

Effects of Low-Protein, and Supplemented Very Low-Protein Diets, on Muscle Protein Turnover in Patients With CKD



Giacomo Garibotto¹, Antonella Sofia¹, Emanuele Luigi Parodi¹, Francesca Ansaldo¹, Alice Bonanni¹, Daniela Picciotto¹, Alessio Signori², Monica Vettore³, Paolo Tessari³ and Daniela Verzola¹

¹Division of Nephrology, Dialysis and Transplantation, Department of Internal Medicine, University of Genoa and Ospedale Policlinico San Martino, Genoa, Italy; ²Department of Health Sciences, Biostatistics Unit, University of Genoa, Genoa, Italy; and ³Metabolism Division, Department of Medicine, University of Padova, Italy

Introduction: Early studies have shown that patients with chronic kidney disease (CKD) are able to maintain nitrogen balance despite significantly lower protein intake, but how and to what extent muscle protein metabolism adapts to a low-protein diet (LPD) or to a supplemented very LPD (sVLPD) is still unexplored.

Methods: We studied muscle protein turnover by the forearm perfusion method associated with the kinetics of ²H-phenylalanine in patients with CKD: (i) in a parallel study in subjects randomized to usual diet (1.1 g protein/kg, *n* = 5) or LPD (0.55 g protein/kg, *n* = 6) (Protocol 1); (ii) in a crossover, self-controlled study in subjects on a 0.55 g/kg LPD followed by a sVLPD (0.45 g/kg + amino/ketoacids 0.1 g/kg, *n* = 6) (Protocol 2).

Results: As compared with a 1.1 g/kg containing diet, a 0.55 g/kg LPD induced the following: (i) a 17% to 40% decrease in muscle protein degradation and net protein balance, respectively, (ii) no change in muscle protein synthesis, (iii) a slight (by approximately 7%, *P* < 0.06) decrease in whole-body protein degradation, and (iv) an increase in the efficiency of muscle protein turnover. As compared with an LPD, an sVLPD induced the following: (i) no change in muscle protein degradation, and (ii) an approximately 50% decrease in the negative net protein balance, and an increase in the efficiency of muscle protein turnover.

Conclusion: The results of these studies indicate that in patients with CKD the adaptation of muscle protein metabolism to restrained protein intake can be obtained via combined responses of protein degradation and the efficiency of recycling of amino acids deriving from protein breakdown.

Kidney Int Rep (2018) 3, 701–710; <https://doi.org/10.1016/j.ekir.2018.01.003>

KEYWORDS: amino acids; chronic kidney disease; ketoacids; low-protein diet; nutrition

© 2018 International Society of Nephrology. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Although protein restriction has been used for decades in the treatment of patients with chronic kidney disease (CKD), there are still several unaddressed issues on the metabolic effects of low-protein diets (LPDs). A major issue is our still incomplete knowledge of the response of muscle protein metabolism to protein restriction in humans.¹ Skeletal muscle is a highly adaptive tissue that responds to nutrient supply, exercise, and hormones, with changes in protein metabolism and, ultimately, in fiber composition and size.¹ However, how muscle protein metabolism

adapts to a low protein intake in humans is still an open question. Nitrogen (N) balance studies have shown that healthy young subjects can maintain neutral or slightly positive balance with protein intakes as low as 0.55 to 0.6 g/kg.^{2–5} Whole-body leucine kinetics studies have shown that adaptation to dietary protein restriction involves a reduction in the rate of leucine flux and oxidation, leading to more efficient use of dietary amino acids (AAs) and reduced ureagenesis.^{3–6} Of note, the concept of “adaptation” to low protein intakes has been separated from the concept of “accommodation”; the latter term implying a decrease in protein synthesis, with development of wasting, when dietary protein intake becomes inadequate (i.e., beyond the limits of the adaptive mechanisms).^{2,5–7}

In CKD, an impaired ability to activate an adaptive response might impair N conservation when an LPD is

Correspondence: Giacomo Garibotto, Department of Internal Medicine, Division of Nephrology, Dialysis and Transplantation, Università di Genova, Viale Benedetto XV, 6, 16132 Genoa, Italy. E-mail: gari@unige.it

Received 25 November 2017; revised 28 December 2017; accepted 8 January 2018; published online 11 January 2018

prescribed.⁸ Nevertheless, studies obtained by the whole-body tracer leucine kinetics have shown that, provided metabolic acidosis is corrected, patients with CKD can efficiently adapt protein turnover to an LPD containing 0.6 to 0.7 g protein/kg.^{9–11} The amount of dietary protein can be reduced further to 0.3 g protein/kg per day with a very low protein diet (VLPD) if a ketoacid (KA) supplement is added to dietary proteins and the essential AA (EAA) skeletons are sufficient to synthesize body proteins.^{12–17} EAA/KA supplements contain substantial amounts of the ketoacid of leucine, which may decrease protein degradation.¹⁴ In addition, EAA, mainly leucine, can increase protein synthesis in muscle.^{13–15} Experimental evidence based on whole-body leucine kinetics shows that a neutral body N balance on a supplemented VLPD (sVLPD) can be attained by long-term reduction of whole-body leucine oxidation and postprandial inhibition of protein degradation.^{16,17}

Despite our understanding of the regulation of whole-body protein metabolism in response to dietary protein restriction, no study so far has examined the effect of low protein intake on muscle protein synthesis and degradation in patients with CKD. The available evidence deriving from whole-body tracer kinetics cannot necessarily be extrapolated to muscle, because muscle protein turnover contributes only 35% to 50% to whole-body protein turnover^{18,19} in humans. Remaining concern is that excessive reduction in dietary protein might accelerate the risk of muscle wasting.²⁰ In addition, in elderly, otherwise healthy subjects, an inadequately low protein intake may lead to a decline in muscle protein synthesis, resulting in sarcopenia.²¹

In this study, we have addressed this issue by measuring muscle protein turnover in 2 cohorts of patients with CKD given 2 levels of dietary protein: the more often used LPD providing 0.55 g protein/kg per day, or a VLPD providing 0.45 g protein/kg per day, supplemented (0.1 g/kg) with EAA and KA. Our data show that in patients with CKD, skeletal muscle responds to a low protein intake via a marked decrease in muscle protein degradation and an increase in the efficiency of muscle protein turnover.

MATERIALS AND METHODS

Study Participants

Subjects were recruited among patients referred for CKD at the Nephrology Division, Department of Internal Medicine, IRCCS AOU San Martino-IST, Genoa, Italy. Subject recruitment started in October 2012 and the study was conducted between January 2013 and June 2015. Subjects all were nonsmokers and moderate alcohol users (<10 drinks per week). None of them had a food allergy, or gained or lost more than 2 kg in the

6 months before the study. The protocols described here were approved by the Ethical Committee of the Department of Internal Medicine of the University of Genoa-IRCCS AOU San Martino-IST, Genoa. Before the patients' participation, the nature, purpose, and risks of the study were reviewed with all patients and their voluntary consent was obtained. Procedures were in accordance with the Helsinki declaration. The study was registered at ISRCTN with identifier ISRCTN64383094.

Seventeen patients with CKD were studied in the 2 protocols (Table 1).

Eleven of these patients participated in Protocol 1 and 6 participated in Protocol 2. Overall, patients were similar for age, sex, race, and laboratory assessments between the study groups; eGFR was lower in patients studied in Protocol 2. Patients younger than 18 and older than 79 years were excluded from the study. The patients were enrolled in the study on a consecutive basis if they did not meet the following exclusion criteria: congestive heart failure or a recent (<12 months) myocardial infarction, liver cirrhosis, infection, malignancy, inflammatory disease, diabetic nephropathy, or fast (>5 ml/min per year) decline in renal function. In addition, patients who did not show evidence of a deep forearm vein for cannulation and sampling were also excluded from the studies. All subjects had a sedentary lifestyle and were instructed

Table 1. Baseline (run-in) characteristics of patients participating to Protocols 1 and 2

Patients	Protocol 1 LPD (0.55 g/kg)	Protocol 1 1.1 g protein/kg	Protocol 2
Number of patients	6	5	6
Age (yr)	62 ± 8	66 ± 7	69 ± 4
Gender (Male/Female)	4/2	4/1	5/1
BMI (kg/m ²)	23 ± 5	25 ± 1	25 ± 2
eGFR (ml/min per 1.73 m ²)	17 ± 4	17 ± 5	12 ± 5 ^a
Etiology of CKD, n (%)			
Hypertension	2 (33)	2 (40)	1 (17)
Glomerulonephritis	3 (50)	2 (40)	3 (50)
Interstitial nephritis	1 (17)	1 (20)	2 (33)
Diuretic use			
(furosemide 25–50 mg)	1 (17)	1 (17)	2 (33)
SGA	6.5 (1)	7 (1)	7 (1)
BUN (mg/dl)	94 ± 7	97 ± 6	99 ± 4
Albumin (g/l)	42 ± 2	43 ± 2	41 ± 1.5
C-reactive protein (mg/l)	2.6 (0.70)	2.8 (0.80)	2.9 (0.60)
[HCO ₃ ⁻] (mmol/l)	24 ± 1.5	25 ± 1	25 ± 1
Cholesterol (mg/dl)	204 ± 35	224 ± 23	170 ± 23
Hemoglobin (g/l)	111 (7)	114 (8)	112 (9)
Phosphate (mg/dl)	4.9 ± 0.32	4.6 ± 0.40	4.3 ± 1.0
Calcium (mg/dl)	9.3 ± 0.34	9.5 ± 0.19	9.5 ± 0.48
Proteinuria (g/d)	0.82 ± 0.21	0.76 ± 0.25	1.5 ± 0.99

BMI, body mass index; BUN, blood urea nitrogen; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; SGA, Subjective Global Assessment.

Variables are described as mean ± SD or median and interquartile range.

^aSignificance of difference versus Protocol 1: *P* < 0.05 or less.

to refrain from physical exercise during the studies. Drugs prescribed as appropriate for each individual were also continued during the studies. All patients received B vitamins and folic acid. Two subjects in Protocol 1 and 2 subjects in Protocol 2 received diuretic therapy (furosemide 25–50 mg/d). Sodium bicarbonate (2–4 g/d) was supplemented as required to maintain plasma bicarbonate into normal ranges. At the time of the studies, all subjects were edema free. Patients did not experience any catabolic event or treatment during the studies. At the run-in, patients received a diet that contained 1.1 to 1.3 g/kg protein and 31 to 34 kcal/kg per day. Residual renal function persisted to be stable (difference between serum creatinine at inclusion and at the end of the studies $<65 \mu\text{mol/l}$) during the studies.

Experimental Protocols and Nutritional Assessment

Calculation of the required sample size was based on the effect size and variance observed in previous studies from our laboratory.^{22–24} Both the qualitative and quantitative effects of an LPD on muscle protein turnover in patients with CKD are unknown. Based on preliminary observations, the sample size calculation suggested a target of 10 subjects per protocol, to have an 80% chance to detect changes in protein degradation and net protein balance. We used the same approach for Protocol 2, although we had no preliminary data on the effects of sVLPD on muscle protein turnover. The studies were terminated after having collected 6 cases per protocol, because the primary objectives of the studies were met.

Schematic diagrams of the protocols used are depicted in Figures 1 and 2. The 2 protocols did not run simultaneously. Protocol 2 followed the completion of studies from Protocol 1. Protocol 1 was designed to study changes of muscle protein metabolism induced by LPD (Figure 1), and was a randomized, parallel design in which subjects were randomly divided into 2 groups that received either a 0.55 g/kg LPD or a 1.1 g/kg per day protein diet.

During the run-in phase, patients were instructed on a stable diet. In the LPD arm, forearm protein turnover was evaluated as follows: (i) after a 6-week period of a 1.1 g/kg per day protein diet, and (ii) 12 to 14 weeks after an LPD (initially 0.8 and subsequently 0.55 g/kg). The amount of dietary protein provided to replace urinary protein excretion was maintained constant during both dietary regimens. On usual protein intake, dietary carbohydrate and lipid represented 40% and 25% of the total energy intake, respectively. On low protein intake, the contribution of lipid to total energy was increased to 35%, by increasing the amount of unsaturated fat, mainly olive oil. At least half of protein

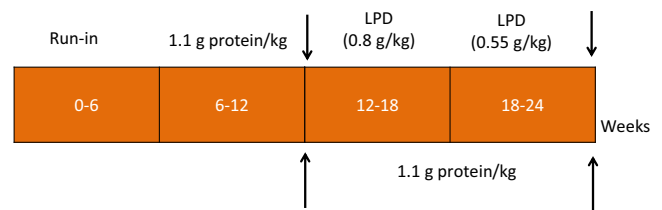


Figure 1. The design of the study in Protocol 1. Protocol 1 was a randomized, parallel design study in which subjects were randomly divided into 2 groups that received either a 0.55-g/kg low-protein diet (LPD) or a 1.1-g/kg per day protein diet. During the run-in phase, patients were instructed on a stable diet. In the LPD arm ($n = 6$), forearm protein turnover was evaluated as follows: (i) after a 6-week period of a 1.1-g/kg per day protein diet, and (ii) 12 weeks after an LPD (initially 0.8 and subsequently 0.55 g/kg). In the 1.1-g protein/kg arm ($n = 5$), muscle protein turnover was studied as follows: (i) after a 6-week period of a 1.1-g/kg per day protein diet, and (ii) 12 weeks after the patients continued the same diet. Arrows indicate forearm protein turnover studies.

of the diet was of high biological value. In the 1.1-g protein/kg arm muscle protein turnover was studied as follows: (i) at the baseline, and (ii) 12 to 14 weeks after the patients continued on a stable, standard 30 to 32 kcal, 1.1 g/kg per day protein diet.

Protocol 2 (Figure 2) was a crossover self-controlled study, which was designed to study the muscle metabolic responses to a supplemented (0.1 g/kg Alfa-Kappa; Fresenius Kabi, Isola, Italy) VLPD (0.45 g/kg), as compared with a 0.55-g/kg LPD. We chose a 3-period, 2-treatment crossover trial because with equivalent treatments a paired analysis provides better power than a parallel group study.^{25,26} Calorie intake was set at 33 kcal/kg per day. One Alfa-Kappa tablet contains 607 mg of a mixture of calcium salts of keto and hydroxy acids (keto-leucine, -isoleucine, -valine, -phenylalanine, and hydroxymethionine), plus 4 EAAs

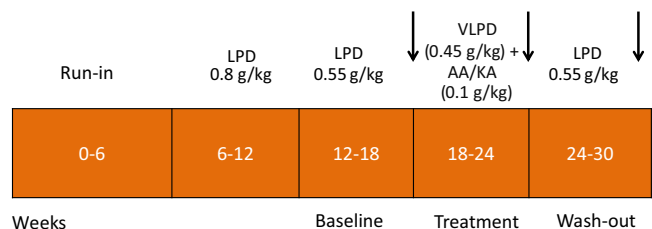


Figure 2. The design of the study in Protocol 2. Protocol 2 was designed to study the muscle metabolic responses to an amino acid/ketoacid (AA/KA)-supplemented (0.1 g/kg) very low protein diet (VLPD) (0.45 g/kg), as compared to a 0.55-g/kg low-protein diet (LPD). This protocol was a prospective, crossover trial, with patients serving as their own controls. The study consisted of three 6-week consecutive periods: the baseline period (LPD, 0.55 g/kg), the treatment period (VLPD 0.45 g/kg + AA/KA 0.1 g/kg), and the washout period (LPD, 0.55 g/kg). Arrows indicate forearm protein turnover studies.

(lysine acetate, threonine, histidine, and tyrosine). The ketoacid prescription was divided into 3 doses taken during meals. After the run-in period (during which patients were instructed on a stable diet), the study consisted of three 6-week consecutive periods: the baseline period (LPD, 0.55 g/kg), the treatment period (VLPD 0.45 g/kg + AA/KA 0.1 g/kg), and the washout period (LPD, 0.55 g/kg). Measurements of forearm and whole-body protein turnover were made at the end of each experimental period. Compliance to supplements was assessed by pill count.

Supplementary Figure S1 shows the CONSORT flow diagram for study 1. After the exclusion of 7 subjects, 6 subjects were randomized to an LPD (0.55 g/kg) or to a 1.1 g protein/kg diet (Protocol 1). All 6 subjects completed the study in the LPD arm, whereas 1 subject dropped out (we were not able to place the catheters) in the 1.1-g protein/kg diet arm. The CONSORT flow diagram for Protocol 2 is shown in Supplementary Figure S2. After the exclusion of 3 subjects, 7 subjects were included into the study. Six subjects completed the study, and 1 subject dropped out (inability to cannulate veins).

For both protocols, dietary interviews were conducted every 2 weeks with each patient before and after the inclusion. Residual renal function persisted to be stable (difference between serum creatinine at inclusion and at the end of the studies $<65 \mu\text{mol/l}$) during the 2 protocols. The dietary surveys included a 3-day food record and a 24-hour urine collection for urea measurement. Lean body mass was evaluated using bioelectrical impedance analysis that was performed using a BCM-Body Composition Monitor (FMC, Bad Homburg, Germany).

Protein Turnover Studies

Muscle protein metabolism was estimated by the forearm perfusion technique associated with ^2H -phenylalanine kinetics.^{22,27–29} The studies were performed in the post-absorptive, overnight-fasted state, at rest, by using a continuous infusion of L-(ring- $^2\text{H}_5$)-phenylalanine (D_5 -Phe) (Euriso-top, Saint Aubin, France) (0.035 $\mu\text{mol/kg}$ per minute) and forearm arteriovenous difference. Blood flow across the forearm was determined after each arteriovenous sampling.²²

Anthropometric Data, Assays, and Calculations

Anthropometric data analysis, assays, and calculations are reported elsewhere and in Supplementary File S1.

Statistical Analyses

Data normality was checked by Kurtosis and Skewness. All data are presented as mean \pm SE or median (interquartile range). Subject clinical characteristics in different groups were compared using the Wilcoxon

rank sum test for continuous variables. Variables found to deviate from normality were log-transformed before applying statistical tests. The 2-tailed t test was used to compare arterial with venous data. In Protocol 1, when the arteriovenous difference was different from 0 ($P < 0.05$), a 2-factor repeated-measures analysis of variance was used to assess differences over time and between treatments for protein turnover. In Protocol 2, a repeated-measures analysis of variance was used to compare the overall changes during the phases of the study. When analysis of variance indicated statistical significance ($P < 0.05$), a *post hoc* F -based test was performed between phases (StatView Statistical Package; Abacus, Berkeley, CA).

RESULTS

Compliance to LPD

Compliance to the diets was obtained after training during the run-in period and thereafter. As shown in Tables 2 and 3, the energy intake of patients was kept within 31–33 kcal/kg per day.

Overall, patients in Protocol 1 progressively reduced their daily protein intake by approximately 50% (Table 2). Their body weight, body mass index, fat-free mass, and muscle area did not change during the experimental periods.

Table 3 shows dietary compliance and body composition during Protocol 2.

nPNA declined from 0.67 g/kg when patients were on an LPD to 0.58 g/kg under the sVLPD. The overall AA/KA supplement dose per patient was 0.149 ± 0.01 tablets/kg body weight per day (i.e., $\sim 91\%$ of prescribed dose). No change in body weight, body mass index, muscle area, and fat-free mass was observed during the study.

Table 2. Compliance, serum biochemistry, and body composition during the adaptation to a low (0.55 g/kg) protein diet in Protocol 1

Parameter	1.1 g/kg	0.8 g/kg	0.55 g/kg
Energy Intake (kcal/kg per day)	33 \pm 3	32 \pm 3	34 \pm 3
Urinary urea (mmol/d)	394 \pm 73	277 \pm 51	168 \pm 38 ^a
nPNA (g/kg per day)	1.26 \pm 0.15	0.95 \pm 0.20	0.66 \pm 0.14 ^a
Body weight (kg)	64.7 \pm 9	64.9 \pm 9	64.9 \pm 9
BMI (kg/m ²)	23.0 \pm 2	22.9 \pm 2	22.8 \pm 2
Mid-arm muscle area (cm ²)	52.0 \pm 6	51.6 \pm 6	51.7 \pm 6
LTM (kg)	44 \pm 3	45 \pm 3	45 \pm 3
eGFR (ml/min per 1.73 m ²)	17 \pm 5	18 \pm 5	17 \pm 5
[HCO ₃ ⁻] _a (mmol/l)	24 \pm 1	24 \pm 2	24 \pm 2
Albumin (g/l)	41 \pm 2.2	42 \pm 2.7	41 \pm 2.6

BMI, body mass index; eGFR, estimated glomerular filtration rate; LTM, lean tissue mass; nPNA, normalized protein nitrogen appearance.

^aSignificance of difference versus 1.1 g/kg diet: $P < 0.01$ or less.

Data are mean \pm SD. Estimation of energy intake was performed through a 3-day dietary record.

Table 3. Compliance and body composition during the 0.55 g/kg low-protein diet (LPD) and the AA/KA-supplemented (0.1 g/kg) very low protein diet (VLPD) (0.45 g/kg)

Parameter	Basal-LPD	VLPD + AA/KA	Washout-LPD
Energy intake (kcal/kg per day)	32 ± 2	32 ± 2	31 ± 3
Urinary urea (mmol/d)	195 ± 31	156 ± 25 ^a	181 ± 30
nPNA (g/kg per day)	0.67 ± 0.2	0.58 ± 0.2 ^a	0.64 ± 0.2
Body weight (kg)	73 ± 13	73 ± 14	73 ± 14
BMI (kg/m ²)	24.5 ± 3.7	24.5 ± 3.7	24.4 ± 3.6
Mid-arm muscle area (cm ²)	51 ± 7	51 ± 6	50 ± 6
FFM (kg)	51 ± 9	51 ± 10	51 ± 9
eGFR (ml/min per 1.73 m ²)	12 ± 5	11 ± 4	12 ± 4
[HCO ₃ ⁻] (mmol/l)	25 ± 2	26 ± 2	24 ± 2
Albumin (g/l)	41 ± 3.8	41 ± 5.2	42 ± 4.2

AA/KA, amino acid/ketoacid; BMI, body mass index; eGFR, estimated glomerular filtration rate; FFM, fat-free mass; nPNA, normalized protein nitrogen appearance. nPNA was estimated by a 24-hour urea collection using Maroni's formula.

^aSignificance of difference versus basal and washout LPD diets: $P < 0.05$. Data are mean ± SD. Estimation of energy intake was performed through a 3-day dietary record.

Effects of an LPD on Skeletal Muscle Protein Synthesis and Degradation

Table 4 shows the effects of the LPD on muscle protein turnover. Individual data are reported in Supplementary Table S1.

At the baseline, protein turnover data were similar in the LPD and 1.1-g protein/kg groups ($P = \text{NS}$). In the postabsorptive, basal state, protein degradation exceeded protein synthesis and net protein balance was negative. This negative balance was markedly decreased (from -17 to -10 nmol/min per 100 ml forearm, -40%, $P < 0.02$) by the LPD (Table 3). This effect was entirely accounted for by a 17% decrease in muscle protein degradation (from 51.0 to 42.5 nmol/min per 100 ml forearm, $P < 0.02$), whereas muscle protein synthesis was not affected by the 0.55-g/kg LPD.

In patients with CKD under the 1.1-g/kg diet, there was no change over time with regard to net protein balance, protein synthesis, and degradation (Table 3).

Effect of an LPD on the Efficiency of Muscle Protein Turnover

In accordance with previous studies,²²⁻²⁴ in the basal period, 66% of phenylalanine that appeared from

muscle protein degradation was cycled back into protein synthesis (Table 3 and Figure 3). During the LPD, this value increased to 76% ($P < 0.05$). No change in the efficiency of protein turnover was observed in patients with CKD who maintained the 1.1-g/kg diet.

Effect of an LPD on Whole-Body Protein Degradation and on the Contribution of Skeletal Muscle to Whole-Body Protein Turnover

As a next step, we compared the effects of an LPD on muscle and whole-body protein degradation. In the postabsorptive state, there is no entry of AAs from dietary sources, and the flux of phenylalanine in the body is derived from entry of phenylalanine released from protein breakdown. Therefore, the measurement of the whole-body rate of appearance of phenylalanine in the postabsorptive state is a measure of the whole-body proteolysis.³⁰ In accordance with previous observations,^{9,10} an LPD induced a slight decrease in whole-body protein degradation (0.64 ± 2 vs. $0.69 \pm 2 \mu\text{mol/kg per minute per } 1.73 \text{ m}^2$ LPD vs. usual protein diet, $P < 0.06$). Considering that the mean fraction of muscle tissue is approximately 0.6,^{31,32} that blood flow in muscle is 70% of total flow, and that muscle is on average 40% of body weight, one could estimate the contribution of skeletal muscle to whole-body degradation to decline from 48% to 39% ($P < 0.03$) during an LPD (Figure 4). Accordingly, extra-muscle tissues appear to be scarcely sensitive to a decrease in protein intake, in comparison with muscle tissue.

Effects of an sVLPD on Skeletal Muscle Protein Synthesis and Degradation and Efficiency of Protein Turnover

The effect of the sVLPD on protein turnover is shown in Table 5. Individual data points are reported in Supplementary Table S2.

At the baseline, when patients were studied under the LPD, protein degradation rates exceeded protein synthesis rates; however, muscle protein degradation rates were low and not different from those observed

Table 4. Effects of a 0.55-g/kg low-protein diet (LPD) on muscle protein synthesis, degradation, and net protein balance in patients with chronic kidney disease (CKD) (phenylalanine uptake or release, nmol/min per 100 ml)

Diet	1.1 g/kg				0.55 g/kg			
	Protein synthesis	Protein degradation	Net protein balance	Efficiency (%)	Protein synthesis	Protein degradation	Net protein balance	Efficiency (%)
CKD-LPD ($n = 6$)	34 (6.0)	51 (5.0)	-17 (2.0) ^a	66 (6.1)	32 (5)	42.5 (4.0) ^b	-10 (5.0) ^{a,b}	76 (7.8) ^c
	1.1 g/kg				1.1 g/kg			
CKD- 1.1 g protein/kg ($n = 5$)	33 (5.6)	48 (9.75)	-16 (3.5) ^a	67 (4.9)	35 (5.75)	51 (4.25)	-17 (3.5) ^a	68 (8.8)

^aSignificance of the arterial-venous difference: $P < 0.01$ or less.

^bTreatment effect: $P < 0.02$ versus the 1.1-g protein/kg diet.

^cTreatment effect: $P < 0.05$.

CKD-LPD subjects were studied after a 6-week period on a 1.1-g protein/kg protein diet and after 6 weeks on a 0.55-g protein/kg protein diet. Patients with CKD on a 1.1-g protein/kg diet were studied twice, at 6-week time points, while they were on a 1.1-g protein/kg diet. Data are expressed as median (interquartile range).

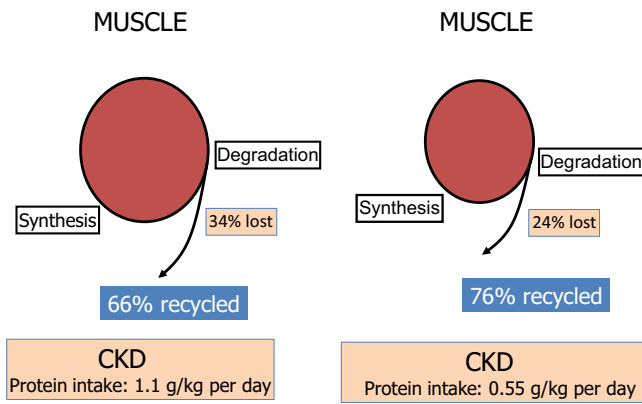


Figure 3. The magnitude of daily protein turnover requires reutilization of amino acids released by protein breakdown for protein synthesis. In patients with chronic kidney disease (CKD) and diets containing 1.1 g/kg protein, 64% of amino acid phenylalanine is recycled into protein synthesis. During a low-protein diet (0.55 g/kg), the entity of amino acid recycling increases to 75%, indicating greater efficiency of protein turnover.

in the Protocol 1 study ($P = NS$). The ingestion of the sVLPD was followed by a less catabolic state, expressed by an approximately 55% decline of the net muscle protein balance ($P < 0.02$) (Table 4) and a further (19%) ($P < 0.05$) increase in the efficiency of muscle protein turnover (Table 4 and Figure 5). This effect was likely the consequence of a combination of mechanisms, including as trend ($P < 0.06$), an 18% increase in muscle protein synthesis, combined with no significant change in muscle protein degradation.

Effects of an sVLPD on Whole-Body Protein Degradation

As compared with the LPD, whole-body protein degradation was not changed by the sVLPD (0.63 ± 0.1 vs. 0.66 ± 0.1 and $0.64 \pm 0.2 \mu\text{mol/kg per min per } 1.73 \text{ m}^2$ in the 3 study periods, $P = NS$).

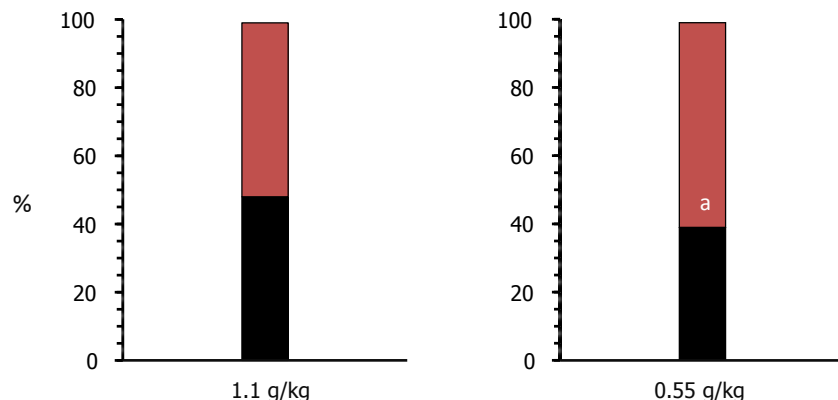


Figure 4. The contribution of muscle to whole-body protein degradation declines markedly during a low-protein diet. a = $P < 0.05$ versus the 1.1-g/kg diet.

DISCUSSION

In the basal, postabsorptive state, muscle protein balance is negative because protein degradation exceeds protein synthesis. The first new observation made from this study is that in patients with CKD in the post-absorptive state, skeletal muscle responds to an LPD through a change in muscle protein dynamics, which is characterized by a less negative protein balance due to the combined effects of reduced protein degradation, unchanged protein synthesis, and overall increased efficiency of protein metabolism. Clearly, these changes serve to minimize muscle N losses and may result in N and muscle mass preservation in response to decreased N intake.

The molecular mechanisms underlying the response of muscle protein metabolism to changes in nutrient intake are only in part known in humans. The modulation of intracellular pathways of protein degradation by changes in AA or protein availability are conserved across different species, including insects and vertebrates.^{1,33} In chicken muscle, daily variations in dietary lysine content alter the expression of several proteolysis-related genes, including atrogin-1 and MuRF-1.³⁴ Similarly, in soleus muscles from growing rats, an LPD decreases muscle protein degradation, an effect that is associated with reductions in the mRNA levels of atrogin-1 and MuRF-1, proteasome, and caspase-3 activity.³⁵ Besides an effect due to reduced AA availability from ingested protein, it is suggested that a decrease in muscle protein degradation pathways follows an increase in insulin sensitivity, with an upregulation of insulin receptor and phosphorylation of Akt.^{36,37} Contrariwise, high protein intakes inhibit other actions of insulin at multiple levels, including the insulin-stimulated tyrosine phosphorylation of insulin receptor-substrate-1 and -2, and insulin-stimulated phosphatidylinositol 3-kinase.³⁸

Table 5. Effects of an AA/KA-supplemented very low protein diet (VLPD) versus a low-protein diet (LPD) on muscle protein synthesis, degradation, and net protein balance in patients with CKD (phenylalanine uptake or release, nmol/min per 100 ml)

Patients	LPD (0.55 g/kg)				VLPD (0.45 g/kg) + EAA/KA (0.1 g/kg)				LPD (0.55 g/kg) washout			
	Protein synthesis	Protein degradation	Net protein balance	Efficiency (%)	Protein synthesis	Protein degradation	Net protein balance	Efficiency (%)	Protein synthesis	Protein degradation	Net protein balance	Efficiency (%)
CKD (n = 6)	32 (4)	45 (5.0)	-12 (1.0)	72 (2.3)	38 (8)	44 (6)	-6.5 (4) ^a	86 (8.3) ^b	32 (4.0)	48 (6)	-15 (5)	67 (8.3)

AA/KA, amino acid/ketoacid, CKD, chronic kidney disease; EAA, essential AA.

^aTreatment effect: $P < 0.01$ or less versus LPD and LPD washout periods.

^b $P < 0.02$ or less versus LPD and LPD washout periods.

Six subjects with CKD were studied after a 6-week period on a 0.55-g protein/kg protein diet (LPD), after 6 weeks on a 0.45-g protein/kg VLPD supplemented with 0.1 g/kg amino and ketoacids, and again after 6 weeks on a 0.55-g protein/kg protein diet (LPD washout). Data are expressed as median and interquartile range.

As a second new finding, we observed that muscle protein turnover after an LPD proved to be more efficient than during a normal protein intake. The magnitude of daily protein turnover, with AA flux several-fold greater than intake,^{2,3} needs large reutilization of AA released by protein breakdown for protein synthesis. According to the model studied here, as much as 60% to 65% of phenylalanine deriving from proteolysis is cycled back into protein synthesis in CKD, a figure similar to what is observed in the normal condition.^{22,27,28} In our study, this percentage increased by 12% when patients ate an LPD, and increased further with an sVLPD. Therefore, during the adaptation to an LPD, protein turnover is more efficient in AA recycling. This observation is in keeping with the preserved nutritional conditions of patients studied here and is part of the response to an LPD. It must be underlined that in patients studied here, metabolic acidosis was corrected by bicarbonate supplementation. One could speculate that the marked decrease in muscle protein degradation observed after the LPD could have been hindered by uncorrected acidosis.

Preservation of muscle protein synthesis is a necessary component of the “adaptation” to a low protein

intake.^{5,6} In our study, muscle protein synthesis was unaffected by a 0.55-g/kg LPD, showing that changes occurring in muscle at this protein intake are truly adaptive. However, it is unclear which protein intake levels may be safe for skeletal muscle, even in healthy subjects. Hursel *et al.*⁷ observed that a 10% to 12% decrease in whole-body protein synthesis and degradation took place during the adaptation to a low (0.4 g/kg) versus high (2.4 g/kg) protein intake; however, the 0.4-g/kg protein diet did not lower fractional muscle protein synthesis. These data suggest that muscle protein synthesis in some healthy young individuals may be unexpectedly maintained at very low protein intakes. However, if not supplemented, a 0.45-g/kg day is not sufficient to maintain protein synthesis and muscle mass in elderly subjects,^{39,40} who now constitute a large majority of patients with CKD in many countries.

As a next step, we studied how muscle protein metabolism adapts to a 0.45-g/kg per day protein, supplemented with 0.1 g/kg EAA and KA, as compared with a 0.55-g/kg LPD. In keeping with previous studies,^{41,42} we observed that the sVLPD was not associated with changes in body weight and composition; however, in terms of muscle protein kinetics, this diet was associated with a lower net muscle catabolism, as compared with the LPD.

The already low muscle protein degradation rates attained during an LPD were not further reduced by the sVLPD, suggesting that adaptation to a more restricted protein intake cannot be attained by further restraining protein degradation. In our study, the decrease in net protein catabolism by the sVLPD was likely an effect of increased efficiency of protein turnover and preservation of muscle protein synthesis, as compared with the LPD. These effects are in keeping with studies showing that higher protein quality intake better stimulates muscle protein anabolism and protein synthesis.^{43,44} Leucine, in particular, has been identified as the key factor stimulating the post-prandial rise in muscle protein synthesis.⁴⁵ Recently, autophagy and apoptosis have been shown to be reduced when an LPD was supplemented with leucine and KA, as compared with an LPD, in rodents with CKD.^{46,47} However, we cannot

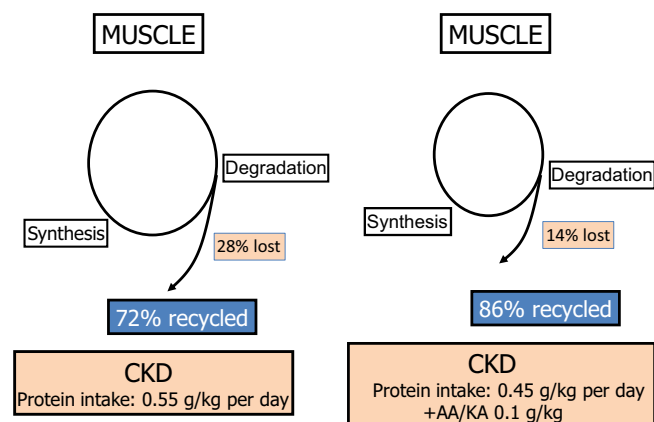


Figure 5. In patients with chronic kidney disease (CKD) and diets containing 0.55 g/kg protein, 72% of amino acid (AA) phenylalanine is recycled into protein synthesis. During a very low-protein diet (0.45 g/kg) supplemented with AA and ketoacids (AA/KA) (0.1 g/kg), the entity of AA recycling increases to 83%, indicating greater efficiency of protein turnover.

exclude the possibility that the persistent suppression of protein degradation and the decrease in net protein balance under the sVLPD was, at least in part, a carry-over effect of previous LPD treatment. In this regard, a significant carryover effect of exposure to an LPD before the treatment period appears unlikely, because protein metabolism fluctuates largely during the day in response to nutrient ingestion and the effects of dietary protein on protein turnover are very fast and reversible.⁴⁸ In addition, the effects of the sVLPD on net protein balance were reversed during the washout LPD period.

It is commonly retained that whole-body measurements of protein turnover are not a sensitive metabolic indicator of adequacy of protein intake.⁴⁹ In our study, although muscle protein metabolism showed to be extremely sensitive to the level of protein intake, extramuscle tissue seemed to be scarcely affected. Because splanchnic organs account for as much as 40% to 50% of whole-body protein degradation, one could assume that protein turnover in these tissues is less sensitive to protein intake than in skeletal muscle. This is also reflected by the finding that liver albumin synthesis is scarcely influenced by protein intakes varying from 0.5 to 1.0 g/kg, if calorie intake is adequate.^{50–52}

A possible limitation of our study is that patients studied here were selected for good compliance to protein-restricted diets. Therefore, the results of our study cannot be extended to patients who are not adherent to dietary treatment. Wasting may develop if not all aspects of the protein-restricted diet are properly implemented or followed or when dietary intake is involuntarily reduced during intercurrent illnesses. In addition, we studied the response to reduced protein intake only in the basal, postabsorptive state. Previous studies have shown that neutral body N balance on an sVLPD can be attained by reduction of whole-body leucine oxidation and postprandial inhibition of protein degradation.^{16,17} However, our study is the first to address the issue of the adaptation of muscle protein turnover to LPDs. Further studies are needed to explore the response of muscle protein turnover to feeding different diets in patients with CKD. A recent observation in older, otherwise healthy subjects shows that the habituation to an LPD (0.7 g/kg) compared with a high protein diet (1.5 g/kg) augments the postprandial availability of dietary protein-derived AAs in the circulation and does not lower basal muscle protein synthesis rates after ingestion of protein.⁵² However, no information is available for patients with CKD.

Another possible limitation of this study is that both methods we used to measure muscle mass, anthropometry and bioimpedance, are influenced by the hydration status. Therefore, it is still possible that during

the study subtle changes in muscle mass may have occurred and passed unnoticed.

In conclusion, we used phenylalanine turnover methodology to examine how uncomplicated, moderately severe CKD affects the ability of patients to adapt to dietary protein restriction in skeletal muscle. Our study demonstrates that both LPD and sVLPD significantly affect forearm protein metabolism. Overall, the results of this study indicate skeletal muscle protein metabolism adaptation to restrained protein intake can be obtained via combined responses of reduced protein degradation and increased efficiency of AA recycling.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

This study was supported by grants from the Fresenius, Kabi, Bad Homburg, Germany (Fresenius Kabi Ketosteril Award 2012) and University of Genoa (Finanziamento di Ateneo).

SUPPLEMENTARY MATERIAL

Figure S1. Flow diagram of studies performed under Protocol 1.

Figure S2. Flow diagram of studies performed under Protocol 2.

Supplementary Methods.

Table S1. Effects of a 0.55-g/kg low-protein diet (LPD) on muscle protein synthesis, degradation, and net protein balance in patients with chronic kidney disease (CKD) (phenylalanine uptake or release, nmol/min per 100 ml).

Table S2. Effects of an amino acid/ketoacid (AA/KA)-supplemented very low protein diet (VLPD) versus a low-protein diet (LPD) on muscle protein synthesis, degradation, and net protein balance in patients with chronic kidney disease (CKD) (phenylalanine uptake or release, nmol/min per 100 ml).

Supplementary material is linked to the online version of the paper at www.kireports.org.

REFERENCES

1. Piccirillo R, Demontis F, Perrimon N, et al. Mechanisms of muscle growth and atrophy in mammals and *Drosophila*. *Dev Dyn*. 2014;243:201–215.
2. Young VR. Kinetics of human acid metabolism: nutritional implications and some lessons. McCollum Award Lecture. *Am J Clin Nutr*. 1987;46:709–725.
3. Waterlow JC, Garlick PJ, Millward DJ. *Protein Turnover in Mammalian Tissues and the Whole Body*. Amsterdam: Elsevier/North-Holland, Biomedical Press; 1978.
4. Waterlow JC. What do we mean by adaptation? In: Blaxter KL, Waterlow JC, eds. *Nutritional Adaptation in Man*. London: John Libbey; 1985:1–10.

5. Motil KJ, Matthews DE, Bier DM, et al. Whole-body leucine and lysine metabolism: response to dietary protein intake in young men. *Am J Physiol.* 1981;240:E712–E721.
6. Westerterp-Plantenga MS, Nieuwenhuizen A, Tome D, et al. Dietary protein, weight loss, and weight maintenance. *Annu Rev Nutr.* 2009;29:21–41.
7. Hursel R, Martens EAP, Gonnissen KJ, et al. Prolonged adaptation to a low or high protein diet does not modulate basal muscle protein synthesis rates – a substudy. *PLoS One.* 2015;10:e0137183.
8. Williams B, Hattersley J, Layward E, et al. Metabolic acidosis and skeletal muscle adaptation to low protein diets in chronic uremia. *Kidney Int.* 1991;40:779–786.
9. Goodship THJ, Mitch WE, Hoerr RA, et al. Adaptation to low-protein diets in renal failure: leucine turnover and nitrogen balance. *J Am Soc Nephrol.* 1990;1:66–75.
10. Bernhard J, Beaufreere B, Laville M, et al. Adaptive response to a low-protein diet in predialysis chronic renal failure patients. *J Am Soc Nephrol.* 2001;12:1249–1254.
11. Mitch WE. Malnutrition: a frequent misdiagnosis for hemodialysis patients. *J Clin Invest.* 2002;12:437–439.
12. Coresh J, Walser M, Hill S. Survival on dialysis among chronic renal failure patients treated with a supplemented low-protein diet before dialysis. *J Am Soc Nephrol.* 1995;6:1379–1385.
13. Walser M. Urea metabolism in chronic renal failure. *J Clin Invest.* 1974;53:1385–1392.
14. Mitch WE, Walser M, Sapir DG. Nitrogen sparing induced by leucine compared with that induced by its keto analogue, alpha-ketoisocaproate, in fasting obese man. *J Clin Invest.* 1981;67:553–562.
15. Mitch WE. Metabolism and metabolic effects of ketoacids. *Am J Clin Nutr.* 1981;33:1642–1648.
16. Masud T, Young VR, Chapman T, et al. Adaptive responses to very low protein diets: the first comparison of ketoacids to essential amino acids. *Kidney Int.* 1994;45:1182–1192.
17. Tom K, Young VR, Chapman T, et al. Long-term adaptive responses to dietary protein restriction in chronic renal failure. *Am J Physiol.* 1995;268:E668–E674.
18. Gelfand RA, Glickman MG, Castellino P, et al. Measurement of L-[1-14C]leucine kinetics in splanchnic and leg tissues in humans. Effect of amino acid infusion. *Diabetes.* 1988;37:1365–1372.
19. Tessari P, Garibotto G, Inchiostro S, et al. Kidney, splanchnic, and leg protein turnover in humans. Insight from leucine and phenylalanine kinetics. *J Clin Invest.* 1996;98:1481–1492.
20. Fouque D, Mitch WE. Low-protein diets in chronic kidney disease: are we finally reaching a consensus? *Nephrol Dial Transplant.* 2015;30:6–8.
21. Castaneda C, Charnley JM, Evans WJ, et al. Elderly women accommodate to a low-protein diet with losses of body cell mass, muscle function, and immune response. *Am J Clin Nutr.* 1995;62:30–39.
22. Garibotto G, Russo R, Sofia A, et al. Skeletal muscle protein synthesis and degradation in patients with chronic renal failure. *Kidney Int.* 1994;45:1432–1439.
23. Garibotto G, Sofia A, Canepa A, et al. Acute effects of peritoneal dialysis with dialysates containing dextrose or dextrose and amino acids on muscle protein turnover in patients with chronic renal failure. *J Am Soc Nephrol.* 2001;12:557–567.
24. Garibotto G, Russo R, Sofia A, et al. Metabolic acidosis and the adaptation of muscle protein metabolism to dietary protein intake in patients with chronic renal failure. *J Am Soc Nephrol.* 1997;8(Suppl S):A0330.
25. Maclure M. The case-crossover design: a method for studying transient effects on the risk of acute events. *Am J Epidemiol.* 1991;133:144–153.
26. Cleophas TJ, de Vogel EM. Crossover studies are a better format for comparing equivalent treatments than parallel-group studies. *Pharma World Sci.* 1998;20:113–117.
27. Louard RJ, Fryburg DA, Gelfand RA, et al. Insulin sensitivity of protein and glucose metabolism in human forearm skeletal muscle. *J Clin Invest.* 1992;90:2348–2354.
28. Garibotto G, Sofia A, Russo R, et al. Insulin sensitivity of muscle protein metabolism is altered in patients with chronic kidney disease and metabolic acidosis. *Kidney Int.* 2015;88:1419–1426.
29. Tessari P, Deferrari G, Robaudo C, et al. Phenylalanine hydroxylation across the kidney in humans. *Kidney Int.* 1999;56:2168–2172.
30. Matthews DE. An overview of phenylalanine and tyrosine kinetics in humans. *J Nutr.* 2007;137(6 Suppl 1):1549S–1555S.
31. Yki-Jarvinen H, Young A, Lamkin C, et al. Kinetics of glucose disposal in whole body and across the forearm in man. *J Clin Invest.* 1987;79:1713–1719.
32. Denne SC, Litchely EA, Liu YM, et al. Proteolysis in skeletal muscle and whole body in response to euglycemic hyperinsulinemia in normal adults. *Am J Physiol.* 1991;261:E809–E814.
33. Sakuma K, Yamaguchi A. Sarcopenia and cachexia: the adaptations of negative regulators of skeletal muscle mass. *J Cachexia Sarcopenia Muscle.* 2012;3:77–94.
34. Tesseraud S, Bouvarel I, Collin A, et al. Daily variations in dietary lysine content alter the expression of genes related to proteolysis in chicken pectoralis major muscle. *J Nutr.* 2009;139:38–43.
35. Batistela E, Pereira MP, Siqueira JT, et al. Decreased rate of protein synthesis, caspase-3 activity, and ubiquitin-proteasome proteolysis in soleus muscles from growing rats fed a low-protein, high-carbohydrate diet. *Can J Physiol Pharmacol.* 2014;92:445–454.
36. Rigalleau V, Blanchetier V, Combe C, et al. Low-protein diet improves insulin sensitivity of endogenous glucose production in predialytic uremic patients. *Am J Clin Nutr.* 1997;65:1512–1516.
37. Dos Santos MP, Batistela E, Pereira MP, et al. Higher insulin sensitivity in EDL muscle of rats fed a low-protein, high-carbohydrate diet inhibits the caspase-3 and ubiquitin-proteasome proteolytic systems but does not increase protein synthesis. *J Nutr Biochem.* 2016;34:89–98.
38. Patti ME, Brambilla E, Luzi L, et al. Bidirectional modulation of insulin action by amino acids. *J Clin Invest.* 1998;101:1519–1529.
39. Castaneda C, Charnley JM, Evans WJ, et al. Elderly women accommodate to a low-protein diet with losses of body cell

- mass, muscle function, and immune response. *Am J Clin Nutr.* 1995;62:30–39.
40. Castaneda C, Dolnikowski GG, Dallal GE, et al. Protein turnover and energy metabolism of elderly women fed a low protein diet. *Am J Clin Nutr.* 1995;62:40–48.
 41. Fouque D, Chen J, Chen W, et al. Adherence to ketoacids/essential amino acids-supplemented low protein diets and new indications for patients with chronic kidney disease. *BMC Nephrol.* 2016;17:6–12.
 42. Shah AP, Kalantar-Zadeh K, Kopple JD. Is there a role for ketoacid supplements in the management of CKD? *Am J Kidney Dis.* 2015;65:659–673.
 43. Symons TB, Sheffield-Moore M, Wolfe RR, et al. A moderate serving of high-quality protein maximally stimulates skeletal muscle protein synthesis in young and elderly subjects. *J Am Diet Assoc.* 2009;109:1582–1586.
 44. Koopman R, Verdijk L, Manders RJ, et al. Co-ingestion of protein and leucine stimulates muscle protein synthesis rates to the same extent in young and elderly lean men. *Am J Clin Nutr.* 2006;84:623–632.
 45. Pennings B, Boirie Y, Senden JM, et al. Whey protein stimulates postprandial muscle protein accretion more effectively than do casein and casein hydrolysate in older men. *Am J Clin Nutr.* 2011;93:997–1005.
 46. Huang J, Wang J, Gu L, et al. Effect of a low-protein diet supplemented with ketoacids on skeletal muscle atrophy and autophagy in rats with type 2 diabetic nephropathy. *PLoS One.* 2013;8:e81464–e81470.
 47. Wang DT, Lu L, Shi Y, Geng ZB, et al. Supplementation of ketoacids contributes to the up-regulation of the Wnt7a/Akt/p70S6K pathway and the down-regulation of apoptotic and ubiquitin-proteasome systems in the muscle of 5/6 nephrectomized rats. *Br J Nutr.* 2014;11:1536–1548.
 48. Arnal M, Oled C, Attaix D, et al. Dietary control of protein turnover. *Diabete Metab.* 1987;13:630–642.
 49. World Health Organization. *Protein and Amino Acid Requirements in Human Nutrition: Report of a Joint WHO/FAO/UNU Expert Consultation.* WHO Technical Report Series, no. 935. Geneva, Switzerland: World Health Organization; 2007. Available at: http://apps.who.int/iris/bitstream/10665/43411/1/WHO_TRS_935_eng.pdf. Accessed February 21, 2018.
 50. Gersovitz M, Munro HN, Udall J, et al. Albumin synthesis in young and elderly subjects using a new stable isotope methodology: response to level of protein intake. *Metabolism.* 1980;29:1075–1086.
 51. Levitt DG, Levitt MD. Human serum albumin homeostasis: a new look at the roles of synthesis, catabolism, renal and gastrointestinal excretion, and the clinical value of serum albumin measurements. *Int J Gen Med.* 2016;9:229–255.
 52. Gorissen SH, Horstman AM, Franssen R, et al. Habituation to low or high protein intake does not modulate basal or postprandial muscle protein synthesis rates: a randomized trial. *Am J Clin Nutr.* 2017;105:332–342.