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Randomized Control Trials

Impact of 3-week citrulline supplementation on postprandial protein metabolism in malnourished older patients: The Ciproage randomized controlled trial

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SUMMARY

Background: Citrulline (CIT), is not extracted by the splanchnic area, can stimulate muscle protein synthesis and could potentially find clinical applications in conditions involving low amino acid (AA) intake, such as in malnourished older subjects.
Objective: Our purpose was to research the effects of CIT supplementation on protein metabolism in particular on non-oxidative leucine disposal (NOLD, primary endpoint), and splanchnic extraction of amino acids in malnourished older patients.
Design: This prospective randomized multicenter study determined whole-body and liver protein synthesis, splanchnic protein metabolism and appendicular skeletal muscle mass (ASMM) in 24 malnourished older patients [80–92 years; 18 women and 6 men] in inpatient rehabilitation units. All received an oral dose of 10 g of CIT or an equimolar mixture of six non-essential amino acids (NEAAs), as isonitrogenous placebo, for 3 weeks.
Results: NOLD and albumin fractional synthesis rates were not different between the NEAA and CIT groups. Splanchnic extraction of dietary amino acid tended to decrease ($p = 0.09$) in the CIT group

Abbreviation: ASMM, appendicular skeletal muscle mass; AUC, area under the curve; BMI, body mass index; CIT, citrulline; CO_2, carbon dioxide; CRP, C-reactive protein; DXA, dual-energy X-ray absorptiometry; EAA, essential amino acids; Endo Ra leu, endogenous leucine flux; FM, fat mass; FSR, fractional synthesis rate; KIC, L-ketoisocaproate; LBM, lean body mass; Leu Ox, leucine oxidation; LM, lean mass; MNA, mini nutritional assessment; MPE, mole percent excess; NEAA, non-essential amino acid; NOLD, non-oxidative leucine disposal; Ra, rate of appearance.

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(45.2%) compared with the NEAA group (60.3%). Total differences in AA and NEAA area under the curves between fed-state and postabsorptive-state were significantly higher in the CIT than in the NEAA group. There were no significant differences for body mass index, fat mass (FM), lean mass (LM) or ASMM in the whole population except for a tendential decrease in FM for the citrulline group ($p = 0.089$). Compared with Day 1, lean mass and ASMM significantly increased (respectively $p = 0.016$ and $p = 0.018$) at Day 20 in CIT-treated women (mean respective increase of 1.7 kg and 1.1 kg), and fat mass significantly decreased ($p = 0.001$) at Day 20 in CIT-group women (mean decrease of 1.3 kg).

Conclusions: Our results demonstrate that CIT supplementation has no effect on whole-body protein synthesis or liver protein synthesis in malnourished older subjects. However, CIT supplementation was associated with a higher systemic AA availability. In the subgroup of women, CIT supplementation increased LM and ASMM, and decreased FM.

1. Introduction

Aging is associated with a loss of skeletal muscle mass and function. Malnutrition can accelerate this process to the point where it becomes pathological: this condition is termed sarcopenia [1]. Sarcopenia is associated with a decreased postprandial rate of muscle protein synthesis indicating a state of anabolic resistance [1]. This blunted muscle protein metabolism response to meal intake is due in part to higher first-pass splanchnic extraction of dietary amino acids, which limits peripheral amino acid availability [2], as shown in humans for leucine [3] and phenylalanine [4], and in aged rats [5,6]. Muscle signaling pathways needed to translate the initiation of protein synthesis may also be impaired [7,8]. The rationale for nutritional supplementation with essential amino acids (EAAs), particularly leucine at high dosages, is their ability to stimulate postprandial muscle protein synthesis [9]. Supplementation with EAAs for 3–6 months has increased lean body mass in healthy older women and sarcopenic older subjects [10,11]. However, EAAs do not escape splanchnic extraction, as evidenced by higher extraction of dietary AAs in older subjects [2]. In addition, long-term use of a leucine-supplemented diet failed to improve muscle mass and function [12,13], maybe because participants were habitually consuming sufficient leucine in their regular diets.

Citrulline (CIT), an amino acid not extracted in the splanchnic area [14], can stimulate muscle protein synthesis via the mTOR signaling pathway [15], and so could potentially find clinical applications in conditions involving low amino acid intake, such as in malnourished older subjects [14,16]. In malnourished aged rats, CIT increases muscle mass and function [17], muscle protein content and synthesis [18], and the expression of myofibrillar proteins [19]. Healthy young adults fed a hypoprotein diet showed increased muscle protein synthesis when given oral CIT compared with iso-nitrogenous non-essential amino acids (NEAAs) [20]. This anabolic action of CIT appeared to be independent of insulin or insulin-like growth factor 1 (IGF-1) action [20,21]. However, in two studies [20,21] in young adults, oral CIT did not modify whole-body protein kinetics or nitrogen balance compared with iso-nitrogenous NEAAs. The fact that action of CIT is muscle-specific may explain why nitrogen balance did not differ between CIT-supplemented diet and NEAA-enriched diet in old malnourished rats [18]. The CIT-supplemented diet increased muscle synthesis, whereas a NEAA-enriched diet increased liver protein synthesis. Oral CIT rapidly increases CIT and arginine availability in adults [22,23] and older patients [24]. CIT administration is safe and well tolerated [25]; the human adult dose most appropriate for clinical practice is 10 g per day [23].

To our knowledge, no study has yet investigated the effects of oral CIT supplementation in older malnourished human subjects to determine whether CIT has a protein anabolic effect in this population. Our study compared the effects of CIT supplementation against NEAA supplementation, as isonitrogenous placebo, on whole-body and liver protein synthesis, and on muscle mass in malnourished older persons. Our main objective was to assess the impact of CIT on postprandial protein synthesis. Secondary objectives were to evaluate the effect of CIT on anthropometric parameters, particularly lean mass, and to determine whether effects of CIT were related to modifications in AA splanchnic sequestration.

2. Subjects and methods

2.1. Materials

Citrulline, alanine, aspartic acid, glycine, histidine, proline and serine were purchased from Kyowa Hakko Kogyo Co. (Tokyo, Japan); L-leucine (1-^{13}C), L-leucine (5,5,5-D_3) and sodium bicarbonate (^{13}C) were from Eurisotop (Saint-Aubin, France). All AAs were checked for isotopic and chemical purity by chromatography–mass spectrometry (Hewlett–Packard 5971A, Palo–Alto, CA). Tracer solutions were tested for sterility, pyrogenicity and stability before use, and were prepared in sterile non-pyrogenic saline. In each experiment, the tracers were filtered through 0.22 μm filters.

2.2. Ethical approval and registration

This study was approved by the hospital ethics committee under number P070127, and conformed to the standards for the use of human subjects in research prescribed in the most recent update of the Declaration of Helsinki. Each participant was informed of the purpose of the study, the experimental procedures, and the potential risks before giving written consent. This prospective, randomized, doubled-blind trial was designed as a proof-of-concept study.

This trial is registered at http://clinicaltrials.gov (study ID number NCT00714675) and was funded by the French Ministry for Health under the Hospital-led Clinical Research Program (PHRC).

2.3. Setting and participants

Patients were screened from seven geriatric rehabilitation care units affiliated to the Greater Paris public hospital system (AP-HP) for an average length of stay of 60 days (intermediate care). These units focus on rehabilitation for daily living activities and prevention of falls. Patients were prospectively selected and consecutively included in the study.

The inclusion criteria were: age >70 years; moderate undernutrition defined according to French health high authority guidelines [26] as albuminemia ≥30 g/l and <35 g/l and/or body mass index (BMI) ≥ 18 and <21 and/or mini nutritional assessment

(MNA) [27] score <17 (but with albuminemia ≥30 g/l), spontaneous energy intake >20 kcal/kg body weight/day and protein intake >0.8 g/kg body weight/day.

Patients were ineligible if they presented severe cognitive impairment (mini mental state score <18), severe inflammation (C-reactive protein (CRP) > 50 mg/l) or diabetes mellitus, if they were being fed by parenteral or enteral nutrition, if they presented severe renal insufficiency (creatinine clearance calculated with the Cockcroft-Gault formula <30 ml/min), class-IV heart failure, severe liver disease, a documented intestinal insufficiency, respiratory failure, chronic infectious or inflammatory disease, corticosteroid medications or progressive cancer.

The trial was managed by the Henri-Mondor University Hospital Clinical Research Unit. Participating sites received initiation, routine monitoring, and closeout visits.

2.4. Diet and treatments

The study was a double-blind, randomized, controlled prospective, multicenter trial of superiority in two parallel groups: one CIT-supplemented and one placebo-supplemented. All patients received standard nutritional care during the study and were given a diet supplying 2000 kcal/day and 66 g/day protein. They also received either an oral dose (double-blind procedure) of 10 g of CIT or an equimolar mixture of six NEAAs (alanine 1.91 g, aspartic acid 2.85 g, glycine 1.61 g, histidine 3.32 g, proline 2.46 g, serine 2.25 g) given iso-nitrogenously every morning from Day 1 to Day 21. We note that histidine is not an NEAA [28], but has no known pharmacological effect on protein metabolism.

No adverse events due to the treatments were recorded.

2.5. Random assignment

Patients were randomly assigned by a centralized computer procedure (Excel 2003, Microsoft Corporation, Washington DC) on a 1:1 basis to receive CIT or NEAAs, stratified by center and by permuted blocks of four. Physicians were blinded to block size. Patients were enrolled by their treating physician, and registered with the central trial coordinating office by fax.

2.6. Endpoints

The primary endpoint was non-oxidative leucine disposal. Secondary endpoints were leucine balance, splanchnic extraction, albumin fractional synthesis rate, changes in body composition, and fed-state *vs*. postabsorptive state differences in AA concentrations.

2.7. Variables studied

2.7.1. Dietary intake

Energy and protein intakes were recorded (food diaries) on study Days 1, 2, 3, 12, 13, 14, 18, 19 and 20. Patients were also asked to rate their appetite on Days 1 and 20 using a 100 mm visual analog scale with “no appetite” and “extremely good appetite” as anchors.

2.7.2. Physical activity

Patients were invited to wear a pedometer (Tanita PD-637, Tokyo, Japan) every day during the study to monitor their step count. Patients were instructed to begin using the pedometer as soon as they started walking and to remove it immediately before going to sleep.

2.7.3. Body composition measurements

Body composition measurements were evaluated at Day 1 and Day 20. Total lean soft-tissue mass (LM), appendicular skeletal muscle mass (ASMM) and fat mass (FM) were determined using dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy Advance, General Electric Medical Systems, Vélizy, France). ASMM was measured as the sum of the LM for arms plus legs. LM index, ASMM index and FM index were calculated as the ratios of LM, ASMM and FM to the square of the person's height in meters. Height correction allows the clinical significance of these values to be interpreted in patients of differing heights.

2.7.4. Blood chemistry

On Day 1, serum albumin, C-reactive protein (CRP), orosomucoid, transthyretin, creatinine, insulin, insulin-like growth factor-1(IGF-1) and plasma AA concentrations were measured in the morning after fasting overnight. Creatinine clearance (ml/min) was calculated using the Cockcroft-Gault formula. On Day 21, plasma AA concentrations were measured in the morning after fasting overnight.

2.8. Measurement of protein turnover and related parameters

On Day 21, after a 10 h overnight fast, a catheter was inserted into a vein of the arm for tracer infusion. A second catheter was inserted retrograde into a dorsal vein of the contralateral hand for blood sampling after first placing the hand in a ventilated box heated to 60 °C. At 7:30 a.m. ($t_0 - 180$ min), a continuous infusion of L-leucine (1-^{13}C) was begun at 0.10 μmol kg lean mass^{-1} min^{-1} and continued for 390 min after a priming dose (8.4 μmol kg lean mass^{-1} min^{-1} of leucine (1-^{13}C) and 6 mg of sodium bicarbonate (^{13}C)) to reach a tracer steady-state during both basal period and fed period (Fig. 1). After 180 min of infusion (i.e. at t_0), a liquid meal was ingested in small boluses (50 ml) given every 20 min to reach a tracer steady-state as previously described [29]. L-Leucine (5,5,5-D_3) was added to the meal to obtain an oral administration rate of 0.10 μmol kg lean mass^{-1} min^{-1}. The treatment (CIT or NEAA) was given only once, with the first bolus. The diet provided 15 kcal kg lean mass^{-1}, 15% as protein (in the form of milk protein concentrate: Protifar Plus®, Nutricia, Rueil-Malmaison, France) containing 10.2% leucine and 3% phenylalanine, 50% as carbohydrate (dextrin maltose) and 35% as fat (in the form of vegetable oil).

As shown in Fig. 1, blood and breath samples were taken before every infusion, at the isotopic plateau of the intravenous tracer before the meal, and after meal ingestion. After centrifugation, the plasma supernatant was separated and stored at −80 °C for subsequent analysis of plasma enrichments in L-leucine (1-^{13}C), L-leucine (5,5,5- D_3), L-ketoisocaproate (KIC) (1-^{13}C) and L-KIC (5,5,5- D_3).

Plasma AA concentrations were measured before meal intake, i.e. at $t_0 - 180$ min and $t_0 - 60$ min, at t_0, and after meal intake, i.e. at $t_0 + 120$ min and $t_0 + 180$ min. As shown in Fig. 1, total carbon dioxide (CO_2) production rates were measured twice for 60 min each time in the postabsorptive state, and in the fed state by open-circuit indirect calorimetry (Deltratrac, Datex, Geneva, Switzerland).

2.9. Analytical methods

Plasma L-leucine (1-^{13}C), L-leucine (5,5,5- D_3), L-ketoisocaproate (KIC) (1-^{13}C) and L-KIC (5,5,5-D_3) enrichments were measured by gas chromatography–mass spectrometry (Hewlett–Packard 5971A, Palo–Alto, CA) using tert-butyldimethylsilyl derivates. Isotopic enrichment of plasma KIC is reported to reflect intracellular leucine enrichment more closely than the isotopic enrichment of plasma leucine.

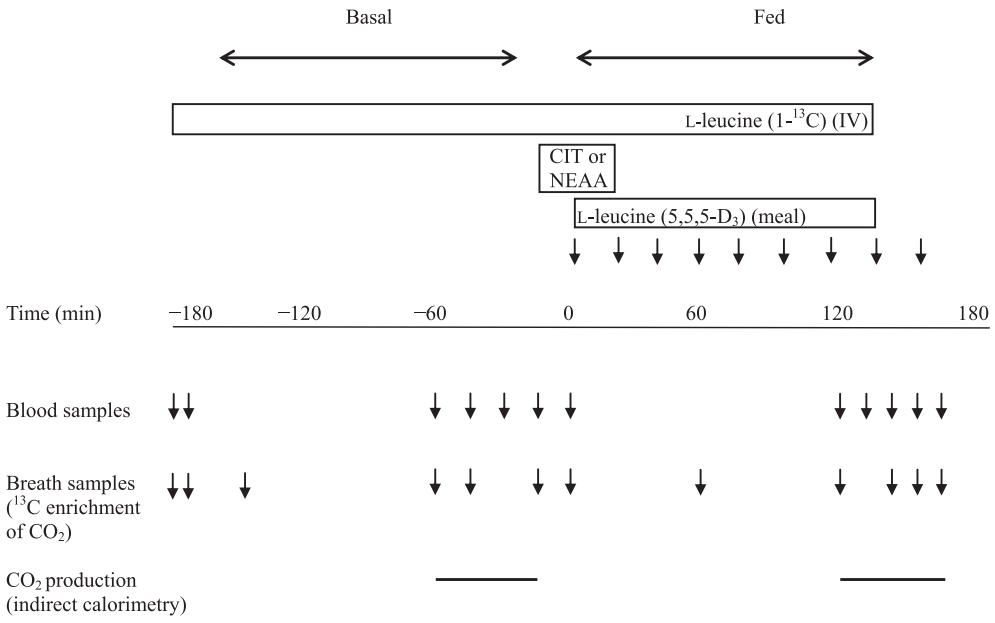

Fig. 1. Study protocol design. There were two successive periods, a basal state from −180 min to t_0, and a fed state from t_0 to +180 min. The basal state was a fasted state, i.e. after an overnight fast. A continuous intravenous infusion of (1-^{13}C) L-leucine was performed after a priming dose to reach a tracer steady state during both the basal and fed periods. Another tracer, (5,5,5-D_3) L-leucine, was added to the liquid meal along with the treatment (CIT or NEAA) in the first meal. The meal was ingested in small aliquots (50 ml) given every 20 min to reach a steady state. Blood and breath sampling and CO_2 production measures were conducted throughout the study. NEAAs: non-essential amino acids; CIT: citrulline.

To determine plasma AA concentrations, plasma was deproteinized with sulfosalicylic acid then centrifuged. The supernatant was stored at −80 °C until analysis. To determine the concentrations of insulin, IGF-1, albumin, transthyretin, glucose, creatinine, and C-reactive protein (CRP), plasma supernatant was separated and stored at −20 °C until analysis. Breath samples were kept in Vacutainers (Becton Dickinson) for further measurements of ^{13}C enrichment of CO_2.

Isotopic albumin enrichments for fractional synthesis rate measurements were analyzed by gas chromatography–combustion–isotope ratio mass spectrometry (GC/C/IRMS) after precipitation, hydrolysis and derivatization as previously described [2].

Plasma AA concentrations were determined by ion-exchange liquid chromatography using an AminoTac JLC-500/V AA analyzer (Jeol, Tokyo, Japan).

Serum albumin, CRP and transthyretin were measured by laser nephelometry (BN2, Siemens, Paris, France).

Plasma IGF-1 concentrations were measured using an immunoradiometric method (IGF1-RIACT, Cisbio Assays, Gif-sur-Yvette, France). Plasma insulin concentrations were determined by fully-automated two-site chemiluminescent immunoenzymatic assay (ACCESS II, Beckman–Coulter, Villepinte, France).

2.10. Calculations

Steady-state equations for whole-body leucine kinetics were used as below for L-leucine (1-^{13}C):

$$\text{Ra } [^{13}\text{C}] \text{ leu} = \text{F } [^{13}\text{C}] \text{ leu}/([^{13}\text{C}] \text{ leu MPE} \times 0.01),$$

where Ra [^{13}C] leu (μmol.kg lean body mass^{-1} min^{-1}) is total leucine flux or rate of appearance (Ra), F [^{13}C] leu (μmol.kg lean body mass^{-1} min^{-1}) is tracer infusion rate corrected for isotope purity, and [^{13}C] leu MPE (μmol kg^{-1} lean body mass.min^{-1}) is plasma enrichment of metabolite [^{13}C] leucine at isotopic plateau.

The resulting mole ratios were converted algebraically into tracer mole percent excess (MPE) after subtracting the corresponding baseline plasma values. The parameters were normalized for lean mass in order to consider differences in body composition between subjects.

The same equation was used to obtain Ra [D_3] leu.

Non-oxidative leucine disposal (NOLD) (μmol kg lean body mass^{-1} min^{-1}), an index of whole-body protein synthesis, is the difference between total leucine Ra and leucine oxidation (Leu Ox). Leu Ox (μmol kg lean body mass^{-1} min^{-1}) was calculated by dividing $^{13}CO_2$ production rate by [^{13}C] KIC enrichment, as KIC is the immediate precursor of irreversible leucine decarboxylation in cells. Where $^{13}CO_2$ production was the product of CO_2 production and $^{13}CO_2$ enrichment, $^{13}CO_2$ enrichment was corrected for incomplete recovery by a factor of 0.8.

Endogenous leucine flux (Endo Ra [^{13}C] leu; μmol kg lean body mass^{-1} min^{-1}), an index of whole-body proteolysis, was calculated as the difference between total leucine flux and tracer infusion rate.

Leucine balance (μmol kg lean body mass^{-1} min^{-1}) was calculated as the difference between NOLD and endogenous leucine fluxes.

During feeding, leucine intake was subtracted from the respective total fluxes to obtain endogenous leucine fluxes. However, as a fraction of dietary AA were extracted by the splanchnic tissues and did not reach the systemic circulation, endogenous leucine flux had to be corrected for actual leucine entry into the systemic circulation, where isotope enrichments were measured to calculate whole-body leucine turnover. Splanchnic extraction of leucine was calculated as follows:

$$\text{Leu splanchnic extraction } (\%) = [1 - ([^{13}\text{C}] \text{ leu}/[\text{D}_3] \text{ leu})] \times 100,$$

where [^{13}C] leu and [D_3] leu are total leucine fluxes calculated according to Balagopal et al. [30] with the intravenous ^{13}C leu or oral D_3 leu tracers, respectively. Splanchnic extraction is the fraction of ingested leucine taken up by the gut or liver during its first pass [3].

Albumin fractional synthesis rate (FSR) (%/day) was calculated by dividing the incorporation rate of [^{13}C] leu into albumin with plasma [^{13}C] leu enrichment as precursor pool.

2.11. Sample size estimation

Based on an expected difference in fed-state protein synthesis of 0.34 μmol kg lean body mass^{-1} min^{-1} of NOLD (corresponding to 0.8 g protein kg lean mass^{-1} day^{-1}) between NEAA group and CIT group and a standard deviation of 0.21 μmol kg lean mass^{-1} min^{-1} of NOLD (corresponding to 0.5 g of protein kg lean mass^{-1} day^{-1}), using two-sided error rate of 5% and a power at 95%, we calculated that we would need $n = 11$ patients in each group to see differences between means. We took the values found by other authors [31,32].

2.12. Statistical analysis

Data were analyzed and a flow diagram was created according to CONSORT guidelines.

Descriptive analysis compared the two randomized groups in terms of baseline demographics, body composition and biological parameters. Only descriptive statistics were used at this stage. Quantitative variables were expressed as median [min–max] or first quartile (25th percentile, 'Q1') and third quartile (75th percentile, 'Q3') according to distribution. Qualitative variables were expressed as numbers [%]. Plasma AA concentrations are expressed as area under the curve (AUC) above the baseline, as calculated by the trapezoidal method, both before (at $t_0 - 180$, $t_0 - 60$, t_0) and after meals (at $t_0 + 120$, $t_0 + 180$), for each AA.

Endpoints were analyzed using the intent-to-treat principle taking into account all the patients who completed the study. Subgroup analyses in women were conducted according to gender owing to gender-related differences in body composition. The results for the subgroup of men were not considered because the number enrolled in the study was too low.

We compared leucine kinetics, albumin synthesis rate and plasma AA concentrations between the two groups (CIT and NEAA), in postabsorptive state and in fed state, using Wilcoxon tests for unpaired samples.

Effect of treatment on BMI, lean mass, muscular appendicular skeletal mass and fat mass was studied using mixed linear regression models (one model for one anthropometric measure) adapted to the repeated nature of the data (D1 and D20 for each anthropometric variable). The response variable was the anthropometric variable at the two times of measure, and the explanatory variables, the group of randomization (i.e. the treatment), the time (D1 or D20), an interaction term between time and group of randomization (assessing the treatment effect) and a supplemental adjustment for baseline anthropometric variable. Similar modeling was done for energy and protein intake and appetite ratings as response variables.

Factors associated with leucine splanchnic extraction were also assessed: leucine splanchnic extraction was the response variable, handled in continuous mode, and the association between several factors (anthropometric and metabolic parameters) and the leucine splanchnic extraction. For this purpose, uni- and multivariate linear regression was used. Factors associated with $p < 0.20$ in univariate analysis were considered for multivariate analysis. The treatment group was introduced into the model as explanatory variable. The interactions were tested, in particular between the treatment group variables and the other explanatory variables. The final multivariate linear model of regression included factors significantly associated with leucine splanchnic extraction. As there are no clear associated factors of leucine extraction in the literature, non-adjustment for non-significant factor was forced in the model. The coefficients of the final multivariate model correspond to the slope of the linear regression.

All tests were two-tailed. Threshold of significance was $p < 0.05$. No multiple imputations for missing data were performed. Statistical analysis was performed using Stata v12.0 (College Station, TX).

3. Results

The study enrolled 29 patients. Figure 2 summarizes the inclusion process. Patient characteristics at baseline are listed in Table 1. Age was 74–97 years, and three-quarters of the sample were women.

Normal residence of patients was own home for 81%. The main patient diagnoses were: neurological disease (38%), rehabilitation after fracture (35%), cardiovascular disease (16%), post-infectious disease (7%), and other medical diseases (4%).

At Day 20 evolution of protein intakes were different between the two groups, with a decrease in the CIT group and an increase in the NEAA group (Table 2), but patient appetite scores were not different between the two groups.

Compliance on pedometer use for step counts was not excellent (n = 17; 11 NEAA/6CIT) and reached 2478 steps [1265; 14822] (median [Q1-Q3]) in the NEAA group and 1741 steps [82; 272 13854] in the CIT group, i.e. not significantly different between groups ($p = 0.48$).

In the postabsorptive state and in fed state, leucine kinetics expressed per kg of lean mass and albumin FSR were not different between groups (Table 3), except for D_3-leucine total flux ($p = 0.01$).

Splanchnic extraction of dietary AAs tended to decrease ($p = 0.09$) in the CIT group (45.2%) compared with the NEAA group (60.3%). Factors associated with leucine splanchnic extraction were albumin FSR (mean decrease of 7.77% in leucine splanchnic extraction for an 1%/d increase in albumin FSR; 95% CI [−10.52; −5.00]; $p = 0.001$), CIT treatment and BMI (mean decrease of 11.52% in leucine splanchnic extraction for a 1 kg/m^2 increase in BMI; 95% CI [−21.51; −1.52]; $p = 0.026$). When adjusted for albumin FSR and changes in BMI, splanchnic extraction of dietary AA decreased ($p = 0.004$) (Table 3) in the CIT group compared with the NEAA group.

Table 4 shows the changes in body composition in the NEAA and CIT groups in the whole population and in women. There were no significant differences for BMI, FM, LM and ASMM in the whole population except for a tendential decrease in FM in the citrulline group ($p = 0.089$). However, there was as significant increase in LM ($p = 0.016$) and ASMM ($p = 0.018$) and a significant decrease in FM in the citrulline group compared with the controlled group in the population of women.

Citrulline, EAA and NEAA concentrations just before and after the treatment are given in Table 5. Citrulline concentrations increased rapidly and had not fallen back to baseline 3 h after the intake.

Table 6 reports the differences in AA AUCs between fed state and postabsorptive state. Apart from the AAs supplied by the treatments, six AA AUC differences between fed state and postabsorptive state showed between-group differences: three were related to CIT metabolism (arginine and ornithine increased in the CIT group, glutamine increased in the NEAA group and decreased in the CIT group), while phenylalanine and lysine increased in the CIT group and asparagine increased in the NEAA group. Total AA and NEAA AUC differences (excluding the AAs of the treatment) increased significantly more in the CIT group than in the NEAA group.

There was no change in postabsorptive-state plasma AAs between Day 21 and Day 1 except for arginine and ornithine, both of which are related to CIT metabolism (arginine: −23.0 μmol/l [−43; 20] in the NEAA group, 28.5 μmol/l [3; 85] in the CIT group, $p = 0.001$ on Wilcoxon test for unpaired samples;

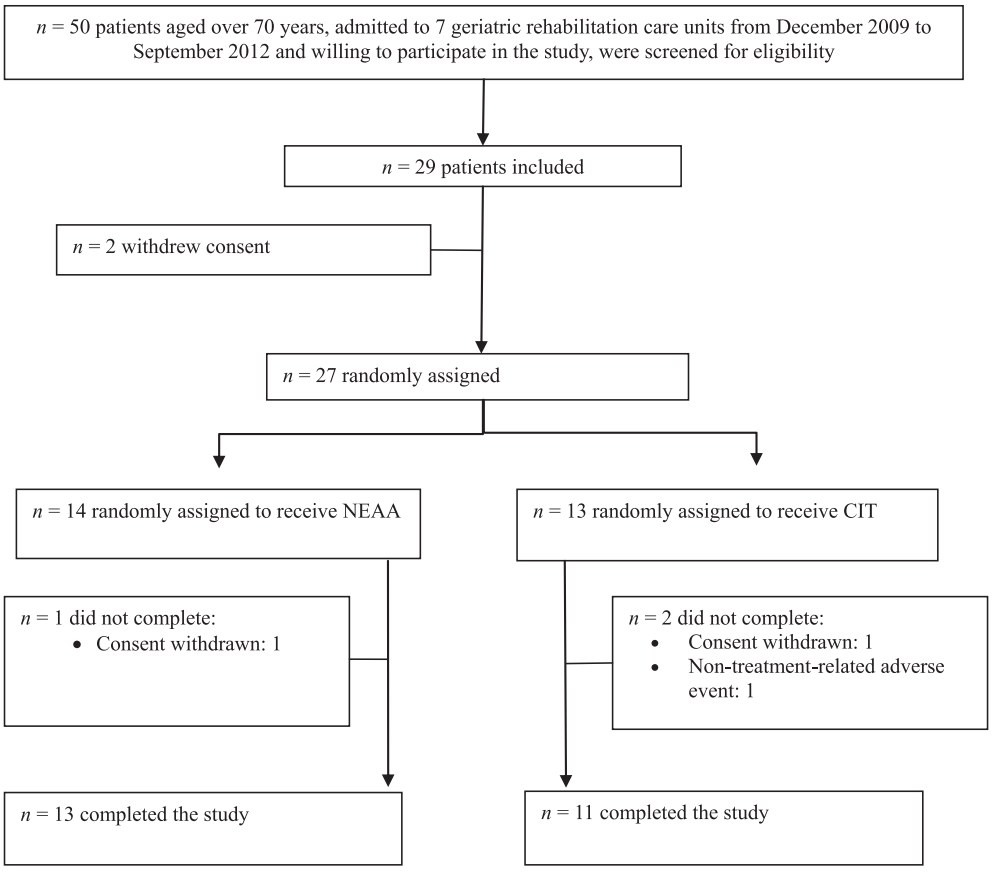

Fig. 2. Flowchart of the CIPROAGE trial. NEAAs: non-essential amino acids; CIT: citrulline.

ornithine: −13.0 μmol/l [−31; 10] in the NEAA group, 44.0 μmol/l [28; 116] in the CIT group, $p < 0.001$).

4. Discussion

Our results show that CIT supplementation has no effect on whole-body protein synthesis or liver protein synthesis in malnourished older subjects. However, CIT supplementation was associated with a higher systemic AA availability. In the subgroup of women, CIT supplementation increased LM and ASMM, and decreased FM. After a 3-week supplementation, this represents a mean increase in lean mass of about 1.7 kg, a mean increase in appendicular skeletal muscle mass of about 1.1 kg and a mean decrease in fat mass of about 1.3 kg. These changes appear to be clinically relevant.

Table 1
Patient characteristics at baseline in the NEAA and CIT groups.

	NEAA $n = 13$	CIT $n = 11$
Age (y)	88 [77; 92]	89 [74; 97]
Sex ratio		
Women *n* (%)	10 (76.9)	8 (72.7)
Men *n* (%)	3 (23.1)	3 (27.3)
Body mass index	21.6 [18.2; 33.2]	19.7 [16.4; 26.5]
Lean mass (kg)	34.2 [28.7; 52.6]	32.0 [27.3; 54.0]
Lean mass index (kg/m^2)	14.3 [12.6; 18.0]	13.8 [10.5; 19.1]
Appendicular skeletal muscle mass (kg)	14.1 [10.9; 21.6]	11.8 [9.9; 20.4]
Appendicular skeletal muscle mass index (kg/m^2)	5.7 [4.8; 7.8]	5.1 [3.6; 7.2]
Fat mass (kg)	13.6 [7.6; 42.0]	14.2 [5.2; 25.0]
Fat mass index (kg/m^2)	5.7 [3.5; 14.4]	5.2 [1.9; 10.7]
Mini Nutritional Assessment score	17.2 [12; 20.5]	16 [10; 19.5]
Creatinine clearance (ml/min)	60 [40; 104]	58 [37; 88]
Albumin (g/l)	31.2 [25.8; 34.8]	31.4 [24; 41.1]
Transthyretin (mg/l)	190 [160; 270]	175 [140; 230]
CRP (mg/l)	4.8 [1.0; 51.8]	4.4 [1; 44.9]
Insulin (μU/ml)	6.4 [1.2; 61.6]	4.7 [1.8; 10.8]
IGF-1 (ng/ml)	127 [81; 280]	102 [46; 171]

Values are median [min−max] unless otherwise stated.
NEAAs: non-essential amino acids; CIT: citrulline; CRP: C-reactive protein; IGF-1: insulin-like growth factor-1.

Table 2
Energy and protein intakes and appetite rating during the study period in the NEAA and CIT groups.

	Days	NEAA $n = 13$	CIT $n = 11$	Between-group differences Regression coefficient [95%CI][a]	p[a]
Energy (kcal kg weight^{-1} d^{-1})	1 to 3	31.5 [15; 51]	35.3 [25; 53]	Reference	
	12 to 14	28.7 [16.2; 53]	31.8 [13.2; 48.5]	−5.86 [−10.1; −1.63]	0.007
	18 to 20	36.0 [19.8; 52.7]	31.6 [11.9; 53.8]	−4.1 [−8.36; 0.13]	0.058
Protein (g kg weight^{-1} d^{-1})	1 to 3	1.1 [0.61; 1.7]	1.3 [0.88; 1.73]	Reference	
	12 to 14	1.2 [0.29; 2.15]	1.1 [0.42; 1.82]	−0.28 [−0.54; −0.01]	0.038
	18 to 20	1.2 [0.71; 2.08]	1.1 [0.37; 1.65]	−0.30 [−0.57; −0.04]	0.026
Appetite rating (mm)	1	45 [6; 97]	52 [9; 88]	Reference	
	20	64 [6; 80]	56 [6; 100]	−5.37 [−25.0; 14.3]	0.59

Energy and protein intakes were recorded (food diaries) on study Days 1 to 3, 12 to 14, and 18 to 20. Patients were also asked to rate their appetite on Days 1 and 20 using a 100 mm visual analog scale with “no appetite” and “extremely good appetite” as anchors.
Values are median [min–max].
NEAAs: non-essential amino acids; CIT: citrulline.
[a] Mixed linear regression models with an interaction term between time and randomization group to estimate the between-group differences.

Table 3
Leucine kinetics and albumin synthesis rate in postabsorptive state and in fed state.

	Postabsorptive state			Fed state		
	NEAA $n = 13$	CIT $n = 11$	p[a]	NEAA n = 13	CIT n = 11	p [a]
Main endpoint						
NOLD (μmol kg LBM^{-1} min^{-1})	2.1 [1.5; 3.4]	2.2 [1.9; 2.3]	0.98	2.1 [1.5; 4.2]	1.7 [1.3; 4.8]	0.54
Secondary endpoints						
^{13}C-Leucine total flux (μmol kg LBM^{-1} min^{-1})	2.6 [1.8; 3.7]	2.5 [1.9; 5.0]	0.84	3.0 [2.1; 5.7]	3.4 [2.0; 6.6]	0.66
Endogenous leucine flux (μmol kg LBM^{-1} min^{-1})	2.5 [1.7; 3.6]	2.4 [1.8; 4.9]	0.79	1.2 [0.61; 3.7]	1.4 [−0.64; 4.8]	0.40
Endogenous leucine flux corrected by splanchnic extraction (μmol kg LBM^{-1} min^{-1})				2.2 [1.6; 4.6]	2.0 [0.9; 4.8]	0.49
Leucine oxidation (μmol kg LBM^{-1} min^{-1})	0.38 [0.28; - 0.48]	0.35 [0.27; −0.48]	0.88	0.88 [0.68; 1.15]	1.13 [0.78; 1.71]	0.23
Leucine balance (μmol kg LBM^{-1} min^{-1})	−0.27 [−0.50; −0.10]	−0.25 [−0.53; −0.14]	0.84	0.94 [0.53; 1.54]	0.84 [0.71; 1.95]	0.43
Leucine balance corrected by splanchnic extraction (μmol kg LBM^{-1} min^{-1})				−0.03 [−0.52; 0.34]	−0.03 [−0.83; 0.996]	0.40
Splanchnic extraction (%)				60.3 [35.0; 85.6]	45.2 [10.6; 93.0]	0.09 0.004[b]
D3-Leucine total flux (μmol kg LBM^{-1} min^{-1})				6.3 [3.86; 12.7]	3.9 [3.4; 19.9]	0.01
Albumin FSR (%/d)	7.0 [3.4; 8.6]	5.9 [2.8; 10.2]	0.40	8.2 [4.2; 10.0]	7.78 [3.3; 12.7]	0.75
Albumin FSR-relative change				26.2 [6.5; 45.5]	35.5 [16.0; 71.7]	0.14

NEAAs: non-essential amino acids; CIT: citrulline; LBM: lean body mass; NOLD: non-oxidative leucine disposal; FSR: fractional synthesis rate.
FSR relative percent change = [[(fed state FSR) − (postabsorptive-state FSR)]/postabsorptive-state FSR].
Values are given as median [min–max].
[a] Wilcoxon test for unpaired samples.
[b] Multivariate regression linear model with the splanchnic extraction as explanatory variable and treatment group as explicative variable adjusted for albumin FSR and changes in BMI.

CIT did not modify either whole-body protein synthesis or albumin FSR in either the post-absorptive or postprandial states.

There are few studies on the effect of CIT on whole-body protein synthesis in humans. Of two studies conducted in healthy young adults in the post-absorptive state, one showed that CIT ingestion in subjects fed a low-protein diet had no significant effect on whole-body protein synthesis compared with NEAAs [20], although it did demonstrate that CIT stimulates muscle protein synthesis. The other study found no effect of CIT on whole-body protein synthesis and nitrogen balance when compared with isonitrogenous placebo [21]. Finally, in old malnourished rats, CIT increased muscle protein synthesis compared with NEAAs, but not liver protein synthesis [18]. Here, CIT did not increase albumin FSR in the liver. As its action is muscle-specific, and muscle protein synthesis contributes to only 25% of whole-body synthesis [20], this may explain why whole-body protein synthesis remained unchanged. A limitation of our study is that we did not measure muscle protein synthesis, largely because at the time the protocol was drafted, neither Jourdan et al. [20] nor Thibault et al. [21] had been published. In addition, obtaining muscle biopsies in older hospitalized patients is delicate. Note that 3 h after the CIT bolus, plasma CIT concentration was still very high (median 1426 μmol/l, see Table 5) compared to the CIT concentration of around 700 μmol/l found at steady-state in Jourdan et al. [20] in which CIT stimulated muscle protein synthesis. Finally, a pharmacokinetic study [23] reports that plasma CIT returned to baseline values 5–8 h post-loading only.

This study also demonstrates for the first time, to the best of our knowledge, that oral administration of CIT is associated with an increased peripheral bioavailability of AAs in the fed state, as shown by the calculated AA AUCs. The systemic availability of dietary AAs is a key determinant of protein synthesis. First-pass splanchnic (i.e. gut and liver) extraction of dietary AAs increases with age and defines age-related splanchnic sequestration of AAs (SSAA) [3]. Age-related SSAA could result from an increase in AA oxidation in the liver, an increase in liver and/or gut protein synthesis, or an increase in inflammation-driven Kupffer cell activity. Some of us have already shown [5] that SSAA in old rats is not related to Kupffer cell activity or to increased urea production. Here we show that splanchnic extraction and albumin FSR are inversely correlated. Increased SSAA is not therefore explained by increased liver protein synthesis [2], or at least not by secreted proteins.

Here CIT supplementation was negatively associated with leucine splanchnic extraction. This induces an increase in the

Table 4
Changes in body composition in the NEAA and CIT groups.

	Day	NEAA All $n = 13$ Women $n = 10$	CIT All $n = 11$ Women $n = 8$	Between-group differences Regression coefficient [95%CI] [a]	p^a
Body mass index (kg/m^2)					
All	D1	21.6 [18.2; 33.2]	19.7 [16.4; 26.5]	Reference	
All	D20	21.3 [18.3; 33.1]	19.6 [16.4; 25.6]	−0.04 [−0.52; 0.43]	0.86
Women	D1	20.7 [18.3; 33.2]	19.9 [17.1; 26.5]	Reference	
Women	D20	20.7 [18.3; 33.1]	19.4 [17.1; 25.6]	−0.20 [−0.54; 0.14]	0.24
Fat mass (kg)					
All	D1	13.6 [7.6; 42.0]	14.2 [5.2; 25.0]	Reference	
All	D20	16.0 [5.6; 42.4]	11.2 [6.8; 23.0]	−0.97 [−2.1; 0.15]	0.089
Women	D1	12.5 [7.6; 42.0]	14.5 [10.4; 25.0]	Reference	
Women	D20	12.6 [5.6; 42.3]	13.3 [9.4; 23.0]	−1.77 [- 2.84; −0.71]	0.001
Lean mass (kg)					
All	D1	34.2 [28.7; 52.6]	32.0 [27.3; 54.0]	Reference	
All	D20	34.4 [29.8; 57.2]	34.6 [28.5; 49.5]	0.55 [−1.21; 2.32]	0.54
Women	D1	32.5 [28.7; 52.6]	31.5 [27.3; 38.9]	Reference	
Women	D20	33.6 [29.8; 49.1]	33.7 [28.5; 39.1]	1.85 [0.35; 3.35]	0.016
Appendicular skeletal muscle mass (kg)					
All	D1	14.1 [10.9; 21.6]	11.8 [9.9; 20.4]	Reference	
All	D20	14.0 [11.6; 24.9]	13.3 [11.4; 18.5]	0.1 [−0.85; 1.06]	0.83
Women	D1	13.4 [10.9; 20.1]	11.5 [9.9; 13.5]	Reference	
Women	D20	13.6 [11.6; 21.3]	12.1 [11.4; 14.5]	0.79 [0.14; 1.45]	0.018

Values are median [min−max].
[a] Mixed linear regression models with an interaction term between days and group of randomization to estimate the between-group differences.

Table 5
Pre- and post-treatment amino acid concentrations in the control (NEAA) and CIT-treated groups.

Amino acids	Group		t_0	$t_0 + 120$	$t_0 + 180$
Citrulline	CIT	$n = 11$	47 [40; 59]	2184 [1346; 2608]	1426 [1278; 1715]
EAAs	CIT	$n = 11$	75 [45; 124]	114 [73; 215]	113 [73; 210]
	NEAA	$n = 12$	102 [52; 154]	123 [71; 198]	107 [74; 206]
NEAAs except for supplemented Citrulline	CIT	$n = 11$	60 [36; 135]	92 [44; 292]	92 [44; 324]
	NEAA	$n = 12$	57 [33; 124]	75 [38; 328]	70 [35; 245]

AAs: essential amino acids; NEAAs: non-essential amino acids; CIT: citrulline.
Values are given as median [Q1-Q3] in μmol/l at t_0, $t_0 + 120$ and $t_0 + 180$.

postprandial systemic bioavailability of AAs, as demonstrated in Table 6. SSAA certainly has an impact on protein metabolism, as it most likely participates in the age-related decrease in lean body mass. The effect of CIT on muscle mass observed in the women may thus be related to the increased availability of AAs. This is in line with the negative correlation between SSAA and changes in BMI.

An important question is how CIT blunts the SSAA of other AAs that are not metabolically linked. One hypothesis is that CIT inhibits hepatocyte uptake of other AAs because it uses a large number of transporters [33]. However, CIT is not taken up by the liver [34], at least in physiological conditions.

The CIT and NEAA groups showed significant differences in fed-state *vs*. postabsorptive-state concentrations for some AAs: two AAs related to CIT metabolism (arginine and ornithine) were significantly increased in the CIT group. Consistent with previous studies, we found that oral CIT resulted in hyperargininemia [20,22,23,35] and hyperornithinemia [20,22,23]. We previously showed that CIT-supplemented malnourished aged rats showed a significant increase in arginine and ornithine plasma concentrations compared with their NEAA-supplemented counterparts [17]. The kidney is the main producer of arginine from CIT [14]. As kidney expresses argininosuccinate synthetase and argininosuccinate lyase, it releases arginine as a function of CIT uptake [14]. Plasma arginine is then converted to ornithine by arginase in the liver. The conversion of CIT to arginine regulates the conversion of arginine to ornithine and *vice versa* [36].

Glutamine increased in the NEAA group and decreased in the CIT group. The same changes in glutamine plasma levels were observed following NEAA or CIT administrations in a study in healthy adults fed a low-protein diet [20]. Quantitatively, the main site of glutamine production in the body is muscle, and the main consumption sites in the physiological state are the gut, liver and kidneys [36]. Hence the observed decrease in postprandial glutamine in the CIT group could be related to an increase in ureagenesis stimulated by the high levels of arginine [37], or to a decrease in glutamine production by muscles. This second hypothesis could explain the increase in lean body mass in the CIT group observed in the women, since muscle glutamine content correlates with protein synthesis rates [38]. Interestingly, postprandial alanine AUC seemed very low in the CIT group compared with our previous results in older malnourished patients without CIT supplementation [39]. Like glutamine, alanine is characterized by a high production rate in muscles.

Lysine, like arginine, increased in the CIT-treated group. As lysine and arginine use the same transport system [40], hyperarginemia may result in a lower lysine uptake and thus higher postprandial lysinemia.

Sarcopenia in older subjects is associated with mobility disorders, increased risk of fall and fracture, less ability to perform daily living activities, loss of independence, and increased risk of death [1,41]. Our findings on the effects of citrulline on body composition in women are in line with results describing a beneficial effect of CIT on lean mass in experimental models. In malnourished aged rats, CIT supplementation (5 g/kg/day) increased muscle mass, muscle protein content and muscle protein synthesis compared with NEAA supplementation [18]. These effects of CIT on muscle

Table 6
Amino acid AUC differences between fed state and postabsorptive state in the control (NEAA) and CIT-treated groups.

	NEAA $n = 12$	CIT $n = 11$	p[a]
Isoleucine	45.3 [18.5; 99.5]	60.0 [6.5; 111.5]	0.32
Leucine	107.3 [−16.5; 205]	136.0 [33; 228.5]	0.22
Lysine	94.3 [43; 188.5]	156.0 [14; 208]	0.02
Methionine	23.8 [7; 40.5]	17.5 [−3.5; 28.5]	0.15
Phenylalanine	27.3 [4; 50.0]	41.5 [−3; 63.5]	0.01
Threonine	63.8 [25.8; 101.0]	55.0 [10.0; 64.0]	0.20
Valine	90.5 [34.5; 180.5]	130.5 [27.5; 238.5.]	0.11
EAAs	430.8 [144; 899]	580.0 [57.5; 948.5]	0.16
Alanine[b]	526.0 [48; 1205]	18.5 [−315.5; 101.5]	<0.001
Arginine	21.0 [5.5; 51]	483.5 [36; 607]	<0.001
Asparagine	50.0 [13.5; 100]	27.5 [−6.5; 45.5]	0.04
Aspartic acid[b]	5.8 [−10; 14.5]	0.5 [−1.0; 5]	<0.01
Citrulline	5.5 [−15.0; 21.5]	3819.0 [157; 5029]	<0.001
Cysteine	8.5 [−3.5; 24]	6.5 [−6.5; 13.0]	0.09
Glutamate	16.8 [−4.5; 103.5]	21.0 [−21; 51]	0.93
Glutamine	173.3 [65.5; 412]	−107.0 [−222.5; 48.5]	<0.001
Glycine[b]	397.8 [1060; 872]	−45.5 [−119.5; −4.0]	<0.001
Histidine[b]	248.8 [65; 938]	19.0 [7; 40.5]	<0.001
Ornithine	35.3 [19; 86]	551.5 [112.5; 817]	<0.001
Proline[b]	771.5 [473.5; 1500]	227.0 [34; 297.5]	<0.001
Serine[b]	386.0 [159.5; 745]	16.0 [−27; 50.5]	<0.001
Taurine	2.5 [−318; 28.0]	−0.5 [−24.5; 29]	0.42
Tyrosine	30.0 [9; 77.5]	57.0 [16.0; 84.5]	0.06
NEAAs	2817.8 [1069.5; 5021]	5299.5 [96.5; 6294.5]	0.01
NEAAs except for supplemented AAs	351.0 [157; 643]	1171.0 [35.5; 1372.5]	0.001
Total AAs except for supplemented AAs	821.0 [301; 1519.5]	1804.0 [93; 2253.5]	<0.01

NEAAs: non-essential amino acids; CIT: citrulline.
Plasma AA concentrations were expressed as area under the curve (AUC) above baseline, as calculated by the trapezoid method, before the meal (at $t_0 - 180$, $t_0 - 60$, t_0) and after the meal (at $t_0 + 120$, $t_0 + 180$).
Values are median [min−max] in μmol h/l.
[a] Wilcoxon test for unpaired samples.
[b] NEAAs contained in the control group supplementation.

mass were also found in healthy aged rats (1 g/kg/day for 12 weeks) that lost fat mass [42]. Aging is characterized by fat mass infiltration leading to a low grade inflammation [43], which accelerates the decrease in lean mass [44]. The decrease in fat mass observed in women in our study may therefore have contributed to the increase in muscle mass. This decrease in fat mass may be explained by the fact that CIT directly increases fatty acid release from rat visceral adipose tissue [45], whereas glyceroneogenesis is downregulated [46]. Moreover, results of proteomics investigations in malnourished aged rats showed that CIT increases the expression of the main myofibrillar proteins and of enzymes involved in glycogenolysis and glycolysis [19].

Behaviors of protein intakes were different between the two groups, with a decrease in the CIT group and an increase in the NEAA group, but patient appetite scores were not different. We cannot explain this effect, but even so observed an increase in lean mass in women.

Up to now, field-research efforts have focused on supplementation with either leucine, EAA or mixtures. However, there are few randomized controlled studies in older subjects yielding results on the effects of AA supplementations on lean mass. Two studies found an effect on lean mass after 3 or 6 months of EAA supplementation [10,11], and another study found an effect on muscle mass after 3 months, but only when combined with exercise [47]. On leucine-rich protein supplement, a recent meta-analysis of 16 randomized controlled studies enrolling a total of 999 older subjects [48] found that leucine-rich supplementation increased lean body mass in sarcopenic subjects. However, the two studies investigating long-term effects (3–6 months) of leucine supplementation (7.5 g/d) in older subjects both reported negative results on muscle mass and strength [12,13]. Note that splanchnic extraction of leucine is about 50% in older subjects [2,3] whereas CIT is not extracted in the splanchnic area [14].

This study was designed as a proof-of-concept study to investigate the protein anabolic effect of CIT in older patients, as such data is lacking, despite a strong rationale for testing this hypothesis. These results also give insight into the physiological disturbances of protein metabolism in very old subjects.

There are some limitations to this study, in particular the low number of patients. Metabolic studies like this one are very difficult to conduct in frail hospitalized older patients. Also, the enrolment of men, who displayed differences from women, was very low: data on men were therefore discarded in the subgroup analysis. Blood was not sampled in the first 2 h because reaching a steady state requires waiting for a homogeneous mixing of the tracer in the tracee pool to ensure that all the calculations are validated. This approach is of course open to debate but it is still robust, especially for the calculation of splanchnic metabolism of AA which is more difficult to approach using a bolus dose of protein.

In conclusion, the results of our study demonstrate that CIT supplementation has no effect on whole-body protein synthesis or liver protein synthesis in malnourished older subjects. However, CIT supplementation was associated with a higher systemic AA availability. These results on ASMM and fat mass in our subgroup of women should now spur further studies on the clinical benefits (on nutritional status, sarcopenia, physical activity, recovery) in older patients suffering from malnutrition, especially during the period of recovery from acute disease.

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Authors' contributions to the manuscript

Aussel C and Bouillanne O designed the research.

Melchior JC, Faure C, Forasassi C, Herbaud S, Le Corvoisier P and Raynaud-Simon A conducted the research.

Paul M, Boirie Y, Astier A, Walrand S provided essential reagents or essential materials.

Canouï-Poitrine F and Guery E analyzed the data or performed statistical analysis.

Bouillanne O, Aussel C and Cynober L wrote the paper.

Neveux N, Chevenne D and Nivet-Antoine V were responsible for the biological analyses.

Conflicts of interest

Luc Cynober and Cécile Faure are shareholders in the Citrage company. Luc Cynober receives honoraria from Citrage. Citrage is a laboratory for research and development specializing in dietary supplements for older adults. None of the other authors have any conflict of interest to declare in relation with this work.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2018.02.017.

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