Published in final edited form as:

Metabolism. 2018 January ; 78: 167–178. doi:10.1016/j.metabol.2017.09.015.

Metabolic phenotyping using kinetic measurements in young and older healthy adults

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Abstract

Background—The aging process is often associated with the presence of sarcopenia. Although changes in the plasma concentration of several amino acids have been observed in older adults, it remains unclear whether these changes are related to disturbances in whole body production and/or interconversions.

Methods—We studied 10 healthy young (~22.7 y) and 17 older adults (~64.8 y) by administering a mixture of stable amino acid tracers in a pulse and in a primed constant infusion. We calculated whole body production (WBP) and metabolite to metabolite interconversions. In addition, we measured body composition, muscle function, and provided questionnaires to assess daily dietary intake, physical activity, mood (anxiety, depression) and markers of cognitive function. Plasma enrichments and metabolite concentrations were measured by GC- and LC-MS/MS and statistics were performed by student t-test.

Results—Older adults had a 11% higher body mass index ($p=0.04$) and 27% reduced peak leg extension force ($p=0.02$) than the younger group, but comparable values for muscle mass, mood and cognitive function. Although small differences in several plasma amino acid concentrations were observed, we found older adults had about 40% higher values of WBP for glutamine (221 ± 27 vs. 305 ± 21 $\mu\text{mol/kg ffm/h}$, $p=0.03$) and tau-methylhistidine (0.15 ± 0.01 vs. 0.21 ± 0.02 $\mu\text{mol/kg ffm/h}$, $p=0.04$), 26% lower WBP value for arginine (59 ± 4 vs. 44 ± 4 $\mu\text{mol/kg ffm/h}$,

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Trial registry: ClinicalTrials.gov; NCT01787682 URL: www.clinicaltrials.gov

Author contributions: NEPD, GAMTH, JT, DW and MPKJE had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. NEPD and MPKJE designed the research and were involved in recruitment of subjects and conduct of the study. NEPD, GAMTH, JT, DW and MPKJE were involved in the data analysis, sample analysis and writing of the manuscript.

Other contributions: We thank Tiffany Smit, Fari Koeman, Cindy Lieben, Agate McNew, Sunday Simbo, Renate Jonker, Barbara van der Meij and Clayton Cruthirds and CTRAL nurse Cassie, Amy, Blair and Liz for assisting in the data collection.

Conflicts of Interest: None of the authors had any financial or personal interest in any company or organization sponsoring the research, including advisory board affiliations.

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p=0.02) and a reduction in WBP (50%; 1.23 ± 0.15 vs. 0.69 ± 0.06 $\mu\text{mol/kg ffm/h}$, p=0.001) and concentration (25%; 3.5 ± 0.3 $\mu\text{mol/l}$ vs. 2.6 ± 0.2 $\mu\text{mol/l}$, p=0.01) for β -Hydroxy β -Methylbutyrate. No differences were observed in protein catabolism. Clearance of arginine was decreased (27%, P=0.03) and clearance of glutamine (58%, p=0.01), leucine (67%, p=0.001) and KIC (76%, p=0.004) were increased in older adults.

Conclusions—Specific differences exist between young and older adults in amino acid metabolism.

Keywords

metabolic phenotype; plasma amino acids; stable tracers; whole body production; aging

1. Introduction

The process of aging is often accompanied by sarcopenia, which is characterized by loss of muscle mass and strength with functional impairment and adverse outcomes (1, 2), including higher risks of falls, disability, hospitalization and death (3–5). In order to understand how metabolism changes during the process of aging, many studies have measured the concentration of metabolites in plasma of younger and older adults. Lower plasma concentrations were found in older subjects for the amino acids glycine and histidine levels (6), alanine, glycine and histidine (7) whereas higher values were found for glutamate and ornithine concentrations (8). On the contrary, a number of studies describe the differences in plasma concentration between both groups as small or undetectable (9, 10).

As plasma amino acid concentration only reflects the disposal capacity of that amino acid by the body (11), studies were conducted using stable tracer infusions in younger and older subjects to measure the whole body rate of appearance (WbRa) of amino acids as a proxy for whole body production (WBP). No differences were found between younger and older adults in WbRa of leucine turnover, Wb oxidation rate (12–14) or WbRa of phenylalanine (14), although some observed a 10–15% reduction in WbRa of Phenylalanine in the older adults (15, 16). Limited information is available on WbRa of non-essential amino acids. We previously found that older adults have a lower glutamate WbRa (17), whereas no changes were observed in WbRa of phenylalanine, leucine, valine, isoleucine (10), or tau-methylhistidine (18).

Clear insight into the effects of aging on amino acid metabolism is, however of crucial importance as aging induced metabolic disturbances might serve as potential therapeutic targets. The data reported were obtained in different groups of older and younger adults with a wide variation in body composition and age and the groups were not gender-matched. To examine whole body protein and amino acid kinetics on an individual level, we recently developed a pulse method combining multiple stable tracers, which makes it possible to unravel multiple metabolic pathways in the same individual simultaneously.

Besides information of the individual's metabolic phenotype, the pulse method has several technical advantages. Amino acid metabolism is usually measured by primed-constant infusion of stable tracers of amino acids (19–22) in which careful priming is required to

obtain tracer steady state. However, this is problematic for a number of amino acids. The WbRa of glutamine (23), taurine (24) and tau-methylhistidine (25) are very difficult to measure as these amino acids have large pool sizes in the body that will take a long time to reach steady-state. In contrast, the pulse method is able to measure WbRa of amino acids with different pool sizes by analyzing the decay curves of the tracer enrichments in plasma using (non)-compartmental modeling (19). In order to combine WbRa measurements of amino acids with different pool sizes, we developed a new approach using a mixture of stable tracers of amino acids. The tracers in this mixture do not interfere with enrichment measurements of other amino acids and can be measured by LC-MS/MS or GC-MS/MS. In the present study, we examined the metabolic phenotype of younger and older subjects with no major health conditions from the MEDIT trial (Metabolism of Disease with Isotope Tracers), a large controlled and still recruiting trial in healthy and diseased subjects in which comprehensive metabolic characterization is performed by combined pulse and primed-constant infusion of multiple stable tracers. We studied younger and older adults in the MEDIT trial, who were carefully characterized by body composition, muscle strength, habitual physical activity, protein intake and markers of mood and cognitive function. We hypothesize that the process of aging in humans affects amino acid whole body production and interconversions, which contributes to differences in nutritional status, mood and functional performance between the older and younger groups.

2. Materials and methods

2.1 Subjects

MEDIT included 10 young adults and 17 older adults (Table 1) who were recruited *via* advertisements in the local community. Medical history and medication use were assessed as part of the screening process. Inclusion criteria 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 (e.g. cancer, heart failure, COPD), ability to walk, sit down and stand up independently, age 55 years or older (older adults group), age between 20–30 years old (younger group). The exclusion criteria were history of untreated metabolic diseases including hepatic or renal disorder, presence of fever within the last 3 days, body mass index $>35 \text{ kg/m}^2$ and $< 18 \text{ kg/m}^2$, use of protein or amino acid containing nutritional supplements within 1 month of study day, highly physically active in daily life (involved in regular strenuous physical activity program). Written informed consent was obtained from all subjects, and the study was approved by the Institutional Review Board of Texas A&M University.

2.2 Study protocol

All subjects were studied in the Clinical Research Unit of the Center for Translational Research on Aging and Longevity at Texas A&M University under postabsorptive conditions (no food after 12am). The study procedures were identical in both groups and the study day lasted approximately 5 hr.

2.2.1 Body composition—In the early morning, body weight was measured by a digital beam scale and height by a stadiometer. BMI was calculated by dividing body weight by

squared height. Furthermore, whole body, trunk and extremity (arms and legs) fat mass (FM) and fat-free mass (FFM) were obtained in the subjects by dual-energy X-ray absorptiometry (DXA) (Hologic QDR 4500/Version 12.7.3.1 (Bedford, MA)) while the subjects were in supine position. The anthropometric and body composition data of all subjects were standardized for height. For this purpose, FM and FFM were divided by squared height (kg/m^2), as suggested by VanItallie (26) to obtain BMI, FFM index (FFMI), and FM index (FMI) and compared to published reference data (27, 28).

2.2.2 Stable tracer infusion by IV pulse or continuous infusion—For stable tracer infusions (continuous and pulse), a peripheral line was placed in a vein of the lower arm and for blood sampling in a superficial dorsal vein of the contralateral hand. The hand was placed in a thermostatically controlled hot box (internal temperature: 60°C), a technique to mimic direct arterial sampling (29). After a venous blood sample was collected to measure baseline enrichment, an IV pulse containing a cocktail of amino acid stable tracers (Cambridge Isotope Laboratories: Woburn, MA, USA) was administered. The labels and concentrations of the different stable tracers are listed in Table 2. In addition, after 90 min, we started a primed constant infusion with stable tracers of phenylalanine, tyrosine and tryptophan. Arterialized-venous blood was sampled at $t = 2, 5, 10, 15, 20, 30, 40, 50, 60, 90, 120, 150, 180, 210, 225,$ and 240 minutes after pulse administration. Samples were processed as described below for analysis of tracer enrichments and concentrations of amino acids.

2.2.3 Muscle function—At the end of the study day to estimate overall muscle function, both respiratory and skeletal (hand/leg) muscle function were measured in all subjects. Maximal expiratory pressure (MEP) and inspiratory pressure (MIP) were assessed by determining the maximal value of at least 3 reliable attempts using a hand-held mouth pressure device (Micro Respiratory Pressure Meter (RPM)). Following pre-test stretching and warm-up, the peak leg torque during one leg reciprocal extensions (at $60^\circ/\text{sec}$) and the peak handgrip force that the subject were able to generate out of 3 reproducible repetitions, with 1 minutes of rest between each attempt, were used as a marker of leg strength and handgrip strength, using Kincom isokinetic dynamometry (Isokinetic International, Chattanooga, TN) and Vernier dynamometry (Vernier software and Technology, Beaverton, OR) respectively. After 5 minutes of rest, leg endurance was assessed by performing 30 maximal single leg extensions (at $120^\circ/\text{sec}$)(30) to assess a decrease in leg force output (31). We used the average force output of the last 3 repetitions as percentage of the first 3 repetitions.

2.2.4 Questionnaires—Habitual dietary intake was assessed by 24-hour dietary recall, a retrospective method that consists of precisely recalling, describing and quantifying the intake of foods and beverages consumed in the 24 hour period prior to the study day. Physical activity level in the week was measured by the Physical Activity Scale (PASE) for the Elderly questionnaire (32). As changes in Tryptophan metabolism could be related to differences in mood and cognition between the young and older groups, the Hospital Anxiety and Depression Scale (HADS) (33) was used to assess levels of anxiety and depression symptom frequencies during the previous week. The Stroop Color Word

Interference Test (34) was used as a measure of executive functioning that assesses selective/focused attention, cognitive flexibility as response inhibition for colored printed words.

2.3 Biochemical analysis

We collected arterialized-venous blood into Li-heparinized or EDTA tubes (Becton Dickinson Vacutainer system, Franklin Lakes, New Jersey, USA), placed tubes immediately on ice and promptly centrifuged them (4°C, 3,120 x *g* for 5 min) to obtain plasma. Plasma was aliquotted with vortexing to tubes containing either 0.1 vol of 33% (w/w) trichloroacetic acid or the residue after evaporation of 0.17 vol of 33% (w/w) 5-sulfosalicylic dihydrate to denature proteins. Storage was at -80°C.

We measured plasma amino acids and the enrichments of their tracers batchwise by stable tracer-dilution LC/MS/MS (22, 35, 36). In brief, we diluted deproteinized plasma supernatants (or no-plasma calibrators) 7.5-fold in reagents required for 9-fluorenylmethoxycarbonyl (Fmoc) reaction, the final recipe being Fmoc-Cl (2.5 mg/mL), acetonitrile (50%), boric acid (233 mM) and sufficient NaOH for an initial pH of 9.5. We stopped reactions after 15 min by adding 1/3rd vol of 1 M acetic acid in 8% v/v acetonitrile. We injected 0.2–1.7 µL into the 24 µL/min pumped flow (microLC 200, Eksigent, part of AB Sciex) of 73:23:4 water-acetonitrile-isopropanol containing 11 mM ammonium acetate, thus onto a 100 x 0.5 mm column packed by Eksigent with 2.7 µm 90 Å HALO C18 fused-core silica beads (Advanced Materials Technology). Fmoc-amino acids eluted over 3.2 min as we displaced this mobile phase with acetonitrile at a rate of 5.6%/min; *bis*-Fmoc-amino acids (ornithine, histidine, lysine and tyrosine) eluted by 4.5 min after we stepped acetonitrile to 56% then reestablished the same gradient slope. Ions were conveyed by heated electrospray (3.8 kV IS; 150°C TEM; 14 GAS1; 25 GAS2) to a 5500 QTRAP (Sciex) for multiple reaction monitoring (MRM) of the loss of the Fmoc moiety or moieties, regenerating the amino acid anion. Collision energy (CE) was de-optimized as needed for analytes that would otherwise risk detector saturation. Peaks were automatically identified and integrated by the SignalFinder1 algorithm in MultiQuant v. 3.0 (Sciex). We used default integration settings except we deselected a saturation correction option and narrowed the retention-time search window half-width to 8 sec. Every integration was then inspected. Manual reintegration (redrawing of baselines and peak boundaries) was disallowed, but manual re-identification was allowed as needed to coerce the automatic reintegration of a missed peak, a misidentified peak (e.g., of ILE as LEU) or a region wherein two peaks previously had been merged or conversely one peak inappropriately split. Peak areas were exported to Excel for calculation of area ratios for 1) concentration measurements using a tracer for the internal standard and 2) enrichment measurements of an exogenous tracer (or tracer metabolite) to the corresponding amino acid. Area ratios of calibrators were regressed against calibrator known tracer-to-tracee ratios (TTR) by ordinary least squares (Prism 7, GraphPad). By reverse prediction, we then converted each plasma sample area ratio to a TTR.

2.4 Calculations

The Rate of Appearance (Ra) of tyrosine, phenylalanine and tryptophan was calculated from the last hour of the primed constant infusion period as: $Ra = \text{Tracer Infusion Rate} / \text{median TTR}$ (22, 37). For all other tracers that were given with the pulse, we measured the Ra with

non-compartmental analysis. Non-compartmental analysis was done using GraphPad Prism (version 7.0). The change in TTR over time was fitted (19). The decay of the TTR of the stable tracers that were injected with the pulse (DOSE) were fitted with either two exponentials: $TTR(t) = a \cdot \exp(-k_1 \cdot t) + b \cdot \exp(-k_2 \cdot t)$ or three exponentials: $TTR(t) = a \cdot \exp(-k_1 \cdot t) + b \cdot \exp(-k_2 \cdot t) + c \cdot \exp(-k_3 \cdot t)$. The area under the curve (AUC) was calculated from the integral of the two or three exponential curve (38). Ra is then calculated as DOSE/AUC. The Ra was calculated as $DOSE/(a/k_1 + b/k_2)$ for 2 exponentials and as $DOSE/(a/k_1 + b/k_2 + c/k_3)$ for 3 exponentials. For metabolites from the injected stable tracers, the change in the TTR during the study was fitted as: $TTR(t) = -a \cdot \exp(-k_1 \cdot t) + b \cdot \exp(-k_2 \cdot t) + c \cdot \exp(-k_3 \cdot t)$ and the integral was calculated to represent the AUC. The exact composition of the pulse of stable tracers or that of the continuous infusion rate are in Table 1.

We calculated the conversion of 1 amino acid into another amino acid by using the Ra of the product amino acid and the ratio between the TTR at plateau or AUC of the TTR from pulse of the product/substrate (38, 39). Thus the conversion of phenylalanine was calculated as $Ra_{TYR} (L-[^{13}C_9-^{15}N]tyrosine) \cdot TTR(L-[ring-^2H_4]tyrosine)/TTR(L-[ring-^2H_5]phenylalanine)$. The same approach is used to calculate the conversion of arginine to citrulline, citrulline to arginine, glutamate to glutamine and leucine to KIC. In addition, we calculated the absolute production of HMB from the fractional synthesis rate (40) of L-[5,5,5- 2H_3]leucine to HMB and multiplying this with the plasma HMB concentration.

Clearance of the stable tracers was calculated as Ra/plasma concentration (19).

2.5 Statistical analysis

Results are expressed as mean $\pm$ standard error (SE). If data failed the normality or equal variance test, they were log-transformed where appropriate. The unpaired Student's t test was used to determine differences in body composition, muscle function, and plasma metabolites between the young and older groups. The statistical package within Graphpad Prism (Version 7.0), and SPSS (version 20) was used for data analysis. The level of significance was set at $p < 0.05$.

3. Results

We studied a total of 27 subjects, 10 young adults and 17 older adults (Table 2). Besides a difference in age, the older adults were characterized by a 11% higher BMI ($P=0.039$) due to a 27% higher fat mass ($P=0.077$). Although total lean mass was not different between the groups, we found a 27% lower peak leg extension force in the older adults ($P=0.017$). No differences were found in physical activity, dietary intake, mood, and cognitive function between the groups.

Postabsorptive plasma concentrations of several amino acids are shown in Table 3. We found significant higher concentrations in the older adults for the non-essential amino acids aspartate (42%, $P=0.029$), glutamate (75%, $P=0.002$), citrulline (37%, $P=0.013$), serine (17%, $P=0.025$) and taurine (24%, $P=0.011$) and a significant lower large neutral amino acids to tryptophan (LNAA/TRP) ratio (19%, $P=0.017$). No significant difference was found

in plasma concentrations of essential amino acids. We also found lower KIC (25%, $P=0.020$) and HMB (25%, $P=0.0098$) concentrations in the older adults.

We also measured the plasma amino acid concentration kinetics in young adults for 4 hours after administering the tracer pulse (Figure 1). Except for glycine, the tracee (unlabeled) concentrations of the amino acids that were included in the pulse did not change.

Although the plasma concentrations of some amino acids were different between younger and older adults, their whole body productions (WBP; Table 4) did not differ. In contrast, we found a higher WBP of glutamine (38%; $P=0.031$) and tau-methylhistidine (40%; $P=0.041$), while the WBP of arginine (26%; $P=0.019$) was lower in older adults. We also found a trend for increased conversion of glutamate to glutamine in older adults (61%; $P=0.052$) that partially could explain the higher WBP of glutamine. Older and young adults had a comparable phenylalanine to tyrosine conversion at about 8% of phenylalanine WBP, suggesting equal net protein breakdown in the postabsorptive state. In the downstream leucine pathway, HMB production was reduced by 50% in the older adults. The WBP and interconversion of several other amino acids were not significantly different between the older and young adults, indicating that amino acid metabolism is surprisingly comparable between young and older adults, except a few exceptions.

The conversion of arginine to citrulline, a proxy of nitric oxide production, was also assessed. After administering a pulse of the arginine stable tracer, the plasma enrichment of citrulline followed a characteristic pattern (Figure 2). Interestingly, the citrulline enrichment remained high for at least 4 hours, suggesting that the pool in which the conversion takes place is large. In contrast, the conversion of citrulline to arginine was rapid and most likely takes place in a smaller pool that is quickly mixed. The arginine *de novo* production was about 54% of citrulline WBP in the young adults and 40% in the older adults, and about 10% of arginine WBP in both the young and older adults. Nitric Oxide (NO) production was about 44% of citrulline WBP in young adults and 27% in older adults, and about 7% of arginine WBP in both the young and older adults.

As we measured both the WBP and the plasma concentrations of several amino acids, we were able to calculate the clearance rates of these amino acids (Table 5). We found a reduced clearance rate of arginine (27%; $p=0.033$), and increased clearance rates of glutamine (58%; $p=0.007$), leucine (67%; $p=0.0007$) and KIC (76%; $p=0.0035$) in the older adults. The clearance rates of all other amino acids were not different between the groups.

We furthermore analyzed whether lower values of muscle mass and function (as markers of sarcopenia) were associated with metabolic changes. Appendicular lean index was associated with plasma concentration of BCAA ($r:0.57$, $p=0.0019$), BCKA ($r:0.47$, $p=0.0223$), tau-methylhistidine ($r:0.47$, $p=0.0136$), HMB ($r:0.39$, $p=0.0628$), and LNAA ($r:0.54$, $p=0.0035$), as well as Ra of KIC ($r:-0.42$, $p=0.0410$), leucine to KIC conversion rate ($r:-0.49$, $p=0.0159$), KIC to leucine conversion ($r:-0.48$, $p=0.0171$), glycine ($r:-0.45$, $p=0.0197$) and glutamate ($r:-0.54$, $p=0.0178$). Handgrip strength was associated with plasma concentrations of BCAA ($r:0.56$, $p=0.0022$), BCKA ($r:0.57$, $p=0.0040$), HMB ($r:0.49$, $p=0.0179$), tau-methylhistidine ($r:0.51$, $p=0.0066$), LNAA ($r:0.54$, $p=0.0038$), and Ra

of KIC ($r:-0.54$, $p=0.0067$), KIC to leucine conversion ($r:-0.44$, $p=0.0306$), glycine ($r:-0.58$, $p=0.0014$) and taurine ($r:-0.54$, $p=0.0162$). Leg strength was associated with plasma concentration of BCAA ($r:0.54$, $p=0.0054$), BCKA ($r:0.59$, $p=0.0050$), HMB ($r:0.59$, $p=0.0046$), tau-methylhistidine ($r:0.44$, $p=0.0295$), LNAA ($r:0.50$, $p=0.0109$), and glutamate to glutamine conversion rate ($r:-0.56$, $p=0.0158$).

4. Discussion

We studied whether plasma concentrations and whole body production of a large set of amino acids differ between younger and older adults. Our new approach to combine many stable tracers in one pulse is innovative as it makes measurement of many important metabolic pathways in the same subject possible. Furthermore, it provides an important tool to unravel the metabolic disturbances present in many conditions and disease states. We found that the metabolic differences between younger and older adults were small and only present for some non-essential amino acids and related metabolites including glutamine, tau-methylhistidine and HMB.

The older group was characterized by lower limb muscle strength and higher fat mass than the younger group. Fat-free mass, mood and marker of cognitive function however were not different between the groups. The average total PASE value for the older adults was above normal values (41) indicating that they were physically active. This might explain that physical activity was not different between the older and younger groups, although we realize that the PASE questionnaire used is developed particularly for the older population.

4.1 Elevated whole body glutamine and tau-methylhistidine production rates in the older adults

In the older adults, we found significant increases in some of the non-essential amino acids, which is in line with other studies (6–10). As plasma concentrations are related to the disposal capacity of the corresponding substrates (11), the higher concentration of some amino acids in the older group could be related to a reduction in clearance rates for these same amino acids (Table 5).

We only found increased WBP rates for glutamine and tau-methylhistidine, and reduced WBP rates for arginine and HMB in the older adults. All other measured WBP were not significantly different. We and others found glutamine WBP rates between 300 and 400 $\mu\text{mol/kg bw/h}$ in older adults (42, 43) and between 250 and 300 $\mu\text{mol/kg bw/h}$ in younger adults (44) when measured by primed-constant infusion protocol. As it remains very difficult to obtain a tracer steady state with GLN tracer infusion, it causes an underestimation (23) of GLN enrichment and thus an overestimation of whole body GLN production. Time to steady state in GLN was calculated to be at least 12 hours after priming because of the large intramuscular glutamine pool. This problem does not exist when administering a pulse of GLN tracer. We therefore conclude that whole body GLN production is higher in older than in younger adults (Table 4). We also found higher rates for tau-methylhistidine production in the older adults, which corresponds with a higher muscle interstitial tau-methylhistidine concentration in older adults (45). The observed negative correlation between appendicular skeletal muscle mass and tau-methylhistidine production, as marker of myofibrillar protein

breakdown, is in line with our previous study in COPD (46). Ninety percent of the total body tau-methylhistidine pool is present in skeletal muscle (47), and the ubiquitin-proteasome pathway is assumed to provide most of the proteolytic activity required for the degradation of myofibrillar protein (48). Although glycine production was not different between older and younger subjects, low values for appendicular muscle mass was associated with elevated glycine production rates. Glycine is a precursor for metabolites such as glutathione and creatine. Although its mechanism of action remains unclear, glycine has the potential to protect muscles in wasting diseases and during dieting or fasting via its anti-inflammatory properties and suppression of production of reactive oxygen species (49, 50). Whether the enhanced glycine production in these adults is a mechanism to protect against sarcopenia needs further investigation.

4.2 Reduced whole body HMB production rate despite elevated leucine and KIC metabolism in the older adults

We found differences in the pathway from leucine to KIC to HMB between younger and older adults. Although comparable values were observed for plasma leucine concentration, WBP of leucine and KIC, and the clearance rates for leucine and KIC were higher in the older adults. Further, the plasma concentration and WBP of HMB were substantially lower in older adults. In older rats lower plasma leucine (51) and HMB concentrations were found (52), but HMB WBP rates in humans have not been reported before.

4.3 Why is conversion of leucine to HMB reduced in older adults?

WBP of leucine depends on whole body protein synthesis/breakdown and the downstream conversion to KIC. No changes were observed in WBP of leucine and KIC, which is in agreement with the unchanged WBP of phenylalanine. However, there is a clear reduction in the conversion of leucine to HMB. At the moment, there is no understanding why this happens (52), but points to a possible intervention with HMB in older adults that could have potential positive effects on, for instance, muscle mass. Studies in C2C12 myoblasts already have shown enhanced viability at 25 μ M levels, but no studies to date have studied the effects of lower HMB concentrations (53). We have shown that when HMB is given as a dietary supplement, it can reduce bedrest-related muscle loss in older adults (54). Furthermore, in the present study muscle weakness was associated with low HMB plasma concentration which is in line with previous data in young and older adults (55). Like for the BCAA, there is evidence that HMB positively influences protein metabolism by increasing muscle protein synthesis and reducing breakdown (56, 57). Moreover, HMB is able to improve functional capacity (strength, power and aerobic performance (58, 59), particularly in those who are untrained (60).

4.4 What are the (dis)advantages of using a stable tracer pulse approach as compared to the commonly used primed-constant and continuous tracer infusion approach?

Our aim was to simultaneously measure the whole body production and interconversion rates of several important amino acids. In the past, we have done a number of studies that combined the measurement of stable tracers to measure protein kinetics and pathways like the arginine pathway (20, 35, 61). We used the primed-constant and continuous infusion protocol as this can be used in situations in which the concentration of the metabolite in

plasma is approximately known to calculate the priming dose. When concentrations are very different between subjects because of their condition or disease, this will pose a problem. This usually is solved by administering a tracer prime but also a tracer infusion of several hours to assure a tracer steady state (40).

The pulse method does not need any knowledge about the pool size (19) and thus can be used in every subject, independent of the disease condition, pool sizes etc. We believe this is a major advantage. The trade-off is that in contrast to the primed- constant and continuous infusion protocol, more blood samples are often required to be able to fit the enrichment decay curves in order to obtain the complete AUC (including the AUC of the first 2 minutes and after 4 hours). On the other hand, by combining a large set of stable tracers in the pulse solution with different decay rates, we need to take an appropriate number of blood samples that covers the decay of all stable tracers in the pulse. In addition, the amount of stable tracers needed for the pulse approach is much less than used for the primed- constant and continuous infusion protocol. Therefore, we think that the pulse approach has more advantages and could be a better approach to measure metabolism on an individual basis.

When all possible metabolic pathways in humans are taken into consideration, we do not see any potential problems of combining the used set of stable tracers. The MS/MS method enables us to measure the enrichment of the different isotopomers. Any future expansion of the pulse with other stable tracers requires careful consideration in relation to the possible metabolic pathways, the possible MS/MS approaches for analysis, and the costs of the tracers.

The absolute values for WBP, measured by the pulse approach seem to be generally different from that obtained by the primed-constant and continuous infusion approach. For instance, the WBP of arginine was 44.0 (4.0) $\mu\text{mol/kg ffm/h}$ in healthy older adults, but within the range of 80–100 $\mu\text{mol/kg ffm/h}$ range in our previous studies (20, 21, 35). Also for citrulline, the WBP was lower (11.1 (0.8) $\mu\text{mol/kg ffm/h}$) than that obtained in previous studies (range of 13–15 $\mu\text{mol/kg ffm/h}$). The same was also observed for citrulline to arginine conversion (arginine *de novo* production). In contrast, the conversion of arginine to citrulline (NO production) was much higher (4.4 (0.5) $\mu\text{mol/kg ffm/h}$) than previously found in healthy subjects, using the primed-constant and continuous infusion tracer technique (0.2 $\mu\text{mol/kg ffm/h}$ (20, 35, 62). However, we and others found NO production rates in healthy subjects in the range of 1–4 $\mu\text{mol/kg ffm/h}$ (21, 40, 63). Although direct comparisons of metabolic fluxes between studies should be done with caution (different study protocols, methods of detection and study groups), these large differences between studies suggest methodological problems such as not having accurately primed the pool of citrulline that is produced from arginine. Furthermore, we have recently shown in the same animal that the rate of appearance (Ra) measured with non-compartmental analysis relates very well with the Ra measured with the primed-constant and continuous infusion tracer technique, albeit higher rates were found for phenylalanine appearance (64). We therefore hypothesize that the differences in the NO production between the different studies is mainly caused by the fact that citrulline enrichment from the arginine to citrulline conversion is often underestimated when using the continuous infusion tracer method. This type of problem can only be resolved by using the pulse approach to measure whole body NO production.

Higher rates of whole body NO production with the pulse approach now show that arginine to citrulline production is in balance with the citrulline to arginine production (Table 4). We think that the pulse method provides a better understanding of the relationship between NO production and arginine *de novo* production, suggesting that when arginine *de novo* production is reduced, often present in critically-ill patients (40), NO production is also reduced. We suggest that the pulse approach is the preferable method particularly when products of amino acids are measured that require more time to reach steady state, which is required to enable calculation of their conversion rates. Previously, this was also suggested for tyrosine produced from phenylalanine (8h to reach steady state). This was solved by simultaneously providing a prime of tyrosine like we did in the present study (65). However, this approach is complicated in situations such as when the phenylalanine to tyrosine conversion is increased (e.g. higher net protein breakdown) that require a large prime amount. The pulse approach does not need any adjustment and can be used in any circumstance as long as the individual is in a physiological steady state like in the fasting state or during sip feeding.

4.5 Strengths and weaknesses

The strengths of the presented work are that using the pulse approach, more in depth knowledge can be obtained about the fluxes and metabolism of metabolites in the same individual in one experiment. A weakness of the present study is that we used non-compartmental analysis to obtain the fluxes and conversions of the substrates, while possible compartmental analysis would give additional insight into the metabolic fluxes and could better highlight the intracellular fluxes. Another weakness is that we did not perform the pulse approach with the phenylalanine and tyrosine stable tracers.

4.6 Conclusions

Our translational research approach to measuring metabolic flux in humans indicates that younger and older adults have specific differences in . amino acid metabolism, when expressed per kg of fat-free mass and measured with the pulse tracer approach.

Acknowledgments

We sincerely thank the healthy older and younger subjects for their willingness to participate in this research study and to those who have made this work possible.

Source of Funding: Research reported in this publication was partially supported by the National Institute of Environmental Health Sciences of the National Institutes of Health [grant number P30ES023512]. "The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health."

Abbreviations

AUC	Area under the curve
BMI	Body Mass Index
BCAA	Sum of the branched-chain amino acids valine, isoleucine and leucine

BCKA	Sum of the branched-chain keto acids: KIC, KIV and KMV.
DXA	dual-energy X-ray absorptiometry
EAA	Sum of the amino acids threonine, valine, methionine, isoleucine, leucine, tryptophan, phenylalanine, histidine and lysine
FM	Fat Mass
FFM	Fat Free Mass
GC-MS/MS	Gas Chromatography–tandem Mass Spectrometry
HADS	The Hospital Anxiety and Depression Scale (HADS) (33)
HMB	β -hydroxy β -methylbutyrate
KIC	α -ketoisocaproic acid
KIV	α -ketoisovalerate
KMV	α -keto- β -methylvalerate
LC-MS/MS	Liquid Chromatography–tandem Mass Spectrometry
LNAA	Sum of the amino acids valine, isoleucine, leucine, phenylalanine and tyrosine
LNAA/TRP	Ratio between the LNAA and tryptophan concentration
MEDIT trial	MEtabolism of Disease with Isotope Tracers
MPE	Maximal expiratory pressure
MIP	Maximal inspiratory pressure
NO	Nitric Oxide
NEAA	Sum of the amino acids aspartate, glutamate, asparagine, glutamine, serine, glycine, arginine, alanine, proline and tyrosine
PASE	Physical Activity Scale for the Elderly questionnaire (32)
Ra	Rate of Appearance
Tau-Methylhistidine	3-methyl-histidine
STROOP	The Stroop Color Word Interference Test (34)
TTR	Tracer-Tracee Ratio
WBP	Whole Body Production

WbRa

Whole body Rate of Appearance

References

1. Morley JE, Abbatecola AM, Argiles JM, Baracos V, Bauer J, Bhasin S, Cederholm T, Coats AJ, Cummings SR, Evans WJ, Fearon K, Ferrucci L, Fielding RA, Guralnik JM, Harris TB, Inui A, Kalantar-Zadeh K, Kirwan BA, Mantovani G, Muscaritoli M, Newman AB, Rossi-Fanelli F, Rosano GM, Roubenoff R, Schambelan M, Sokol GH, Storer TW, Vellas B, von Haehling S, Yeh SS, Anker SD. Society on Sarcopenia C, Wasting Disorders Trialist W. Sarcopenia with limited mobility: an international consensus. *J Am Med Dir Assoc*. 2011; 12(6):403–9. DOI: 10.1016/j.jamda.2011.04.014 [PubMed: 21640657]
2. Lang T, Streeper T, Cawthon P, Baldwin K, Taaffe DR, Harris TB. Sarcopenia: etiology, clinical consequences, intervention, and assessment. *Osteoporos Int*. 2010; 21(4):543–59. DOI: 10.1007/s00198-009-1059-y [PubMed: 19779761]
3. Landi F, Cruz-Jentoft AJ, Liperoti R, Russo A, Giovannini S, Tosato M, Capoluongo E, Bernabei R, Onder G. Sarcopenia and mortality risk in frail older persons aged 80 years and older: results from the SIRENTE study. *Age Ageing*. 2013; 42(2):203–9. DOI: 10.1093/ageing/afs194 [PubMed: 23321202]
4. Landi F, Liperoti R, Russo A, Giovannini S, Tosato M, Capoluongo E, Bernabei R, Onder G. Sarcopenia as a risk factor for falls in elderly individuals: results from the SIRENTE study. *Clinical nutrition (Edinburgh, Scotland)*. 2012; 31(5):652–8. DOI: 10.1016/j.clnu.2012.02.007
5. Bianchi L, Ferrucci L, Cherubini A, Maggio M, Bandinelli S, Savino E, Brombo G, Zuliani G, Guralnik JM, Landi F, Volpato S. The Predictive Value of the EWGSOP Definition of Sarcopenia: Results From the InCHIANTI Study. *The journals of gerontology*. 2016; 71(2):259–64. DOI: 10.1093/geron/glv129 [PubMed: 26333772]
6. Van Eijk HM, Dejong CH, Deutz NE, Soeters PB. Influence of storage conditions on normal plasma amino-acid concentrations. *Clin Nutr*. 1994; 13(6):374–80. Epub 1994/12/01. [PubMed: 16843417]
7. Katsanos CS, Kobayashi H, Sheffield-Moore M, Aarsland A, Wolfe RR. Aging is associated with diminished accretion of muscle proteins after the ingestion of a small bolus of essential amino acids. *Am J Clin Nutr*. 2005; 82(5):1065–73. Epub 2005/11/11. doi: 82/5/1065 [pii]. [PubMed: 16280440]
8. Volpi E, Mittendorfer B, Wolf SE, Wolfe RR. Oral amino acids stimulate muscle protein anabolism in the elderly despite higher first-pass splanchnic extraction. *The American journal of physiology*. 1999; 277(3 Pt 1):E513–20. Epub 1999/09/14. [PubMed: 10484364]
9. Walrand S, Short KR, Bigelow ML, Sweatt AJ, Hutson SM, Nair KS. Functional impact of high protein intake on healthy elderly people. *American journal of physiology Endocrinology and metabolism*. 2008; 295(4):E921–8. DOI: 10.1152/ajpendo.90536.2008 [PubMed: 18697911]
10. Engelen MP, De Castro CL, Rutten EP, Wouters EF, Schols AM, Deutz NE. Enhanced anabolic response to milk protein sip feeding in elderly subjects with COPD is associated with a reduced splanchnic extraction of multiple amino acids. *Clin Nutr*. 2012; 31(5):616–24. Epub 2012/06/12. DOI: 10.1016/j.clnu.2012.04.006 [PubMed: 22682082]
11. Deutz NE. The 2007 ESPEN Sir David Cuthbertson Lecture: amino acids between and within organs. The glutamate-glutamine-citrulline-arginine pathway. *Clin Nutr*. 2008; 27(3):321–7. Epub 2008/05/27. DOI: 10.1016/j.clnu.2008.03.010 [PubMed: 18501998]
12. Boirie Y, Gachon P, Beaufrere B. Splanchnic and whole-body leucine kinetics in young and elderly men. *Am J Clin Nutr*. 1997; 65(2):489–95. [PubMed: 9022534]
13. Kullman EL, Campbell WW, Krishnan RK, Yarasheski KE, Evans WJ, Kirwan JP. Age attenuates leucine oxidation after eccentric exercise. *Int J Sports Med*. 2013; 34(8):695–9. DOI: 10.1055/s-0032-1327659 [PubMed: 23325713]
14. Moreau K, Walrand S, Boirie Y. Protein redistribution from skeletal muscle to splanchnic tissue on fasting and refeeding in young and older healthy individuals. *J Am Med Dir Assoc*. 2013; 14(9):696–704. DOI: 10.1016/j.jamda.2013.06.005 [PubMed: 24011660]
15. Koopman R, Walrand S, Beelen M, Gijsen AP, Kies AK, Boirie Y, Saris WH, van Loon LJ. Dietary protein digestion and absorption rates and the subsequent postprandial muscle protein synthetic

- response do not differ between young and elderly men. *J Nutr.* 2009; 139(9):1707–13. Epub 2009/07/25. jn.109.109173 [pii]. DOI: 10.3945/jn.109.109173 [PubMed: 19625697]
16. Pennings B, Koopman R, Beelen M, Senden JM, Saris WH, van Loon LJ. Exercising before protein intake allows for greater use of dietary protein-derived amino acids for de novo muscle protein synthesis in both young and elderly men. *Am J Clin Nutr.* 2011; 93(2):322–31. Epub 2010/11/19. DOI: 10.3945/ajcn.2010.29649 [PubMed: 21084649]
 17. Rutten EP, Engelen MP, Castro CL, Wouters EF, Schols AM, Deutz NE. Decreased whole-body and splanchnic glutamate metabolism in healthy elderly men and patients with chronic obstructive pulmonary disease in the postabsorptive state and in response to feeding. *J Nutr.* 2005; 135(9): 2166–70. [PubMed: 16140893]
 18. Sheffield-Moore M, Dillon EL, Randolph KM, Casperson SL, White GR, Jennings K, Rathmacher J, Schuette S, Janghorbani M, Urban RJ, Hoang V, Willis M, Durham WJ. Isotopic decay of urinary or plasma 3-methylhistidine as a potential biomarker of pathologic skeletal muscle loss. *Journal of cachexia, sarcopenia and muscle.* 2014; 5(1):19–25. DOI: 10.1007/s13539-013-0117-7
 19. Wolfe, RR., Chinkes, DL. *Isotope Tracers in Metabolic Research: Principles and Practice of Kinetic Analysis.* New York: Wiley, New York, New York; 2005. p. 274
 20. Jonker R, Deutz NE, Erbland ML, Anderson PJ, Engelen MP. Alterations in whole-body arginine metabolism in chronic obstructive pulmonary disease. *Am J Clin Nutr.* 2016; 103(6):1458–64. DOI: 10.3945/ajcn.115.125187 [PubMed: 27146652]
 21. Luiking YC, Poeze M, Deutz NE. Arginine infusion in patients with septic shock increases nitric oxide production without haemodynamic instability. *Clin Sci (Lond).* 2015; 128(1):57–67. DOI: 10.1042/CS20140343 [PubMed: 25036556]
 22. Engelen MP, Safar AM, Bartter T, Koeman F, Deutz NE. High anabolic potential of essential amino acid mixtures in advanced nonsmall cell lung cancer. *Annals of oncology : official journal of the European Society for Medical Oncology /ESMO.* 2015; 26(9):1960–6. DOI: 10.1093/annonc/mdv271
 23. Van Acker BA, Hulsewe KW, Wagenmakers AJ, Deutz NE, Van Kreel BK, Halliday D, Matthews DE, Soeters PB, Von Meyenfeldt MF. Absence of glutamine isotopic steady state: implications for the assessment of whole-body glutamine production rate. *Clin Sci (Lond).* 1998; 95(3):339–46. Epub 1998/09/09. [PubMed: 9730854]
 24. Rakotoambinina B, Marks L, Badran AM, Iglaki F, Thuillier F, Crenn P, Messing B, Darmaun D. Taurine kinetics assessed using [1,2-¹³C₂]taurine in healthy adult humans. *American journal of physiology Endocrinology and metabolism.* 2004; 287(2):E255–62. DOI: 10.1152/ajpendo.00333.2003 [PubMed: 15010336]
 25. Rathmacher JA, Nissen SL. Development and application of a compartmental model of 3-methylhistidine metabolism in humans and domestic animals. *Advances in experimental medicine and biology.* 1998; 445:303–24. Epub 1998/10/22. [PubMed: 9781398]
 26. VanItallie TB, Yang MU, Heymsfield SB, Funk RC, Boileau RA. Height-normalized indices of the body's fat-free mass and fat mass: potentially useful indicators of nutritional status. *Am J Clin Nutr.* 1990; 52(6):953–9. Epub 1990/12/01. [PubMed: 2239792]
 27. Demerath EW, Schubert CM, Maynard LM, Sun SS, Chumlea WC, Pickoff A, Czerwinski SA, Towne B, Siervogel RM. Do changes in body mass index percentile reflect changes in body composition in children? Data from the Fels Longitudinal Study. *Pediatrics.* 2006; 117(3):e487–95. DOI: 10.1542/peds.2005-0572 [PubMed: 16510627]
 28. Schutz Y, Kyle UU, Pichard C. Fat-free mass index and fat mass index percentiles in Caucasians aged 18–98 y. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity.* 2002; 26(7):953–60. Epub 2002/06/25. DOI: 10.1038/sj.ijo.0802037
 29. Abumrad NN, Rabin D, Diamond MP, Lacy WW. Use of a heated superficial hand vein as an alternative site for the measurement of amino acid concentrations and for the study of glucose and alanine kinetics in man. *Metabolism: clinical and experimental.* 1981; 30(9):936–40. Epub 1981/09/01. [PubMed: 7022111]
 30. Engelen M, Klimberg VS, Allasia A, Deutz NE. Presence of early stage cancer does not impair the early protein metabolic response to major surgery. *Journal of cachexia, sarcopenia and muscle.* 2017; 8(3):447–56. DOI: 10.1002/jcsm.12173

31. Saey D, Debigare R, LeBlanc P, Mador MJ, Cote CH, Jobin J, Maltais F. Contractile leg fatigue after cycle exercise: a factor limiting exercise in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2003; 168(4):425–30. DOI: 10.1164/rccm.200208-856OC [PubMed: 12714348]
32. Washburn RA, Smith KW, Jette AM, Janney CA. The physical activity scale for the elderly (PASE): Development and evaluation. *Journal of clinical epidemiology*. 1993; 46(2):153–62. DOI: 10.1016/0895-4356(93)90053-4 [PubMed: 8437031]
33. Zigmond AS, Snaith RP. The hospital anxiety and depression scale. *Acta Psychiatr Scand*. 1983; 67(6):361–70. [PubMed: 6880820]
34. Stroop JR. Studies of interference in serial verbal reactions. *Journal of Experimental Psychology*. 1935; 18(6):643–62. DOI: 10.1037/0096-3445.121.1.15
35. Engelen MP, Safar AM, Bartter T, Koeman F, Deutz NE. Reduced arginine availability and nitric oxide synthesis in cancer is related to impaired endogenous arginine synthesis. *Clin Sci (Lond)*. 2016; 130(14):1185–95. DOI: 10.1042/CS20160233 [PubMed: 27129191]
36. Walker DK, Thaden JJ, Wierchowska-McNew A, Engelen MP, Deutz NE. Determination of beta-hydroxy-beta-methylbutyrate concentration and enrichment in human plasma using chemical ionization gas chromatography tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2017; 1040:233–8. DOI: 10.1016/j.jchromb.2016.11.010
37. Jonker R, Deutz NE, Erbland ML, Anderson PJ, Engelen MP. Effectiveness of essential amino acid supplementation in stimulating whole body net protein anabolism is comparable between COPD patients and healthy older adults. *Metabolism: clinical and experimental*. 2017; 69:120–9. DOI: 10.1016/j.metabol.2016.12.010 [PubMed: 28285641]
38. Mason A, Engelen MP, Ivanov I, Toffolo GM, Deutz NE. A Four Compartments Compartmental Model to Assess Net Whole Body Protein Breakdown Using a Pulse of Phenylalanine and Tyrosine Stable Isotopes in Humans. *American journal of physiology*. 2017; ajpendo 00362 2016. doi: 10.1152/ajpendo.00362.2016
39. Clarke JT, Bier DM. The conversion of phenylalanine to tyrosine in man. Direct measurement by continuous intravenous tracer infusions of L-[³H] phenylalanine and L-[¹⁴C] tyrosine in the postabsorptive state. *Metabolism: clinical and experimental*. 1982; 31(10):999–1005. [PubMed: 7132735]
40. Luiking YC, Poeze M, Ramsay G, Deutz NE. Reduced citrulline production in sepsis is related to diminished de novo arginine and nitric oxide production. *Am J Clin Nutr*. 2009; 89(1):142–52. DOI: 10.3945/ajcn.2007.25765 [PubMed: 19056593]
41. Washburn RA, McAuley E, Katula J, Mihalko SL, Boileau RA. The physical activity scale for the elderly (PASE): evidence for validity. *J Clin Epidemiol*. 1999; 52(7):643–51. [PubMed: 10391658]
42. Kao C, Hsu J, Bandi V, Jahoor F. Alterations in glutamine metabolism and its conversion to citrulline in sepsis. *American journal of physiology Endocrinology and metabolism*. 2013; 304(12):E1359–64. DOI: 10.1152/ajpendo.00628.2012 [PubMed: 23612995]
43. Brinkmann SJH, Buijs N, Vermeulen MAR, Oosterink E, Schierbeek H, Beishuizen A, de Vries J-PPM, Wisselink W, van Leeuwen PA. Perioperative glutamine supplementation restores the disturbed renal arginine synthesis after open aortic surgery, a randomized controlled clinical trial. *American Journal of Physiology - Renal Physiology*. 2016; doi: 10.1152/ajprenal.00340.2015
44. Darmaun D, Matthews DE, Bier DM. Glutamine and glutamate kinetics in humans. *The American journal of physiology*. 1986; 251(1 Pt 1):E117–26. [PubMed: 2873746]
45. Trappe T, Williams R, Carrithers J, Raue U, Esmarck B, Kjaer M, Hickner R. Influence of age and resistance exercise on human skeletal muscle proteolysis: a microdialysis approach. *The Journal of physiology*. 2004; 554(3):803–13. DOI: 10.1113/jphysiol.2003.051755 [PubMed: 14608013]
46. Rutten EP, Franssen FM, Engelen MP, Wouters EF, Deutz NE, Schols AM. Greater whole-body myofibrillar protein breakdown in cachectic patients with chronic obstructive pulmonary disease. *Am J Clin Nutr*. 2006; 83(4):829–34. Epub 2006/04/08. [PubMed: 16600935]
47. Ballard FJ, Tomas FM. 3-Methylhistidine as a measure of skeletal muscle protein breakdown in human subjects: the case for its continued use. *Clin Sci (Lond)*. 1983; 65(3):209–15. [PubMed: 6872455]

48. Hasselgren PO, Fischer JE. Muscle cachexia: current concepts of intracellular mechanisms and molecular regulation. *Annals of surgery*. 2001; 233(1):9–17. [PubMed: 11141219]
49. Ham DJ, Murphy KT, Chee A, Lynch GS, Koopman R. Glycine administration attenuates skeletal muscle wasting in a mouse model of cancer cachexia. *Clin Nutr*. 2014; 33(3):448–58. DOI: 10.1016/j.clnu.2013.06.013 [PubMed: 23835111]
50. Koopman R, Caldwor MK, Ham DJ, Lynch GS. Glycine metabolism in skeletal muscle: implications for metabolic homeostasis. *Curr Opin Clin Nutr Metab Care*. 2017; 20(4):237–42. DOI: 10.1097/MCO.0000000000000383 [PubMed: 28375879]
51. Jourdan M, Deutz NE, Cynober L, Aussel C. Consequences of age-related splanchnic sequestration of leucine on interorgan glutamine metabolism in old rats. *J Appl Physiol* (1985). 2013; 115(2): 229–34. DOI: 10.1152/japplphysiol.01230.2012 [PubMed: 23640600]
52. Shreeram S, Ramesh S, Puthan JK, Balakrishnan G, Subramanian R, Reddy MT, Pereira SL. Age associated decline in the conversion of leucine to β -Hydroxy- β -Methylbutyrate in rats. *Experimental gerontology*. 2016; 80:6–11. doi: <http://dx.doi.org/10.1016/j.exger.2016.03.021>. [PubMed: 27073170]
53. Vallejo J, Spence M, Cheng AL, Brotto L, Edens NK, Garvey SM, Brotto M. Cellular and Physiological Effects of Dietary Supplementation with beta-Hydroxy-beta-Methylbutyrate (HMB) and beta-Alanine in Late Middle-Aged Mice. *PLoS One*. 2016; 11(3):e0150066.doi: 10.1371/journal.pone.0150066 [PubMed: 26953693]
54. Deutz NE, Pereira SL, Hays NP, Oliver JS, Edens NK, Evans CM, Wolfe RR. Effect of beta-hydroxy-beta-methylbutyrate (HMB) on lean body mass during 10 days of bed rest in older adults. *Clin Nutr*. 2013; 32(5):704–12. DOI: 10.1016/j.clnu.2013.02.011 [PubMed: 23514626]
55. Kuriyan R, Lokesh DP, Selvam S, Jayakumar J, Philip MG, Shreeram S, Kurpad AV. The relationship of endogenous plasma concentrations of beta-Hydroxy beta-Methyl Butyrate (HMB) to age and total appendicular lean mass in humans. *Experimental gerontology*. 2016; 81:13–8. DOI: 10.1016/j.exger.2016.04.013 [PubMed: 27108182]
56. Wilkinson DJ, Hossain T, Hill DS, Phillips BE, Crossland H, Williams J, Loughna P, Churchward-Venne TA, Breen L, Phillips SM, Etheridge T, Rathmacher JA, Smith K, Szewczyk NJ, Atherton PJ. Effects of leucine and its metabolite beta-hydroxy-beta-methylbutyrate on human skeletal muscle protein metabolism. *The Journal of physiology*. 2013; 591(Pt 11):2911–23. DOI: 10.1113/jphysiol.2013.253203 [PubMed: 23551944]
57. Knitter AE, Panton L, Rathmacher JA, Petersen A, Sharp R. Effects of beta-hydroxy-beta-methylbutyrate on muscle damage after a prolonged run. *J Appl Physiol*. 2000; 89(4):1340–4. Epub 2000/09/28. [PubMed: 11007567]
58. Vukovich MD, Dreifort GD. Effect of beta-hydroxy beta-methylbutyrate on the onset of blood lactate accumulation and V(O)₂ peak in endurance-trained cyclists. *Journal of strength and conditioning research /National Strength & Conditioning Association*. 2001; 15(4):491–7. Epub 2001/12/01.
59. Kraemer WJ, Hatfield DL, Volek JS, Fragala MS, Vingren JL, Anderson JM, Spiering BA, Thomas GA, Ho JY, Quann EE, Izquierdo M, Hakkinen K, Maresh CM. Effects of amino acids supplement on physiological adaptations to resistance training. *Medicine and science in sports and exercise*. 2009; 41(5):1111–21. Epub 2009/04/07. DOI: 10.1249/MSS.0b013e318194cc75 [PubMed: 19346975]
60. Rowlands DS, Thomson JS. Effects of β -hydroxy- β -methylbutyrate supplementation during resistance training on strength, body composition, and muscle damage in trained and untrained young men: A meta-analysis. *The Journal of Strength & Conditioning Research*. 2009; 23(3):836–46. [PubMed: 19387395]
61. de Betue CTI, Garcia Casal XC, van Waardenburg DA, Schexnayder SM, Joosten KFM, Deutz NEP, Engelen M. 24-Hour protein, arginine and citrulline metabolism in fed critically ill children - A stable isotope tracer study. *Clin Nutr*. 2017; 36(3):876–87. DOI: 10.1016/j.clnu.2016.12.023 [PubMed: 28089618]
62. Castillo L, Beaumier L, Ajami AM, Young VR. Whole body nitric oxide synthesis in healthy men determined from [15N] arginine-to-[15N]citrulline labeling. *Proc Natl Acad Sci U S A*. 1996; 93(21):11460–5. [PubMed: 8876157]

63. Tessari P, Cecchet D, Cosma A, Vettore M, Coracina A, Millioni R, Iori E, Puricelli L, Avogaro A, Vedovato M. Nitric oxide synthesis is reduced in subjects with type 2 diabetes and nephropathy. *Diabetes*. 2010; 59(9):2152–9. Epub 2010/05/21. DOI: 10.2337/db09-1772 [PubMed: 20484137]
64. Ten Have GAM, Engelen M, Wolfe RR, Deutz NEP. Phenylalanine isotope pulse method to measure effect of sepsis on protein breakdown and membrane transport in the pig. *American journal of physiology Endocrinology and metabolism*. 2017; 312(6):E519–E29. DOI: 10.1152/ajpendo.00351.2016 [PubMed: 28292760]
65. Thompson GN, Pacy PJ, Merritt H, Ford GC, Read MA, Cheng KN, Halliday D. Rapid measurement of whole body and forearm protein turnover using a [2H5]phenylalanine model. *American journal of physiology Endocrinology and metabolism*. 1989; 256(5):E631–9.

FIGURE 1.

Tracee concentration after a pulse that contained tracer of the indicated amino acids

FIGURE 2.

TTR of Arginine [guanidino- $^{15}\text{N}_2$] and Citrulline [Ureido- ^{15}N] (left panel) and Citrulline [$5\text{-}^{13}\text{C};5,5\text{-}^2\text{H}_2$] and Arginine [$5\text{-}^{13}\text{C};5,5\text{-}^2\text{H}_2$].

Table 1

Subject Characteristics

	Young Adults (n=10)	Older Adults (n=17)	T-test (p)
Age (years)	22.7 (0.6)	64.8 (1.7)	<0.001
Sex (Female/Male)	5/5	6/8	0.743
Body Weight (kg)	70.6 (3.1)	78.5 (3.2)	0.110
Body Mass Index (kg/m ²)	24.3 (0.8)	27.0 (0.8)	0.039
Total Lean Mass (kg)	49.56 (3.52)	51.19 (2.76)	0.725
Total Fat Mass (kg)	17.58 (1.66)	22.45 (1.76)	0.077
Total Appendicular Mass (kg)	7.40 (0.43)	7.32 (0.34)	0.886
BMD Spine (z-score)	-0.31 (0.38)	0.39 (0.36)	0.218
Maximal Handgrip Force (N)	302.2 (23.9)	283.8 (15.3)	0.504
Maximal Leg Extension Force (N)	494.9 (40.4)	361.3 (31.8)	0.017
Habitual Caloric intake (kcal/day)	1931 (112)	1587 (218)	0.261
Habitual Protein intake (gram/day)	87.8 (9.6)	66.52 (8.5)	0.123
Habitual Carbohydrate intake (gram/day)	240 (21)	189 (28)	0.212
Habitual Fat intake (gram/day)	66.6 (8.1)	60.4 (9.4)	0.656
POMS (score)	-0.6 (7.2)	-5.7 (3.7)	0.491
PASE (score)	154 (10)	151 (6)	0.872
HADS anxiety (score)	3.8 (0.8)	4.3 (0.8)	0.687
STROOP interference (score)	24.0 (2.6)	44.4 (6.8)	0.035

Data are mean (SE). Statistics are by t-test, bold is P<0.1.

Table 2

Composition of the infused stable tracers

<i>Primed-constant infusion</i>	<i>Prime ($\mu\text{mol/kg ffm; mean (SD)}$)</i>	<i>Continuous infusion ($\mu\text{mol/kg ffm/h; mean (SD)}$)</i>
L-Phenylalanine [ring- $^2\text{H}_5$]	75% of hourly infusion rate	0.167 (0.042)
L-Tyrosine [$^{13}\text{C}_9$ - ^{15}N]	75% of hourly infusion rate	0.0053 (0.0013)
L-Tryptophan [indole- $^2\text{H}_5$]	75% of hourly infusion rate	0.0074 (0.0018)
<i>Pulse</i>	<i>Pulse ($\mu\text{mol/kg ffm; mean (SD)}$)</i>	
L-Hydroxyproline [2- ^2H] or [2,5,5- $^2\text{H}_3$]	0.253 (0.089)	
Glycine [2,2- $^2\text{H}_2$]	9.37 (2.91)	
L-Taurine [1,2,- $^{13}\text{C}_2$]	0.729 (0.194)	
L-Glutamine [5- ^{15}N]	6.25 (1.67)	
L-Leucine [5,5,5,- $^2\text{H}_3$]	1.56 (0.47)	
L-Glutamate [1,2,- $^{13}\text{C}_2$]	0.782 (0.208)	
L-Arginine [guanidino- $^{15}\text{N}_2$]	2.08 (0.62)	
L-Citrulline [5- ^{13}C ;5,5- $^2\text{H}_2$]	0.335 (0.099)	
L-tau-MethylHistidine [methyl- $^2\text{H}_3$]	0.061 (0.0181)	
Keto-Isocaproic Acid [1- ^{13}C]	2.08 (0.62)	

Table 3**Plasma Amino Acid and Keto Acid Concentrations**

	Young Adults (n=10)	Older Adults (n=17)	T-test (p)
Aspartate	1.2 (0.1)	1.7 (0.1)	0.029
Glutamate	25.2 (3.0)	44.2 (4.2)	0.002
Hydroxyproline	15.4 (3.5)	10.8 (0.8)	0.112
Asparagine	55.2 (2.8)	50.6 (2.5)	0.264
Glutamine	453.9 (25.5)	458.7 (18.1)	0.879
Citrulline	22.0 (1.4)	30.2 (2.2)	0.013
Serine	87.1 (6.1)	72.3 (3.2)	0.025
Glycine	271.1 (54.3)	187.1 (10.5)	0.064
Arginine	61.4 (3.4)	66.1 (2.9)	0.315
tau-methylhistidine	4.6 (0.5)	5.4 (0.3)	0.208
Threonine	110.5 (9.9)	107.5 (6.8)	0.796
Alanine	236.2 (28.0)	285.1 (21.3)	0.216
Taurine	30.1 (1.9)	37.4 (1.7)	0.011
Proline	152.0 (17.3)	152.0 (10.8)	0.999
Valine	181.2 (18.3)	184.5 (8.2)	0.850
Methionine	18.5 (1.1)	17.4 (0.7)	0.382
Isoleucine	60.1 (6.5)	54.7 (2.9)	0.400
Leucine	104.4 (10.7)	99.4 (5.1)	0.636
Tryptophan	41.3 (3.5)	34.3 (1.7)	0.057
Phenylalanine	40.2 (2.3)	43.1 (2.2)	0.394
Ornithine	33.7 (3.3)	39.4 (1.7)	0.105
Histidine	58.4 (2.7))	56.6 (2.8)	0.673
Lysine	142.0 (11.5)	147.8 (8.2)	0.628
Tyrosine	38.7 (3.0)	47.4 (2.9)	0.063
BCAA	345.7 (34.9)	338.6 (15.9)	0.836
EAA	756.6 (50.6)	745.4 (20.0)	0.841
NEAA	1382.0 (67.4)	1362.0 (48.4)	0.809
LNAA	424.7 (36.2)	429.1 (18.9)	0.905
LNAA/TRP	0.100 (0.007)	0.081 (0.004)	0.017
α -ketoisocaproic acid (KIC)	27.4 (2.5)	20.7 (1.4)	0.0206
α -ketoisovalerate (KIV)	14.7 (1.2)	13.4 (0.7)	0.3318
α -keto- β -methylvalerate (KMV)	13.7 (1.4)	10.5 (0.7)	0.0516
β -hydroxy β -methylbutyrate (HMB)	3.5 (0.3)	2.6 (0.2)	0.0098
BCKA	59.3 (5.0)	47.3 (2.9)	0.0392

Data are in $\mu\text{mol/l}$, except LNAA/TRP (mean (SE)). BCAA = Sum of the branched-chain amino acids valine, isoleucine and leucine. EAA = Sum of the amino acids threonine, valine, methionine, isoleucine, leucine, tryptophan, phenylalanine, histidine and lysine. NEAA = Sum of the amino acids aspartate, glutamate, asparagine, glutamine, serine, glycine, arginine, alanine, proline and tyrosine, LNAA = Sum of the amino acids valine,

isoleucine, leucine, phenylalanine and tyrosine, LNAA/TRP = Ratio between the LNAA and tryptophan concentration. BCKA - Sum of the branched-chain keto acids: α -ketoisocaproic acid, KIV and KMV. Statistics are by t-test, bold is $P < 0.1$.

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Table 4

Rate of Appearance/Whole body Production and interconversions

	Young Adults (n=10)	Older Adults (n=17)	T-test (p)
Glutamate (n=10&6)	101.1 (14.2)	91.1 (8.9)	0.6200
Hydroxyproline	4.9 (1.0)	3.3 (0.5)	0.2290
Glutamine (n=10&8)	220.8 (26.8)	304.8 (21.2)	0.0310
Citrulline	9.8 (0.5)	11.1 (0.8)	0.2578
Glycine	201.1 (35.3)	158.4 (17.7)	0.2394
Arginine	59.2 (4.1)	44.0 (4.0)	0.0194
tau-methylhistidine	0.15 (0.01)	0.21 (0.02)	0.0411
Taurine	31.7 (4.0)	26.3 (2.7)	0.2868
KIC	29.4 (3.6)	29.4 (3.3)	0.9993
Leucine	81.2 (4.2)	70.8 (6.3)	0.2546
Tryptophan	13.8 (4.2)	12.6 (0.8)	0.4028
Phenylalanine	87.9 (2.3)	93.5 (4.6)	0.3852
Tyrosine	53.8 (3.6)	63.7 (4.8)	0.1639
Citrulline to Arginine conversion rate (Arginine <i>de novo</i> production)	5.3 (0.3)	4.5 (0.4)	0.1925
Arginine to Citrulline conversion rate (NO production)	4.4 (0.5)	3.0 (0.5)	0.0935
Glutamate to Glutamine conversion rate (n=10&6)	10.9 (2.0)	17.6 (2.2)	0.0528
Phenylalanine to Tyrosine conversion rate (net protein breakdown)	8.0 (0.8)	7.1 (0.6)	0.4301
Leucine to KIC conversion rate (Leucine transamination)	24.6 (4.6)	20.7 (4.2)	0.5188
KIC to Leucine conversion rate (Leucine re-amination) (n=6&15)	23.5 (3.4)	26.2 (2.3)	0.4957
Absolute synthesis rate of HMB from Leucine	1.23 (0.15)	0.69 (0.06)	0.0013
LNAA/TRP	0.071 (0.006)	0.065 (0.003)	0.3655

Data are in $\mu\text{mol/kg ffm/h}$, except LNAA/TRP (mean (SE)). LNAA/TRP = Ratio between the sum of the Ra of Leucine, Phenylalanine and Tyrosine divided by the tryptophan Ra. Statistics are by unpaired t-test, bold is $P < 0.1$.

Table 5

Clearance of Amino acids

	Young Adults (n=10)	Older Adults (n=18)	T-test (p)
Glutamate	3.84 (0.75)	2.03 (0.21)	0.0787
Hydroxyproline	0.37 (0.05)	0.25 (0.03)	0.1159
Glutamine	0.48 (0.04)	0.76 (0.08)	0.007
Citrulline	0.46 (0.03)	0.39 (0.02)	0.1161
Glycine	0.86 (0.16)	0.92 (0.08)	0.7174
Arginine	1.02 (0.12)	0.74 (0.06)	0.0331
tau-methylhistidine	0.038 (0.005)	0.039 (0.003)	0.8873
Taurine	1.10 (0.17)	0.73 (0.06)	0.0692
KIC	0.94 (0.11)	1.66 (0.16)	0.0035
Leucine	0.80 (0.07)	1.34 (0.11)	0.0007
Tryptophan	0.36 (0.05)	0.36 (0.02)	0.9935
Phenylalanine	2.38 (0.21)	2.22 (0.12)	0.4943
Tyrosine	1.29 (0.05)	1.40 (0.07)	0.3382

Data are in liter/h. Statistics are by t-test, bold is $P < 0.1$.