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

Olivier Bouillanne^{1,2}, Jean-Claude Melchior^{2,3}, Cécile Faure², Muriel Paul⁴, Florence Canouï-Poitrine^{5,6}, Yves Boirie^{7,8,9}, Didier Chevenne¹⁰, Christine Forasassi¹¹, Ester Guery^{5,6,12}, Stéphane Herbaud¹³, Philippe Le Corvoisier¹⁴, Nathalie Neveux^{2,15}, Valérie Nivet-Antoine^{2,16}, Alain Astier⁴, Agathe Raynaud-Simon^{2,17}, Stéphane Walrand^{7,8}, Luc Cynober^{2,15}, Christian Ausset^{2,18}

¹ Assistance Publique Hôpitaux de Paris (AP-HP), Groupe Hospitalier (GH) Henri-Mondor, Hôpital Emile-Roux, Département de Gériatologie, 94456 Limeil-Brévannes, France;

² Université Paris-Descartes, Faculté des Sciences Pharmaceutiques et Biologiques, EA 4466, Laboratoire de Biologie de la Nutrition, 75006 Paris, France;

³ AP-HP, Hôpitaux Universitaires (HU) Paris-Ile-de-France-Ouest, Hôpital Raymond-Poincaré, Unité de Nutrition clinique-TCA, 92380 Garches, France.

⁴ AP-HP, Hôpital Henri-Mondor, Département de Pharmacie, 94000 Créteil, France;

⁵ Université Paris-Est, UPEC, DHU A-TVB, IMRB, EA 7376, CEpiA (Clinical Epidemiology and Aging), 94000 Créteil, France;

⁶ AP-HP, Hôpital Henri-Mondor, Service de Santé Publique, 94000 Créteil, France;

⁷ INRA, CRNH Auvergne, UMR 1019, Unité de Nutrition Humaine, 63009 Clermont-Ferrand, France;

⁸ Université d'Auvergne, Clermont Université, Unité de Nutrition Humaine, 63009 Clermont-Ferrand, France;

⁹ Centre Hospitalier Universitaire Clermont-Ferrand, Département de Nutrition Clinique, 63003 Clermont-Ferrand, France;

¹⁰ AP-HP, Hôpital Robert Debré, Département de Biochimie et d'Hormonologie, 75019 Paris, France;

¹¹ AP-HP, HU Est-Parisien, Hôpital Rothschild, Service de Gériatrie, 75012 Paris, France;

¹² AP-HP, Hôpital Henri-Mondor, Unité de Recherche Clinique (URC-Mondor), 94000 Créteil, France;

¹³ AP-HP, GH Henri-Mondor, Hôpital Albert-Chenevier, Département de Gériatologie, 94000 Créteil, France;

¹⁴ INSERM, Centre d'Investigation Clinique 1430, Plateforme de Ressources Biologiques, 94000 Créteil, France;

35 ¹⁵ AP-HP, HU Paris-Centre, Hôpital Cochin, Service de Biochimie, 75014 Paris, France;

36 ¹⁶ AP-HP, HU Paris-Ouest, Hôpital Georges Pompidou, Département de Biochimie, 75015

37 Paris, France;

38 ¹⁷ AP-HP, HU Paris-Nord-Val-de-Seine, Hôpital Bichat, Département de Gériatrie, 75018

39 Paris, France;

40 ¹⁸ AP-HP, Hôpital Henri-Mondor, Département de Pharmacie, Unité de Nutrition, 94000

41 Créteil, France;

42

43 **Corresponding author:**

44 Bouillanne Olivier, Hôpital Emile-Roux, 94456 Limeil-Brévannes, France. Tel.: + 33 1 45 95

45 83 20; e-mail: olivier.bouillanne@erx.aphp.fr

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47 **This trial is registered** at <http://clinicaltrials.gov> (study ID number NCT00714675).

48

49 **Abbreviation used:**

50 ASMM, appendicular skeletal muscle mass; AUC, area under the curve; BMI, body mass

51 index; CIT, citrulline; CO₂, carbon dioxide; CRP, C-reactive protein; DXA, dual-energy X-ray

52 absorptiometry; EAA, essential amino acids; Endo Ra leu, endogenous leucine flux; FM, fat

53 mass; FSR, fractional synthesis rate; KIC, L-ketoisocaproate; LBM, lean body mass; Leu Ox,

54 leucine oxidation; LM, lean mass; MNA, mini nutritional assessment; MPE, mole percent

55 excess; NEAA, non-essential amino acid; NOLD, non-oxidative leucine disposal; Ra, rate of

56 appearance.

57 Abstract

58 **Background:** Citrulline (CIT), is not extracted by the splanchnic area, can stimulate muscle
59 protein synthesis and could potentially find clinical applications in conditions involving low
60 amino acid (AA) intake, such as in malnourished older subjects.

61 **Objective:** Our purpose was to research the effects of CIT supplementation on protein
62 metabolism in particular on non-oxidative leucine disposal (NOLD, primary endpoint), and
63 splanchnic extraction of amino acids in malnourished older patients.

64 **Design:** This prospective randomized multicenter study determined whole-body and liver
65 protein synthesis, splanchnic protein metabolism and appendicular skeletal muscle mass
66 (ASMM) in 24 malnourished older patients [80–92 years; 18 women and 6 men] in inpatient
67 rehabilitation units. All received an oral dose of 10 g of CIT or an equimolar mixture of six
68 non-essential amino acids (NEAAs), as isonitrogenous placebo, for 3 weeks.

69 **Results:** NOLD and albumin fractional synthesis rates were not different between the NEAA
70 and CIT groups. Splanchnic extraction of dietary amino acid tended to decrease ($p = 0.09$) in
71 the CIT group (45.2%) compared with the NEAA group (60.3%). Total differences in AA and
72 NEAA area under the curves between fed-state and postabsorptive-state were significantly
73 higher in the CIT than in the NEAA group. There were no significant differences for body
74 mass index, fat mass (FM), lean mass (LM) or ASMM in the whole population except for a
75 tendential decrease in FM for the citrulline group ($p = 0.089$). Compared with Day 1, lean
76 mass and ASMM significantly increased (respectively $p = 0.016$ and $p = 0.018$) at Day 20 in
77 CIT-treated women (mean respective increase of 1.7 kg and 1.1 kg), and fat mass significantly
78 decreased ($p = 0.001$) at Day 20 in CIT-group women (mean decrease of 1.3 kg).

79 **Conclusions:** Our results demonstrate that CIT supplementation has no effect on whole-body
80 protein synthesis or liver protein synthesis in malnourished older subjects. However, CIT

81 supplementation was associated with a higher systemic AA availability. In the subgroup of
82 women, CIT supplementation increased LM and ASMM, and decreased FM.

83

84 **Keywords:** citrulline, malnutrition, lean mass, protein metabolism, amino acids, older adults

85

86 **Introduction**

87 Aging is associated with a loss of skeletal muscle mass and function. Malnutrition can
88 accelerate this process to the point where it becomes pathological: this condition is termed
89 sarcopenia (1). Sarcopenia is associated with a decreased postprandial rate of muscle protein
90 synthesis indicating a state of anabolic resistance (1). This blunted muscle protein metabolism
91 response to meal intake is due in part to higher first-pass splanchnic extraction of dietary
92 amino acids, which limits peripheral amino acid availability (2), as shown in humans for
93 leucine (3) and phenylalanine (4), and in aged rats (5,6). Muscle signaling pathways needed to
94 translate the initiation of protein synthesis may also be impaired (7,8). The rationale for
95 nutritional supplementation with essential amino acids (EAAs), particularly leucine at high
96 dosages, is their ability to stimulate postprandial muscle protein synthesis (9).
97 Supplementation with EAAs for 3 to 6 months has increased lean body mass in healthy older
98 women and sarcopenic older subjects (10,11). However, EAAs do not escape splanchnic
99 extraction, as evidenced by higher extraction of dietary AAs in older subjects (2). In addition,
100 long-term use of a leucine-supplemented diet failed to improve muscle mass and function
101 (12,13), maybe because participants were habitually consuming sufficient leucine in their
102 regular diets.

103 Citrulline (CIT), an amino acid not extracted in the splanchnic area (14), can stimulate muscle
104 protein synthesis via the mTOR signaling pathway (15), and so could potentially find clinical
105 applications in conditions involving low amino acid intake, such as in malnourished older
106 subjects (14,16). In malnourished aged rats, CIT increases muscle mass and function (17),
107 muscle protein content and synthesis (18), and the expression of myofibrillar proteins (19).
108 Healthy young adults fed a hypoprotein diet showed increased muscle protein synthesis when
109 given oral CIT compared with isonitrogenous non-essential amino acids (NEAAs) (20). This
110 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.

Subjects and methods

Materials

Citrulline, alanine, aspartic acid, glycine, histidine, proline and serine were purchased from Kyowa Hakko Kogyo Co. (Tokyo, Japan); L-leucine ($1\text{-}^{13}\text{C}$), L-leucine ($5,5,5\text{-D}_3$) 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 $0.22\text{ }\mu\text{m}$ filters.

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).

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 $\geq 30\text{ g/L}$ and $< 35\text{ g/L}$ 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.

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 isonitrogenously 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.

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.

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.

Variables studied

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.

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.

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.

200 *Blood chemistry*

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

206 **Measurement of protein turnover and related parameters**

207 On Day 21, after a 10 h overnight fast, a catheter was inserted into a vein of the arm for tracer
 208 infusion. A second catheter was inserted retrograde into a dorsal vein of the contralateral hand
 209 for blood sampling after first placing the hand in a ventilated box heated to 60 °C. At 7:30
 210 a.m. ($t_0 - 180$ min), a continuous infusion of L-leucine ($1-^{13}\text{C}$) was begun at $0.10 \mu\text{mol.kg}$
 211 $\text{lean mass}^{-1}.\text{min}^{-1}$ and continued for 390 min after a priming dose ($8.4 \mu\text{mol.kg lean}$
 212 $\text{mass}^{-1}.\text{min}^{-1}$ of leucine ($1-^{13}\text{C}$) and 6 mg of sodium bicarbonate (^{13}C)) to reach a tracer steady-
 213 state during both basal period and fed period (**Fig.1**). After 180 min of infusion (i.e. at t_0), a
 214 liquid meal was ingested in small boluses (50 mL) given every 20 min to reach a tracer
 215 steady-state as previously described (29). L-Leucine ($5,5,5\text{-D}_3$) was added to the meal to
 216 obtain an oral administration rate of $0.10 \mu\text{mol.kg lean mass}^{-1}.\text{min}^{-1}$. The treatment (CIT or
 217 NEAA) was given only once, with the first bolus. The diet provided 15 kcal. kg lean mass⁻¹,
 218 15% as protein (in the form of milk protein concentrate: Protifar Plus®, Nutricia, Rueil-
 219 Malmaison, France) containing 10.2% leucine and 3% phenylalanine), 50% as carbohydrate
 220 (dextrin maltose) and 35% as fat (in the form of vegetable oil).

221 As shown in Fig. 1, blood and breath samples were taken before every infusion, at the
 222 isotopic plateau of the intravenous tracer before the meal, and after meal ingestion. After
 223 centrifugation, the plasma supernatant was separated and stored at -80°C for subsequent

analysis of plasma enrichments in L-leucine ($1\text{-}^{13}\text{C}$), L-leucine ($5,5,5\text{-D}_3$), L-ketoisocaproate (KIC) ($1\text{-}^{13}\text{C}$) and L-KIC ($5,5,5\text{-D}_3$).

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).

Analytical methods

Plasma L-leucine ($1\text{-}^{13}\text{C}$), L-leucine ($5,5,5\text{-D}_3$), L-ketoisocaproate (KIC) ($1\text{-}^{13}\text{C}$) and L-KIC ($5,5,5\text{-D}_3$) enrichments were measured by gas chromatography–mass spectrometry (Hewlett-Packard 5971A, Palo-Alto, CA) using tert-butyldimethylsilyl derivatives. Isotopic enrichment of plasma KIC is reported to reflect intracellular leucine enrichment more closely than the isotopic enrichment of plasma leucine.

To determine plasma AA concentrations, plasma was deproteinized with sulfosalicylic acid then centrifuged. The supernatant was stored at $-80\text{ }^\circ\text{C}$ 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 $-20\text{ }^\circ\text{C}$ 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).

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

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

254 **Calculations**

255 Steady-state equations for whole-body leucine kinetics were used as below for L-leucine (1-
256 ^{13}C):

$$257 \quad \text{Ra } [^{13}\text{C}] \text{ leu} = \text{F } [^{13}\text{C}] \text{ leu} / ([^{13}\text{C}] \text{ leu MPE} \times 0.01),$$

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

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

265 The same equation was used to obtain $\text{Ra } [\text{D}_3] \text{ leu}$.

266 Non-oxidative leucine disposal (NOLD) ($\mu\text{mol.kg lean body mass}^{-1}.\text{min}^{-1}$), an index of whole-
267 body protein synthesis, is the difference between total leucine Ra and leucine oxidation (Leu
268 Ox). Leu Ox ($\mu\text{mol.kg lean body mass}^{-1}.\text{min}^{-1}$) was calculated by dividing $^{13}\text{CO}_2$ production
269 rate by $[^{13}\text{C}] \text{ KIC}$ enrichment, as KIC is the immediate precursor of irreversible leucine
270 decarboxylation in cells. Where $^{13}\text{CO}_2$ production was the product of CO_2 production and
271 $^{13}\text{CO}_2$ enrichment, $^{13}\text{CO}_2$ enrichment was corrected for incomplete recovery by a factor of 0.8.

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

Leucine balance ($\mu\text{mol.kg lean body mass}^{-1}.\text{min}^{-1}$) 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.

Sample size estimation

Based on an expected difference in fed-state protein synthesis of $0.34 \mu\text{mol.kg lean body mass}^{-1}.\text{min}^{-1}$ of NOLD (corresponding to $0.8 \text{ g protein.kg lean mass}^{-1}.\text{day}^{-1}$) between NEAA group and CIT group and a standard deviation of $0.21 \mu\text{mol.kg lean mass}^{-1}.\text{min}^{-1}$ of NOLD (corresponding to $0.5 \text{ g of protein.kg lean mass}^{-1}.\text{day}^{-1}$), 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).

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).

335 **Results**

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

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

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

345 Compliance on pedometer use for step counts was not excellent ($n=17$; 11 NEAA/6CIT) and
346 reached 2478 steps [1265; 14822] (median [Q1-Q3]) in the NEAA group and 1741 steps [82;
347 272 13854] in the CIT group, i.e. not significantly different between groups ($p = 0.48$).

348 In the postabsorptive state and in fed state, leucine kinetics expressed per kg of lean mass and
349 albumin FSR were not different between groups (**Table 3**), except for D₃-leucine total flux
350 ($p = 0.01$).

351 Splanchnic extraction of dietary AAs tended to decrease ($p = 0.09$) in the CIT group (45.2%)
352 compared with the NEAA group (60.3%). Factors associated with leucine splanchnic
353 extraction were albumin FSR (mean decrease of 7.77% in leucine splanchnic extraction for an
354 1%/d increase in albumin FSR; 95% CI [-10.52; -5.00]; $p = 0.001$), CIT treatment and BMI
355 (mean decrease of 11.52% in leucine splanchnic extraction for a 1 kg/m² increase in BMI;
356 95% CI [-21.51; -1.52]; $p = 0.026$). When adjusted for albumin FSR and changes in BMI,
357 splanchnic extraction of dietary AA decreased ($p = 0.004$) (**Table 3**) in the CIT group
358 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 a 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 3h 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: $-23.0 \mu\text{mol/l}$ [-43 ; 20] in the NEAA group, $28.5 \mu\text{mol/l}$ [3 ; 85] in the CIT group, $p = 0.001$ on Wilcoxon test for unpaired samples; ornithine: $-13.0 \mu\text{mol/l}$ [-31 ; 10] in the NEAA group, $44.0 \mu\text{mol/l}$ [28 ; 116] in the CIT group, $p < 0.001$).

382 **Discussion**

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

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

392 There are few studies on the effect of CIT on whole-body protein synthesis in humans. Of two
 393 studies conducted in healthy young adults in the post-absorptive state, one showed that CIT
 394 ingestion in subjects fed a low-protein diet had no significant effect on whole-body protein
 395 synthesis compared with NEAAs (20), although it did demonstrate that CIT stimulates muscle
 396 protein synthesis. The other study found no effect of CIT on whole-body protein synthesis and
 397 nitrogen balance when compared with iso-nitrogenous placebo (21). Finally, in old
 398 malnourished rats, CIT increased muscle protein synthesis compared with NEAAs, but not
 399 liver protein synthesis (18). Here, CIT did not increase albumin FSR in the liver. As its action
 400 is muscle-specific, and muscle protein synthesis contributes to only 25% of whole-body
 401 synthesis (20), this may explain why whole-body protein synthesis remained unchanged. A
 402 limitation of our study is that we did not measure muscle protein synthesis, largely because at
 403 the time the protocol was drafted, neither Jourdan *et al.* (20) nor Thibault *et al.* (21) had been
 404 published. In addition, obtaining muscle biopsies in older hospitalized patients is delicate.
 405 Note that 3 hours after the CIT bolus, plasma CIT concentration was still very high (median
 406 1426 $\mu\text{mol/l}$, see Table 5) compared to the CIT concentration of around 700 $\mu\text{mol/l}$ 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 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 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 to 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 hours 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

504 patients suffering from malnutrition, especially during the period of recovery from acute
505 disease.
506

Conflict of interest statement:

Conflict 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.

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.

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Figure legends:

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 ($1\text{-}^{13}\text{C}$) L-leucine was performed after a priming dose to reach a tracer steady state during both the basal and fed periods. Another tracer, ($5,5,5\text{-D}_3$) 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.

Fig. 2. Flowchart of the CIPROAGE trial

NEAAs: non-essential amino acids; CIT: citrulline

Fig. 1.

Fig. 2.

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

	NEAA <i>n</i> = 13	CIT <i>n</i> = 11
Age (y)	88 [77; 92]	89 [74; 97]
Sex ratio		
Women <i>n</i> (%)	10 (76.9)	8 (72.7)
Men <i>n</i> (%)	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 <i>n</i> = 13	CIT <i>n</i> = 11	Between-group differences Regression coefficient [95%CI] ¹	<i>p</i> ¹
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].

¹ Mixed linear regression models with an interaction term between time and randomization group to estimate the between-group differences

NEAAs: non-essential amino acids; CIT: citrulline.

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

	Postabsorptive state			Fed state		
	NEAA <i>n</i> = 13	CIT <i>n</i> = 11	<i>p</i> ¹	NEAA <i>n</i> = 13	CIT <i>n</i> = 11	<i>p</i> ¹
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 ²
D ₃ -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]

¹ Wilcoxon test for unpaired samples

² 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.

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

		NEAA All <i>n</i> = 13 Women <i>n</i> = 10	CIT All <i>n</i> = 11 Women <i>n</i> = 8	Between-group differences Regression coefficient [95%CI] ¹	<i>p</i> ¹
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]

¹ 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]

EAAs: essential amino acids; NEAAs: non-essential amino acids; CIT: citrulline

Values are given as median [Q1-Q3] in $\mu\text{mol/L}$ at t_0 , $t_0 + 120$ and $t_0 + 180$.

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

	NEAA <i>n</i> = 12	CIT <i>n</i> = 11	<i>p</i> ¹
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
EAAAs	430.8 [144; 899]	580.0 [57.5; 948.5]	0.16
Alanine *	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 *	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 *	397.8 [1060; 872]	−45.5 [−119.5; −4.0]	<0.001
Histidine *	248.8 [65; 938]	19.0 [7; 40.5]	<0.001
Ornithine	35.3 [19; 86]	551.5 [112.5; 817]	<0.001
Proline *	771.5 [473.5; 1500]	227.0 [34; 297.5]	<0.001
Serine *	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 $\mu\text{mol.h/L}$

¹ Wilcoxon test for unpaired samples.

* NEAAs contained in the control group supplementation.