 0.02 g carbohydrates × kg ffm^{-1} (phase 1, t = 120–240 min, average intake: 1.96 g protein and 0.98 g carbohydrates). During the second two hours, participants received 0.10 g protein × kg ffm^{-1} and 0.05 g carbohydrates × kg ffm^{-1} (phase 2, t = 240–360 min, average intake: 4.90 g protein and 2.45 g carbohydrates), and during the last two hours participants received 0.30 g protein × kg ffm^{-1} and 0.15 g carbohydrates × kg ffm^{-1} (phase 3, t = 360–480 min, average intake: 14.70 g protein and 7.35 g carbohydrates). The doses of protein intake were based on our pilot studies to get a good distribution of the intake to be able to create the regression line. The total amount of protein and carbohydrates provided during each 2-h period was divided over 6 sips, and given at 20 min intervals. We used hydrolyzed caseinate as the protein source (amino acid composition is listed in Supplemental Table 1), and maltodextrin as the carbohydrate source. All components were dissolved in non-caloric soda (2.4 mL/kg ffm).

2.4. Other measures

We measured forced expiratory volume in 1 s and forced vital capacity in all participants, with the best attempt out of at least 3 technically acceptable maneuvers being used. Spirometric reference values were those of a US population [24]. Height and weight were determined using a stadiometer and beam scale, respectively. Dual-energy X-ray absorptiometry (DXA) (Hologic QDR 4500) was carried out to measure body composition, including whole body FFM.

To determine habitual energy, protein, carbohydrate and fat consumption, subjects were instructed to complete a 3-day dietary record. Isometric strength in the dominant hand was assessed using a hand dynamometer (Vernier; Beaverton, OR). Maximum grip strength was determined by measuring the maximally developed strength of the flexors of the fingers during 5 s, with the highest value from ≥3 technically acceptable maneuvers being used. Maximally developed strength of the flexors of the fingers measured during 40 maneuvers in one minute was used to determine endurance strength, expressed as an average value or as a percentage of loss between the first and middle three (first half loss), or first and last three (total loss) maneuvers. Administration of the physical activity scale for the elderly (PASE) [25] and hospital anxiety and depression scale (HADS) [26] were used to assess the level of physical activity and levels of anxiety and depression, respectively among participants. In the COPD group, the St. George's respiratory questionnaire for COPD patients (SGRQ-C) was administered to measure the impact of COPD on overall health, daily life, and perceived well-being, and the modified medical research council dyspnea scale (mMRC) was used as a grading system to assess the level of dyspnea.

2.5. Calculations

For the calculation of whole body PS, PB, net PB, net PS, and net protein balance we used the standard steady state tracer dilution equations. Plasma tracer enrichment values were expressed as tracer to tracee ratio corrected for natural abundance (cTTR).

2.5.1. Phase 0 (fasted)

- Whole body rate of appearance (WbRa) PHE[$^{2}H_5$] and TYR [$^{13}C_9$,^{15}N] = Inf ÷ cTTR
- PB = WbRa PHE[$^{2}H_5$]
- Hydroxylation of PHE to TYR = WbRa TYR[$^{13}C_9$,^{15}N] × (cTTR [$^{2}H_4$]-TYR ÷ cTTR [$^{2}H_5$]–PHE)
- PS = whole body rate of disappearance (WbRd) PHE [$^{2}H_5$] = WbRa PHE[$^{2}H_5$] under steady state conditions) [27] − hydroxylation of PHE to TYR
- net PB = PS–PB

where WbRa PHE[$^{2}H_5$] and TYR[$^{13}C_9$,^{15}N] (μmol × kg ffm^{-1} × h^{-1}) represent the total systemic rate of appearance of PHE and TYR calculated from the intravenous infusion (Inf) of [$^{2}H_5$]–PHE and [$^{13}C_9$,^{15}N]-TYR, respectively. Hydroxylation of PHE to TYR represents the conversion PHE into TYR by the enzyme PHE hydroxylase.

Net protein balance was calculated as follows [28]:

- net protein balance = total PHE intake–hydroxylation of PHE to TYR

Net protein balance is equal to net PB in the postabsorptive state.

2.5.2. Phase 1–3 (fed)

PS, hydroxylation of PHE to TYR and net protein balance in phases 1, 2 and 3 of the study were calculated using the equations as stated above, but with enrichment values of PHE and TYR as obtained in each phase. For the calculation of splanchnic PHE extraction, PB, and net PS we used the following equations:

- WbRa PHE[^{15}N] = Oral intake [^{15}N]–PHE ÷ cTTR [^{15}N]–PHE
- Splanchnic PHE extraction = 1–(WbRa PHE[$^{2}H_5$] ÷ WbRa PHE [^{15}N])
- Exogenous WbRa PHE = Total PHE intake × (1–splanchnic extraction PHE)
- PB = WbRa PHE[$^{2}H_5$]–exogenous WbRa PHE
- Net PS = PS–PB

where WbRa PHE[^{15}N] (μmol × kg ffm^{-1} × h^{-1}) represents the total systemic rate of appearance of PHE calculated from the oral intake of [^{15}N]–PHE. Splanchnic PHE extraction represents the fraction of ingested PHE that is metabolized by the gut or liver during its first pass, either via oxidation or PS. Total PHE intake is the sum of unlabeled dietary PHE and labeled [^{15}N]–PHE ingested with the protein-carbohydrate mixture. Exogenous WbRa PHE (μmol × kg ffm^{-1} × h^{-1}) represents the systemic appearance of PHE coming from dietary protein. PB is equal to endogenous WbRa PHE.

2.6. Description of terms

The terms “anabolic threshold” and “anabolic capacity” are depicted in Fig. 2. Anabolic threshold is the minimal level of protein intake where after protein anabolism can be anticipated, thus the level of intake at which the whole body net protein balance is zero. Anabolic capacity is the efficiency with which protein intake increases net protein balance, defined as the change in net protein balance in proportion to the change in protein intake.

2.7. Statistical analysis

Results are expressed as means ± standard errors (SEs) or median (min–max). It was not possible to do a power calculation, because of the use of a newly developed method. Instead, we choose a sample size that is commonly used in metabolic (stable isotope) studies [13]. We compared clinical characteristics of the study populations using the unpaired Student's t-test and Fisher's exact test. Attainment of plasma isotopic steady state (plateau) in each phase of the study was evaluated, using linear regression analysis. Isotopic steady state was considered present if the slope of the regression line was not different from zero. A similar evaluation was performed for the plasma amino acid concentrations. For the assessment of plasma cTTR's and amino acid concentrations, we used the mean value of the three measurements obtained during each phase. We also performed linear regression analysis to examine the association between dietary protein intake and plasma amino acid and insulin concentrations, and whole body net protein balance. We used the coefficients of each individual's linear interpolation to derive the estimate of the intercept, i.e. the anabolic threshold. Two-factor repeated measures analysis of variance with “group” and “study phase” was used to test for effects and interactions between controls and patients and study phases. The two independent variables (study phase and group) interact if the effect of one of the variables differs depending on the level of the other variable. If applicable, Tukey's post hoc testing was used to identify differences between study phases. A significance level of $p < 0.05$ was chosen. Data analysis was done using the statistical program Graphpad Prism (version 7.0a).

3. Results

We studied twenty-two participants (12 COPD patients and 10 healthy older adults). Clinical characteristics and baseline measurements of the study populations are listed in Table 1. According to the GOLD staging system [20], two COPD patients were classified as GOLD II, six as GOLD III, and four as GOLD IV. Except for one patient with long-term exposure to secondhand smoking, all patients had a history of smoking, in comparison to only one healthy control ($p < 0.001$). Furthermore, COPD patients were characterized by lower values for lung function ($p < 0.0001$) and PASE ($p < 0.05$), and higher values for handgrip endurance strength loss ($p < 0.05$), HADS score for depression ($p < 0.01$), and habitual dietary fat intake ($p < 0.05$). Five controls versus four patients were normal weight ($18.5 \geq$ BMI ≤ 24.9 kg/m^2), three controls versus four patients were overweight ($25 \geq$ BMI ≤ 29.9 kg/m^2), and two controls versus 4 patients were obese (BMI ≥ 30 kg/m^2). On a group level, body weight and composition measures were comparable but gender distribution was slightly different.

3.1. Changes in plasma amino acid concentrations

The effect of dietary protein intake on the plasma amino acid concentrations of PHE, LEU, and TYR is shown in Supplemental Fig. 1. On a group level, the slope of the line derived from the

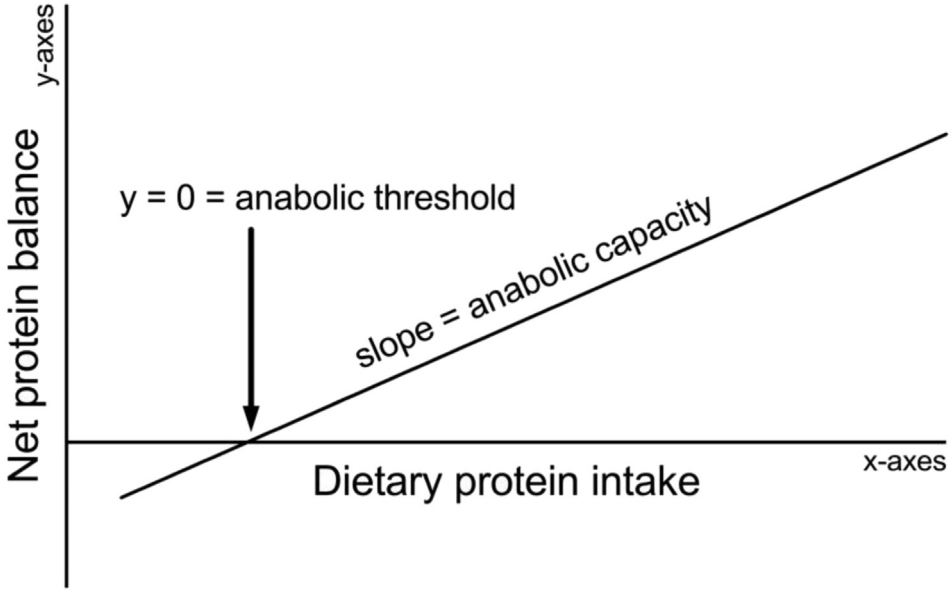

Fig. 2. Depiction of the terms anabolic threshold and capacity.

Table 1
Clinical characteristics and baseline measurements of the study populations.[1]

	Healthy (n = 10)	COPD (n = 12)
General characteristics		
Sex (m/f)	3/7	7/5
Age (y)	67 ± 3	68 ± 3
Smoking history (yes/no)	1/9	11/1***
Years of smoking	30 ± 0	32 ± 3
Smoking status (smoker/non-smoker)	0/10	1/11
Weight (kg)	72 ± 4	79 ± 6
BMI (kg/m^2)	26.1 ± 0.9	28.0 ± 1.5
Pulmonary function and COPD related measures		
FEV_1 (% of predicted)[2]	102 ± 5	36 ± 3****
FVC (% of predicted)[3]	100 ± 4	54 ± 3****
FEV_1/FVC	0.79 ± 0.02	0.50 ± 0.03****
Years since initial diagnosis		10 ± 2
Self-reported years of COPD related symptoms		13 ± 2
mMRC dyspnea grade[4#]		2.4 ± 0.3
0–1 exacerbation per year and no hospitalization for exacerbation (yes/no)		7/5
≥2 exacerbations per year or ≥1 hospitalization for exacerbation (yes/no)		5/7
SGRQ-C total score[5#]		60 ± 3
Body composition		
Fat-free mass (kg)	46.6 ± 3.5	51.0 ± 3.6
Fat-free mass index (kg/m^2)[6]	16.8 ± 0.8	18.1 ± 0.9
Appendicular skeletal muscle index (kg/m^2)[7]	6.9 ± 0.4	7.0 ± 0.4
Total fat (%)	34 ± 2	33 ± 2
Android fat (%)[8]	37 ± 3	37 ± 2
Gynoid fat (%)[8]	37 ± 3	35 ± 2
Android/Gynoid percentage fat	1.0 ± 0.1	1.1 ± 0.1
VAT mass (g)[9]	625 ± 64	874 ± 128
Bone mineral density hip neck (g/cm^2)	0.73 ± 0.04	0.70 ± 0.03
Muscle strength and questionnaires		
Handgrip maximum strength (N/kg ffm arm)	104 ± 5	92 ± 5
Handgrip average endurance strength (N/kg ffm arm)	73 ± 3	66 ± 5
Handgrip endurance strength first half loss (%)[&]	17 ± 2	24 ± 3
Handgrip endurance strength total loss (%)[&]	22 ± 2	30 ± 3*
PASE[10#]	210 ± 36	108 ± 20*
HADS score for anxiety[11]	5.3 ± 1.1	5.5 ± 0.9
HADS score for depression	2.6 ± 0.7	6.5 ± 1.0**
Habitual dietary intake		
Calories (kcal)	1804 ± 107	1565 ± 125
Fat (energy%)	30.7 ± 2.8	38.1 ± 1.3*
Protein (energy%)	17.8 ± 1.2	15.3 ± 0.9
Protein (g/kg BW)	1.1 ± 0.1	0.9 ± 0.2
Carbohydrates (energy%)	51.7 ± 3.0	45.3 ± 1.4

[1]Values are means ± SEs. [2]FEV_1: forced expiratory volume in one second. [3]FVC: forced vital capacity, [4]mMRC: modified medical research council dyspnea scale. [5]SGRQ-C: St. George respiratory questionnaire for COPD patients. [6]Fat-free mass index: (muscle mass + bone mineral content)/height^2. [7]Appendicular skeletal muscle index: (muscle mass legs + muscle mass arms)/height^2. [8]Android fat and gynoid fat correspond to central and peripheral fat distribution, respectively. [9]VAT: visceral adipose tissue. [10]PASE: physical activity scale for the elderly. [11]HADS: hospital anxiety and depression scale. For comparison of numerical values, statistics were performed using the unpaired Student's t-test. Alternatively, categorical values were compared using Fisher's exact test. [#]n = 11. [&]n = 9 for controls and n = 10 for COPD patients. COPD patients significantly different from controls, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. COPD: chronic obstructive pulmonary disease.

three data points collected during each phase was equal to zero for all measured amino acids (suggesting steady state concentrations), except for the TYR concentration in the COPD group in phase 2 (p = 0.0312). We did not find between group differences or study phase-by-group interactions. For all three amino acids, the concentration increased in each subsequent study phase (p < 0.05), except for the TYR concentration between phase 0 and 1 (Supplemental Table 2). Furthermore, there was a linear relationship between dietary protein intake and the plasma concentrations of all three amino acids in both groups (p < 0.01), without any differences in the slopes of the lines between the groups (Fig. 3).

3.2. Changes in plasma insulin concentration

For the plasma insulin concentration, we found a group effect (p = 0.0476), which indicated a higher insulin concentration in COPD patients. Furthermore, we found a study phase effect (p < 0.0001), and study phase-by-group interaction (p = 0.0294) (Supplemental Table 2). In both groups, it was only in phase 3 that the insulin concentration significantly increased above postabsorptive values (p = 0.0003 and p = 0.0133 in patients and controls, respectively). Only in the control group we observed a linear relationship between dietary protein intake and the plasma insulin concentration (p = 0.0080) (Fig. 4).

3.3. Changes in plasma isotope enrichments

The effect of dietary protein intake on the plasma enrichment of the different tracers in each phase of the study is shown in Fig. 5. On a group level, the slope of the line derived from the three data points collected during each phase was equal to zero for all measured tracers (suggesting steady state enrichments), except for [$^2H_5$]–PHE in the COPD group and [^{15}N]-TYR in the control group at the highest level of protein intake (phase 3) (p = 0.0225 and p = 0.0031, respectively).

3.4. Whole body protein metabolism

We did not find between group differences or study phase-by-group interactions for PS, PHE hydroxylation, net PS and SPE

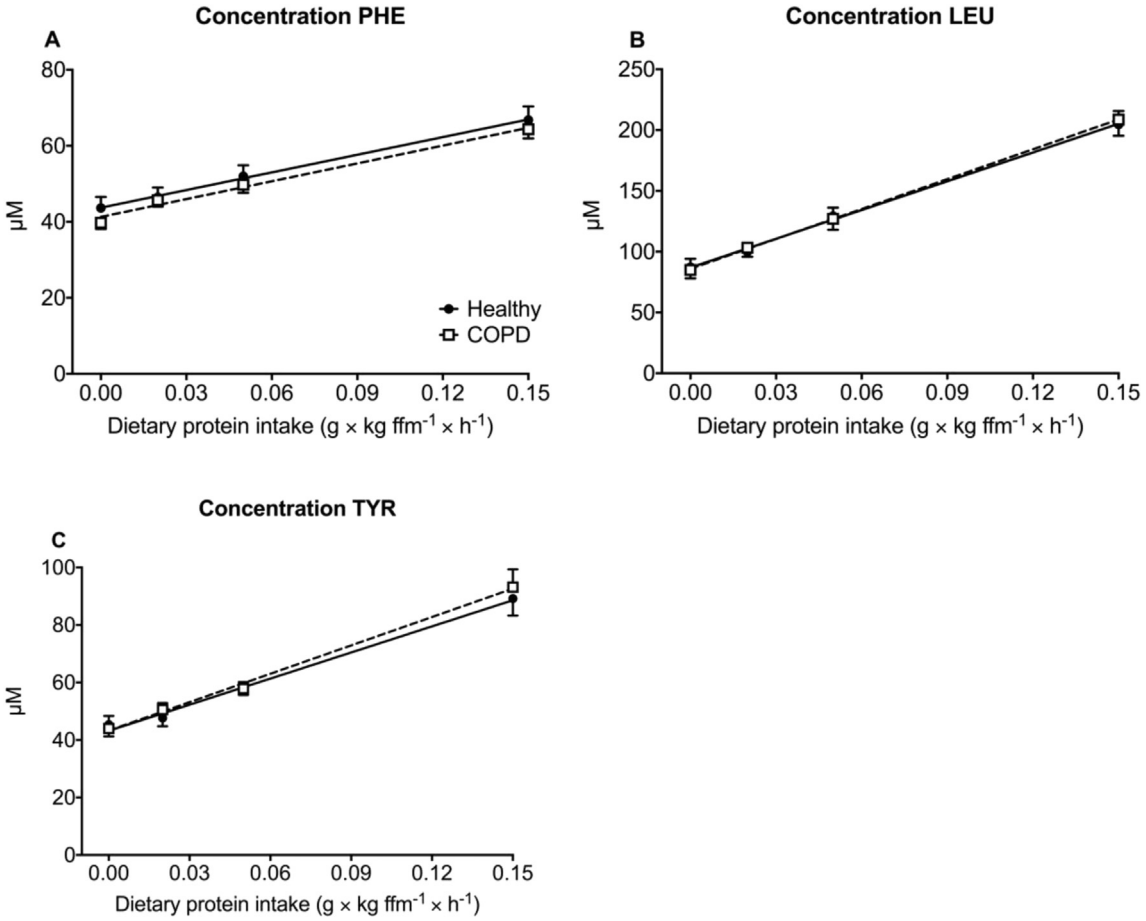

Fig. 3. Plasma amino acid concentrations of PHE (**A**), LEU (**B**), and TYR (**C**) as a function of dietary protein intake in COPD patients (n = 12) and healthy age matched controls (n = 10). PHE control group: y = 155x + 44, R^2 = 0.9987, p = 0.0007. PHE COPD group: y = 156x + 41, R^2 = 0.9864, p = 0.0068. LEU control group: y = 788x + 87, R^2 = 0.9981, p = 0.0010. PHE COPD group: y = 819x + 86, R^2 = 0.9998, p = 0.0001. TYR control group: y = 303x + 43, R^2 = 0.9928, p = 0.0036. TYR COPD group: y = 329x + 43, R^2 = 0.9966, p = 0.0017. Slopes are similar between groups for all three amino acids. Statistics were done using linear regression analysis. COPD: chronic obstructive pulmonary disease. ffm: fat-free mass. LEU: leucine. PHE: phenylalanine. TYR: tyrosine.

(Supplemental Table 3). PS in phase 1 and 2 were comparable to the rate in the postabsorptive state (phase 0), while PS increased in phase 3 of the study (p < 0.0001) (Fig. 6A). We found no between group differences for PB. Furthermore, PB was reduced in each subsequent phase of the study (p < 0.0001), and we found a study phase-by-group interaction (p = 0.0110) (Fig. 6B). Net PS increased in each subsequent phase of the study (phase 0–1: p < 0.05, phase 1–2 and 2–3: p < 0.0001) (Supplemental Table 3). The rate of PHE hydroxylation in phase 1 was similar to the rate observed in the postabsorptive state, while PHE hydroxylation increased 17% in phase 2 compared to phase 1 (p < 0.05), and increased 37% in phase 3 compared to phase 2 (p < 0.0001) (Fig. 6C). Net protein balance increased in each subsequent phase of the study (p < 0.0001) (Fig. 6D). SPE remained comparable throughout the different phases (Fig. 6E).

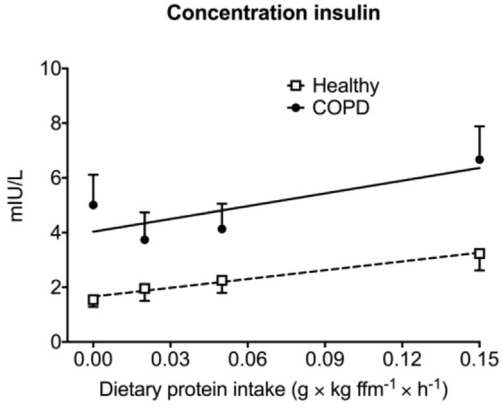

Fig. 4. Plasma insulin concentration as a function of dietary protein intake in COPD patients (n = 12) and healthy age matched controls (n = 10). Control group: y = 10.7x + 1.7, R^2 = 0.9987, p = 0.0080 (slope is significantly different from zero). COPD group: y = 15.5x + 4.0, R^2 = 0.6318, p = 0.2051 (slope is not significantly different from zero). Slopes are similar between groups. Statistics were done using linear regression analysis. COPD: chronic obstructive pulmonary disease. ffm: fat-free mass.

3.5. Anabolic threshold and capacity

In Fig. 7 we show on a group level the linear relation between oral PHE intake and net protein balance in healthy controls (R^2 = 1, p < 0.0001) and COPD patients (R^2 = 1, p < 0.0001).

On an individual level, the linear relation was also significant for all participants (R^2 range 0.9988–1.0, p ≤ 0.0006) (Supplemental Fig. 2). The anabolic threshold was found at an average intake of 4.20 ± 0.36 µmol PHE × kg ffm^{-1} × h^{-1} (range 2.4–5.9) in healthy controls and 3.82 ± 0.31 µmol PHE × kg ffm^{-1} × h^{-1} (range 2.7–5.9) in COPD patients (Fig. 8A). This translated to a protein intake of 0.013 ± 0.001 g × kg ffm^{-1} × h^{-1} (range 0.007–0.018) and 0.012 ± 0.001 g × kg ffm^{-1} × h^{-1} (range 0.008–0.018) in

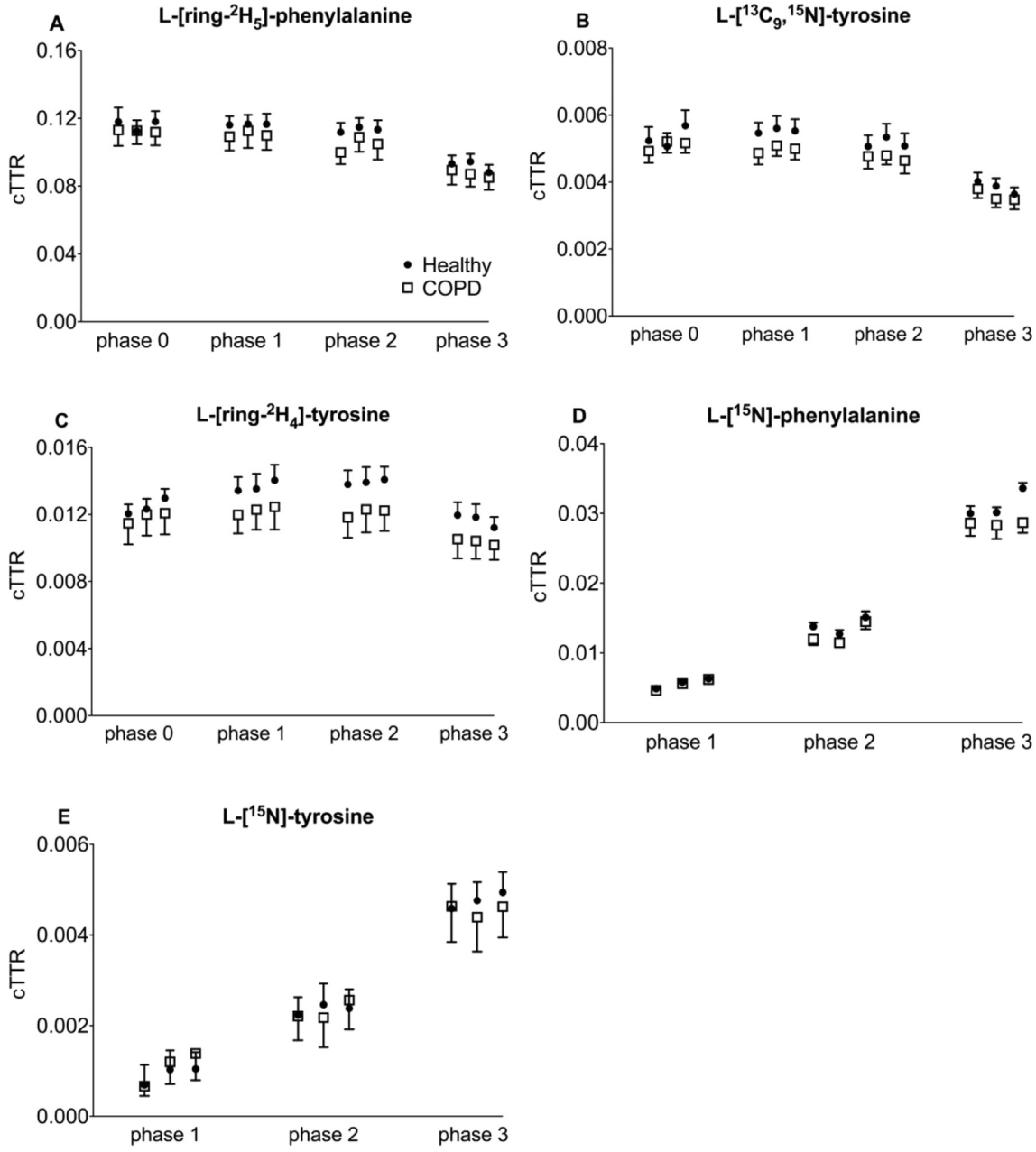

Fig. 5. Mean (±SE) plasma isotope enrichments of L-[ring-$^2H_5$]-phenylalanine (**A**), L-[U-$^{13}C_9$, ^{15}N]-tyrosine (**B**), L-[ring-$^2H_4$]-tyrosine (**C**), L-[^{15}N]-phenylalanine (**D**), L-[^{15}N]-tyrosine (**E**) in COPD patients (n = 12) and healthy age matched controls (n = 10) during four study phases, in which we examined four levels of intake of a protein-carbohydrate mixture. Phase 0 = fasted. Phase 1 = 0.04 g protein × kg ffm^{-1} × 2 h^{-1}. Phase 2 = 0.10 g protein × kg ffm^{-1} × 2 h^{-1}. Phase 3 = 0.30 g protein × kg ffm^{-1} × 2 h^{-1}. COPD: chronic obstructive pulmonary disease.

controls and patients, respectively. On average, we found no significant difference between the groups. On an individual level, the anabolic threshold was situated between phase 0 and 1 for all participants. The average anabolic capacity was 0.954 ± 0.004 (range 0.94–0.98) for controls and 0.952 ± 0.007 (range 0.90–0.99) for patients (Fig. 8B). There was no significant difference between the groups.

4. Discussion

In this study, we applied a newly developed stable tracer approach that is completed in one session for the assessment of whole body protein metabolism in response to feeding. We showed that clinically and weight stable COPD patients do not have changes in the anabolic threshold and/or anabolic capacity as compared to healthy older adults.

4.1. Confirmation of the applicability of a novel stable isotope approach

Our novel approach is not meant to be a replacement of other validated methods like the nitrogen balance technique, but to introduce a less laborious method that can be used to compare different patient groups to examine whether differences are present between the anabolic threshold and capacity when habitual protein intake is not changed. Previously we showed in healthy

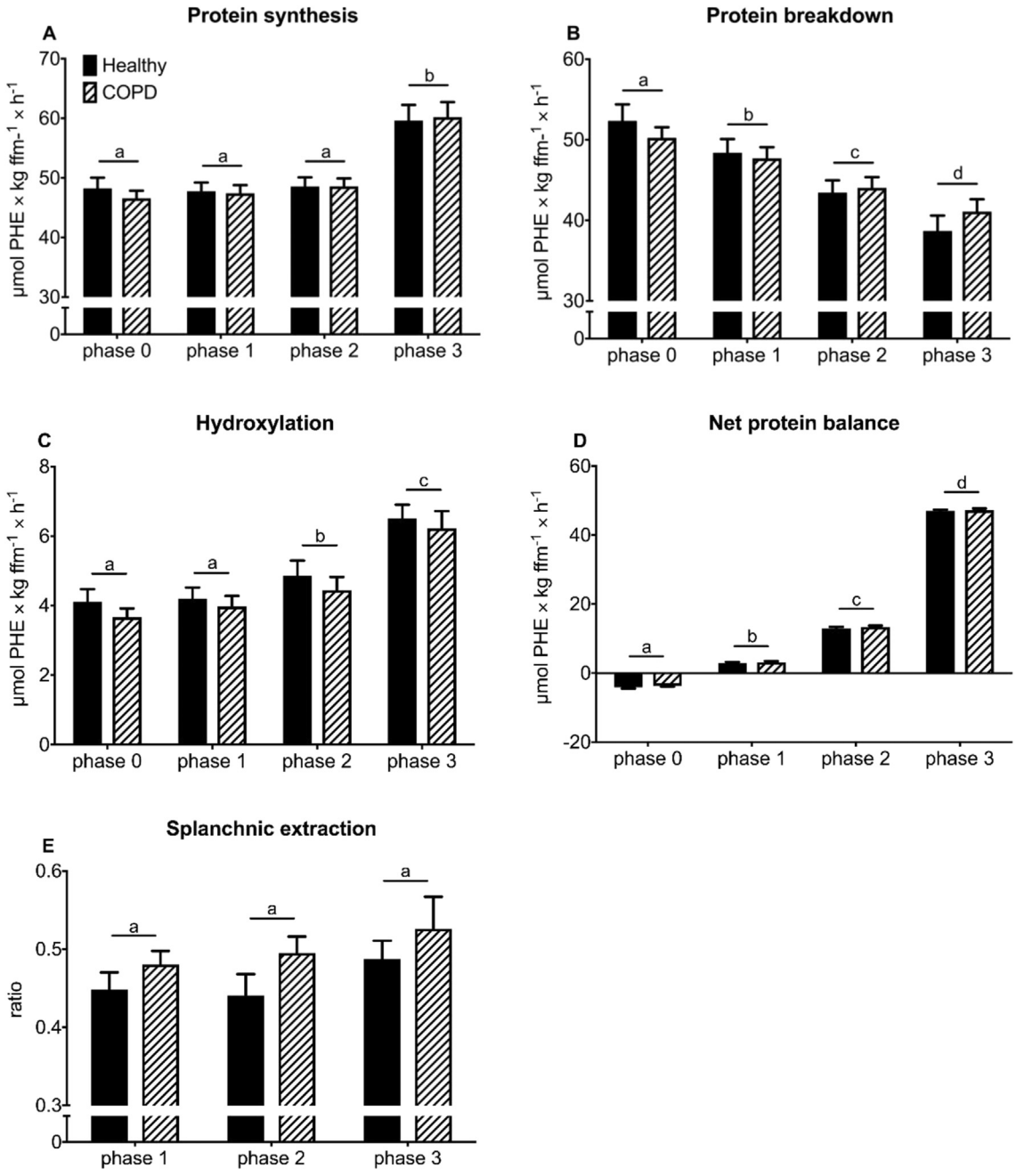

Fig. 6. Mean (±SE) whole body protein synthesis (**A**), breakdown (**B**), hydroxylation (**C**), net protein balance (**D**), and splanchnic PHE extraction (**E**) in COPD patients (n = 12) and healthy age matched controls (n = 10) during the four study phases, in which we examined four levels of intake of a protein-carbohydrate mixture. Phase 0 = fasted. Phase 1 = 0.04 g protein × kg ffm^{-1} × 2 h^{-1}. Phase 2 = 0.10 g protein × kg ffm^{-1} × 2 h^{-1}. Phase 3 = 0.30 g protein × kg ffm^{-1} × 2 h^{-1}. Two-factor repeated measures analysis of variance showed a significant study phase effect for all parameters ($p < 0.0001$), except for SPE. No group effects were observed, but a study phase-by-group interaction was observed for protein breakdown ($p = 0.0110$). Different letters (a,b,c,d) indicate statistical differences between phases, at $p < 0.0001$, except for the difference in hydroxylation between phase 1 and 2, which was significant at $p < 0.05$. Hydroxylation is the conversion of PHE to tyrosine by the enzyme PHE hydroxylase. Splanchnic extraction is the fraction of PHE that is extracted by the splanchnic area during its first pass after consumption. COPD: chronic obstructive pulmonary disease. ffm: fat-free mass. PHE: phenylalanine.

young and older adults, and in weight losing and stable patients with different chronic diseases, that whole body net PS increased linearly as a function of dietary essential amino acid intake (using PHE as a surrogate for all essential amino acids) [13–18]. This relation appears to exist for protein intakes up to at least 90 g/meal [16]. In the current study, we built upon this knowledge and showed that with our newly developed stable tracer approach, it is possible to establish a linear fit between relatively low amounts of PHE (representing 2, 5 and 15 g protein in total per 2 h period for an average person of 50 kg FFM) and whole body net protein balance, and that this relationship can be established on an individual level. It is noteworthy that with sip feeding, as used in this study, plasma (essential) amino acid concentrations also increased linearly as a function of dietary protein intake, and therefore appear to function as a global measure of changes in net protein balance. This is in line with our group-based findings for changes in the plasma PHE concentration and net PS after bolus feeding [13].

The applicability of our approach was dependent on a steady state in plasma tracer enrichment during each 2 h period. We used a highly hydrolyzed enzymatic digest of casein, which facilitated

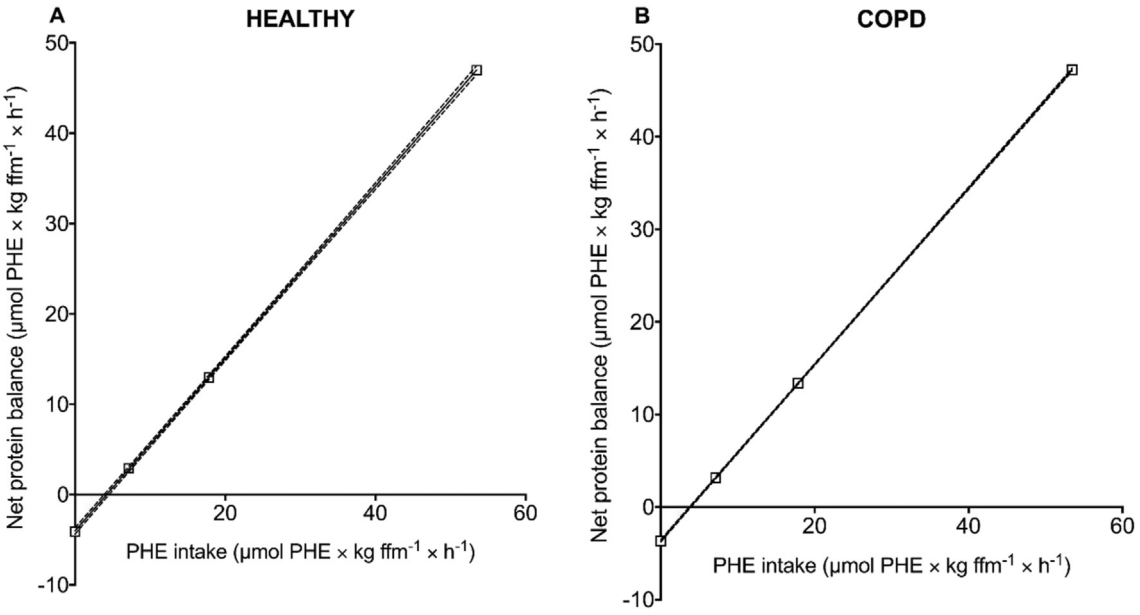

Fig. 7. Whole body net protein balance as a function of PHE intake (labeled and unlabeled) in healthy age matched controls (n = 10) (**A**), and COPD patients (n = 12) (**B**). Control group: y = 0.9536x−4.0, R^2 = 1, p < 0.0001. COPD group: y = 0.9475x−3.6, R^2 = 1, p < 0.0001. Both slopes are significantly different from zero. Statistics were done using linear regression analysis. Dotted lines represent the 95% confidence interval. COPD: chronic obstructive pulmonary disease. ffm = fat-free mass. Net protein balance = PHE intake - PHE hydroxylation. PHE: phenylalanine.

rapid gut absorption and shortened the time needed to obtain steady state in tracer enrichment. Nevertheless, the absolute increase in protein intake between phases 2 and 3 was greater than the absolute increase between phase 0 and 1, or phase 1 and 2, and we assume that as a result the plasma tracer enrichment of [$^2H_5$]−PHE in the COPD group and [^{15}N]-TYR in the control group in phase 3 did not reach perfect steady state. Therefore, we hypothesize that our model would be strengthened by the use of a lower protein intake in phase 3.

4.2. Changes in protein metabolism following graded protein intake

We found no differences between COPD patients and controls regarding the mechanisms (PS and PB) that increased net PS following graded protein intake. At protein intakes of 0.04 and 0.10 g × kg ffm^{-1} × 2 h^{-1}, which were both intakes around the anabolic threshold, we found that net PS was primarily affected by reductions in PB, without significant increases in PS from post-absorptive values. It was only at a higher protein intake of 0.30 g × kg ffm^{-1} × 2 h^{-1} that we also observed a stimulation of PS. The significant decrease in PB despite no sudden increase in insulin might possibly be explained by the intracellular rise of amino acids derived from amino acid intake that provides a signal to the body to inhibit protein breakdown. These results suggest that there may be a lower limit for protein intake, below which the stimulation of PS is not part of the mechanism that improves net PS. On muscle level, a rise in blood essential amino acid concentrations through dietary intake acts as a signal for the stimulation of PS [29].

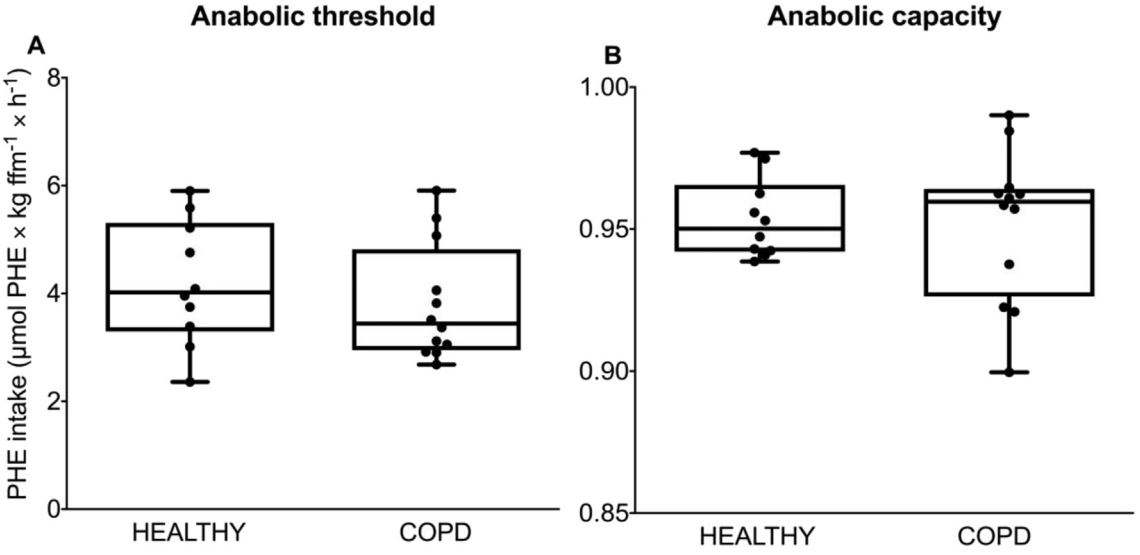

Fig. 8. Anabolic threshold (**A**) and anabolic capacity (**B**) in COPD patients (n = 12) and healthy age matched controls (n = 10). Data are depicted as a box and whiskers (min to max). Depiction of the terms anabolic threshold and anabolic capacity can be found in Fig. 2. No significant differences were found between the groups for either the anabolic threshold or anabolic capacity. Statistics were done using the unpaired Student's t-test. COPD: chronic obstructive pulmonary disease. ffm: fat-free mass.

Possibly, in our study the rise in blood essential amino acid concentrations (Fig. 3A and B) in phase 1 and 2 was too small to be sensed, and therefore did not signal an increase in PS. Moreover, at protein intakes greater than 20–30 g (given as a one-time bolus) it is also a reduction in PB, rather than a stimulation of PS that improves net PS [30,31]. We recently found that the relation between protein intake and net PS remains linear up to 90 g of protein in a meal [16,32], indicating that high amounts of protein intake are not necessary in order to assess anabolic threshold and capacity. Logistically, we decided not to give a large amount of protein as it could be anticipated that steady state between intake and net PS would not be obtained within a 2 h time frame of sip feeding as the stomach emptying, absorption and digestion becomes limited causing a delay in the uptake of the nutrient. It is important to highlight that the total amount of protein/amino acids and not the delivery pattern is important in inducing protein anabolism as equivalent skeletal muscle anabolic responses were observed after intake of bolus vs pulse of consumed EAA in older individuals despite some differences in the overall EAA exposure [33]. Furthermore, feeding regimes (leucine-rich EAA vs. bolus of whey protein) were able to stimulate muscle anabolism equivalently in older women [34].

4.3. Between group anabolic threshold and capacity

We found no evidence of an increased anabolic threshold or reduced anabolic capacity (as a measure of disease related anabolic resistance) in COPD patients. However, patients in this study were clinically and weight stable, had a normal to obese body type, a habitual protein intake that was comparable to controls, and were not preselected on the basis of COPD subtype (i.e. chronic bronchitis or emphysema) [35]. All of the above could have an impact on the anabolic threshold and/or capacity. Therefore, it remains to be studied whether only particular metabolic phenotypes [36], e.g. the cachectic patient who is often characterized by emphysema and hyperinflation, loss of muscle and fat mass and increased muscle protein breakdown [37], are subject to changes in the anabolic threshold and/or capacity. On the other hand, we previously found in advanced NSCLC patients [15] and children with CF hospitalized for an acute exacerbation [18] that the relation between protein intake and net PS was comparable to that of healthy subjects and independent of the presence of nutritional depletion and/or recent weight loss.

4.4. Anabolic threshold as proxy for protein requirement?

Our interpretation of the anabolic threshold in this study is that it represents the minimal amount of a high-quality protein in a meal that is to be consumed to avoid protein loss. The extent to which this value represents an approximation of daily protein requirement requires further validation, including 24-h assessments. During the day, meals provide anabolism, while between meals there is catabolism. Still, with a FFM of 65% of total body weight in both groups and assuming that the anabolic response remains at the same level during 24 h, the conversion of PHE intake at the anabolic threshold from μmol × kg ffm^{-1} × h^{-1} to g protein × kg BW^{-1} × d^{-1} resulted in a value of about 0.19 g protein × kg BW^{-1} × d^{-1} (range 0.11–0.28). This value for minimal daily protein requirement is much less than values established by nitrogen balance and IAAO techniques, which suggest an average protein requirement of 0.66 and 0.96 g protein × kg BW^{-1} × d^{-1} in older adults, respectively [6,10].

We consider the formula used to calculate PHE to TYR hydroxylation to be a limitation, since it is based on the ratio between TYR and PHE tracers in plasma. However, PHE hydroxylation to TYR takes place largely in the liver, and thus by using tracer enrichments in plasma we overestimate the PHE enrichment and underestimate the TYR enrichment derived from PHE at the actual site of hydroxylation, consequently this leads to an underestimation of the hydroxylation rate of PHE to TYR [6], and an underestimation of the protein requirement. This remains a critical weakness of the use of stable PHE and TYR tracers. Furthermore, it is important to recognize that the anabolic threshold is not synonym for what may be considered optimal protein intake for older adults, especially in the presence of a chronic condition such as COPD. Functional parameters, including muscle strength, cardiovascular health, and bone health may all benefit from a protein intake above the anabolic threshold [38].

Although there is no direct evidence that protein anabolic capacity and threshold are affected by gender and age, the study would have benefitted from an equal gender distribution and a younger control group. No sex differences were observed in the anabolic response to nutritional stimuli in middle-aged adults [39], but sexual dimorphism was found in the anabolic response to feeding in obese older adults [40]. Furthermore, smoking is known to affect protein metabolism as it impairs the processes of muscle protein synthesis and maintenance [41].

Besides the need for 24-h assessments to derive daily protein requirement from the anabolic threshold, there are several other methodological limitations that need to be addressed, including the use of continuous instead of bolus feeding which mimics more closely a normal pattern of protein intake, and the role of protein catabolism in between feedings. That said, we believe our approach already provides a relative indication of protein requirement, and is unique in providing information on an individual basis within a single study day. Furthermore, there is no gold standard for the estimation of protein requirements. Established methods are subject to methodological limitations as well [9], including time constraints, and for these reasons are not feasible in many diseased populations. It is however in these populations that more insight in protein requirements is crucial.

In conclusion, in this study we examined the applicability of a novel stable tracer approach to estimate what we defined as the protein anabolic threshold and capacity. Applying this technique in clinically and weight stable COPD patients in comparison to healthy older adults revealed no changes in the protein anabolic threshold and capacity in this subgroup of patients suggesting no disease related anabolic resistance and/or increased requirements. As our stable tracer approach can be applied on an individual level, it may be a new and promising tool to define changes in the ability to become anabolic after feeding in response to aging and diseases associated with nutritional deficiencies such as sarcopenia and cachexia, and in specific (metabolic) phenotypes. More studies are needed to further validate this new approach.

Conflict of interest

The authors have no conflict of interest to declare.

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Author contributions: RJ, ND and ME had full access to all of the data in the study and are accountable for the integrity of the data and the quality of data analysis. RJ, ND, GL, and ME designed and conducted the research. RJ, ND, and ME were involved in the data analysis and writing of the manuscript. GL reviewed the

manuscript. EV, RH, and AZ were involved in the recruitment of study participants.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2018.07.018.

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