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► To cite this version:

O. Bouillanne, J. C. Melchior, C. Faure, M. Paul, F. Canoui-Poitrine, et al.. Impact of 3-week citrulline supplementation on postprandial protein metabolism in malnourished older patients: The Ciproage randomized controlled trial. *Clinical Nutrition*, 2019, 38 (2), pp.564-574. 10.1016/j.clnu.2018.02.017 . hal-02154521

HAL Id: hal-02154521

<https://hal.science/hal-02154521>

Submitted on 22 Oct 2021

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

3

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46

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

111 (IGF-1) action (20,21). However, in two studies (20,21) in young adults, oral CIT did not
112 modify whole-body protein kinetics or nitrogen balance compared with iso-nitrogenous
113 NEAAs. The fact that action of CIT is muscle-specific may explain why nitrogen balance did
114 not differ between CIT-supplemented diet and NEAA-enriched diet in old malnourished rats
115 (18). The CIT-supplemented diet increased muscle synthesis, whereas a NEAA-enriched diet
116 increased liver protein synthesis. Oral CIT rapidly increases CIT and arginine availability in
117 adults (22,23) and older patients (24). CIT administration is safe and well tolerated (25); the
118 human adult dose most appropriate for clinical practice is 10 g per day (23).

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

127

128 **Subjects and methods**

129 **Materials**

130 Citrulline, alanine, aspartic acid, glycine, histidine, proline and serine were purchased from
131 Kyowa Hakko Kogyo Co. (Tokyo, Japan); L-leucine ($1\text{-}^{13}\text{C}$), L-leucine ($5,5,5\text{-D}_3$) and sodium
132 bicarbonate (^{13}C) were from Eurisotop (Saint-Aubin, France). All AAs were checked for
133 isotopic and chemical purity by chromatography–mass spectrometry (Hewlett-Packard
134 5971A, Palo-Alto, CA). Tracer solutions were tested for sterility, pyrogenicity and stability
135 before use, and were prepared in sterile non-pyrogenic saline. In each experiment, the tracers
136 were filtered through $0.22\ \mu\text{m}$ filters.

137 **Ethical approval and registration**

138 This study was approved by the hospital ethics committee under number P070127, and
139 conformed to the standards for the use of human subjects in research prescribed in the most
140 recent update of the Declaration of Helsinki. Each participant was informed of the purpose of
141 the study, the experimental procedures, and the potential risks before giving written consent.
142 This prospective, randomized, doubled-blind trial was designed as a proof-of-concept study.
143 This trial is registered at <http://clinicaltrials.gov> (study ID number NCT00714675) and was
144 funded by the French Ministry for Health under the Hospital-led Clinical Research Program
145 (PHRC).

146 **Setting and participants**

147 Patients were screened from seven geriatric rehabilitation care units affiliated to the Greater
148 Paris public hospital system (AP-HP) for an average length of stay of 60 days (intermediate
149 care). These units focus on rehabilitation for daily living activities and prevention of falls.
150 Patients were prospectively selected and consecutively included in the study.
151 The inclusion criteria were: age > 70 years; moderate undernutrition defined according to
152 French health high authority guidelines (26) as albuminemia $\geq 30\ \text{g/L}$ and $< 35\ \text{g/L}$ and/or

153 body mass index (BMI) ≥ 18 and < 21 and/or mini nutritional assessment (MNA) (27) score
154 < 17 (but with albuminemia ≥ 30 g/L), spontaneous energy intake > 20 kcal/kg body
155 weight/day and protein intake > 0.8 g/kg body weight/day.

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

162 The trial was managed by the Henri-Mondor University Hospital Clinical Research Unit.

163 Participating sites received initiation, routine monitoring, and closeout visits.

164 **Diet and treatments**

165 The study was a double-blind, randomized, controlled prospective, multicenter trial of
166 superiority in two parallel groups: one CIT-supplemented and one placebo-supplemented. All
167 patients received standard nutritional care during the study and were given a diet supplying
168 2000 kcal/day and 66 g/day protein. They also received either an oral dose (double-blind
169 procedure) of 10 g of CIT or an equimolar mixture of six NEAAs (alanine 1.91 g, aspartic
170 acid 2.85 g, glycine 1.61 g, histidine 3.32 g, proline 2.46 g, serine 2.25 g) given iso-
171 nitrogenously every morning from Day 1 to Day 21. We note that histidine is not an NEAA
172 (28), but has no known pharmacological effect on protein metabolism.

173 No adverse events due to the treatments were recorded.

174 **Random assignment**

175 Patients were randomly assigned by a centralized computer procedure (Excel 2003, Microsoft
176 Corporation, Washington DC) on a 1:1 basis to receive CIT or NEAAs, stratified by center

177 and by permuted blocks of four. Physicians were blinded to block size. Patients were enrolled
178 by their treating physician, and registered with the central trial coordinating office by fax.

179 **Endpoints**

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

183 **Variables studied**

184 *Dietary intake*

185 Energy and protein intakes were recorded (food diaries) on study Days 1, 2, 3, 12, 13, 14, 18,
186 19 and 20. Patients were also asked to rate their appetite on Days 1 and 20 using a 100 mm
187 visual analog scale with “no appetite” and “extremely good appetite” as anchors.

188 *Physical activity*

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

192 *Body composition measurements*

193 Body composition measurements were evaluated at Day 1 and Day 20. Total lean soft-tissue
194 mass (LM), appendicular skeletal muscle mass (ASMM) and fat mass (FM) were determined
195 using dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy Advance, General Electric
196 Medical Systems, Vélizy, France). ASMM was measured as the sum of the LM for arms plus
197 legs. LM index, ASMM index and FM index were calculated as the ratios of LM, ASMM and
198 FM to the square of the person’s height in meters. Height correction allows the clinical
199 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

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

226 Plasma AA concentrations were measured before meal intake, i.e. at $t_0 - 180$ min and t_0
227 $- 60$ 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,
228 total carbon dioxide (CO_2) production rates were measured twice for 60 min each time in the
229 postabsorptive state, and in the fed state by open-circuit indirect calorimetry (Deltratrac,
230 Datex, Geneva, Switzerland).

231 **Analytical methods**

232 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
233 ($5,5,5\text{-D}_3$) enrichments were measured by gas chromatography–mass spectrometry (Hewlett-
234 Packard 5971A, Palo-Alto, CA) using tert-butyldimethylsilyl derivates. Isotopic enrichment
235 of plasma KIC is reported to reflect intracellular leucine enrichment more closely than the
236 isotopic enrichment of plasma leucine.

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

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

246 Plasma AA concentrations were determined by ion-exchange liquid chromatography using an
247 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 Ra $[^{13}\text{C}]$ leu ($\mu\text{mol.kg lean body mass}^{-1}.\text{min}^{-1}$) is total leucine flux or rate of appearance
259 (Ra), F $[^{13}\text{C}]$ leu ($\mu\text{mol.kg lean body mass}^{-1}.\text{min}^{-1}$) is tracer infusion rate corrected for isotope
260 purity, and $[^{13}\text{C}]$ leu MPE ($\mu\text{mol.kg}^{-1}$ 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 Ra $[\text{D}_3]$ 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 $[^{13}\text{C}]$ 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.

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

275 Leucine balance ($\mu\text{mol.kg lean body mass}^{-1}.\text{min}^{-1}$) was calculated as the difference between
276 NOLD and endogenous leucine fluxes.

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

$$283 \quad \text{Leu splanchnic extraction (\%)} = [1 - ([^{13}\text{C}] \text{ leu} / [\text{D}_3] \text{ leu})] \times 100,$$

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

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

289 **Sample size estimation**

290 Based on an expected difference in fed-state protein synthesis of $0.34 \mu\text{mol.kg lean body}$
291 $\text{mass}^{-1}.\text{min}^{-1}$ of NOLD (corresponding to $0.8 \text{ g protein.kg lean mass}^{-1}.\text{day}^{-1}$) between NEAA
292 group and CIT group and a standard deviation of $0.21 \mu\text{mol.kg lean mass}^{-1}.\text{min}^{-1}$ of NOLD
293 (corresponding to $0.5 \text{ g of protein.kg lean mass}^{-1}.\text{day}^{-1}$), using two-sided error rate of 5% and
294 a power at 95%, we calculated that we would need $n = 11$ patients in each group to see
295 differences between means. We took the values found by other authors (31,32).

296 **Statistical analysis**

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

298 Descriptive analysis compared the two randomized groups in terms of baseline demographics,
299 body composition and biological parameters. Only descriptive statistics were used at this
300 stage. Quantitative variables were expressed as median [min-max] or first quartile (25th
301 percentile, 'Q1') and third quartile (75th percentile, 'Q3') according to distribution.

302 Qualitative variables were expressed as numbers [%]. Plasma AA concentrations are
303 expressed as area under the curve (AUC) above the baseline, as calculated by the trapezoidal
304 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.

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

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

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

320 Factors associated with leucine splanchnic extraction were also assessed: leucine splanchnic
321 extraction was the response variable, handled in continuous mode, and the association
322 between several factors (anthropometric and metabolic parameters) and the leucine splanchnic
323 extraction. For this purpose, uni- and multivariate linear regression was used. Factors
324 associated with $p < 0.20$ in univariate analysis were considered for multivariate analysis. The
325 treatment group was introduced into the model as explanatory variable. The interactions were
326 tested, in particular between the treatment group variables and the other explanatory variables.
327 The final multivariate linear model of regression included factors significantly associated with
328 leucine splanchnic extraction. As there are no clear associated factors of leucine extraction in
329 the literature, non-adjustment for non-significant factor was forced in the model. The
330 coefficients of the final multivariate model correspond to the slope of the linear regression.
331 All tests were two-tailed. Threshold of significance was $p < 0.05$. No multiple imputations for
332 missing data were performed. Statistical analysis was performed using Stata v12.0 (College
333 Station, TX).

334

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.

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

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

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

376 There was no change in postabsorptive-state plasma AAs between Day 21 and Day 1 except
377 for arginine and ornithine, both of which are related to CIT metabolism (arginine: -23.0
378 $\mu\text{mol/l}$ [-43 ; 20] in the NEAA group, $28.5 \mu\text{mol/l}$ [3 ; 85] in the CIT group, $p = 0.001$ on
379 Wilcoxon test for unpaired samples; ornithine: $-13.0 \mu\text{mol/l}$ [-31 ; 10] in the NEAA group,
380 $44.0 \mu\text{mol/l}$ [28 ; 116] in the CIT group, $p < 0.001$).

381

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

407 steady-state in Jourdan et al. (20) in which CIT stimulated muscle protein synthesis. Finally, a
408 pharmacokinetic study (23) reports that plasma CIT returned to baseline values 5–8 h post-
409 loading only.

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

421 Here CIT supplementation was negatively associated with leucine splanchnic extraction. This
422 induces an increase in the postprandial systemic bioavailability of AAs, as demonstrated in
423 Table 6. SSAA certainly has an impact on protein metabolism, as it most likely participates in
424 the age-related decrease in lean body mass. The effect of CIT on muscle mass observed in the
425 women may thus be related to the increased availability of AAs. This is in line with the
426 negative correlation between SSAA and changes in BMI.

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

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

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

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

457 Sarcopenia in older subjects is associated with mobility disorders, increased risk of fall and
458 fracture, less ability to perform daily living activities, loss of independence, and increased risk
459 of death (1,41). Our findings on the effects of citrulline on body composition in women are in
460 line with results describing a beneficial effect of CIT on lean mass in experimental models. In
461 malnourished aged rats, CIT supplementation (5 g/kg/day) increased muscle mass, muscle
462 protein content and muscle protein synthesis compared with NEAA supplementation (18).
463 These effects of CIT on muscle mass were also found in healthy aged rats (1 g/kg/day for 12
464 weeks) that lost fat mass (42). Aging is characterized by fat mass infiltration leading to a low
465 grade inflammation (43), which accelerates the decrease in lean mass (44). The decrease in fat
466 mass observed in women in our study may therefore have contributed to the increase in
467 muscle mass. This decrease in fat mass may be explained by the fact that CIT directly
468 increases fatty acid release from rat visceral adipose tissue (45), whereas glyceroneogenesis is
469 downregulated (46). Moreover, results of proteomics investigations in malnourished aged rats
470 showed that CIT increases the expression of the main myofibrillar proteins and of enzymes
471 involved in glycogenolysis and glycolysis (19).

472 Behaviors of protein intakes were different between the two groups, with a decrease in the
473 CIT group and an increase in the NEAA group, but patient appetite scores were not different.

474 We cannot explain this effect, but even so observed an increase in lean mass in women.

475 Up to now, field-research efforts have focused on supplementation with either leucine, EAA
476 or mixtures. However, there are few randomized controlled studies in older subjects yielding
477 results on the effects of AA supplementations on lean mass. Two studies found an effect on
478 lean mass after 3 or 6 months of EAA supplementation (10,11), and another study found an

479 effect on muscle mass after 3 months, but only when combined with exercise (47). On
480 leucine-rich protein supplement, a recent meta-analysis of 16 randomized controlled studies
481 enrolling a total of 999 older subjects (48) found that leucine-rich supplementation increased
482 lean body mass in sarcopenic subjects. However, the two studies investigating long-term
483 effects (3 to 6 months) of leucine supplementation (7.5 g/d) in older subjects both reported
484 negative results on muscle mass and strength (12,13). Note that splanchnic extraction of
485 leucine is about 50% in older subjects (2,3) whereas CIT is not extracted in the splanchnic
486 area (14).

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

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

499 In conclusion, the results of our study demonstrate that CIT supplementation has no effect on
500 whole-body protein synthesis or liver protein synthesis in malnourished older subjects.
501 However, CIT supplementation was associated with a higher systemic AA availability. These
502 results on ASMM and fat mass in our subgroup of women should now spur further studies on
503 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

507 **Conflict of interest statement:**

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

512

513 **Authors' contributions to the manuscript:**

514 Aussel C and Bouillanne O designed the research.

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

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

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

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

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

521

522 **Funding sources:**

523 This work was supported by the French Ministry of Health via the *Programme Hospitalier de*
524 *Recherche Clinique* (PHRC Hospital-led Clinical Research Program) DRRC: AOM07014-
525 P070127

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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 ($1-^{13}\text{C}$) 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

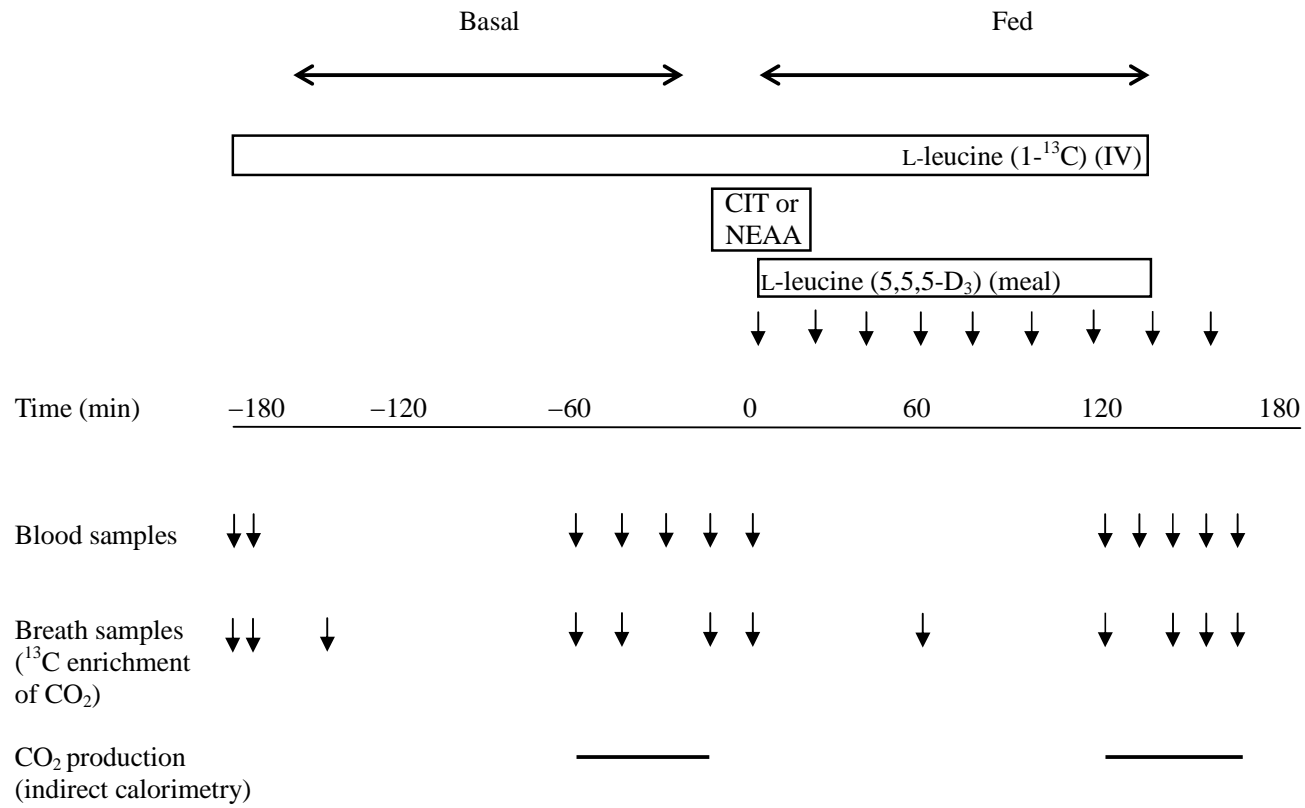


Fig. 1.

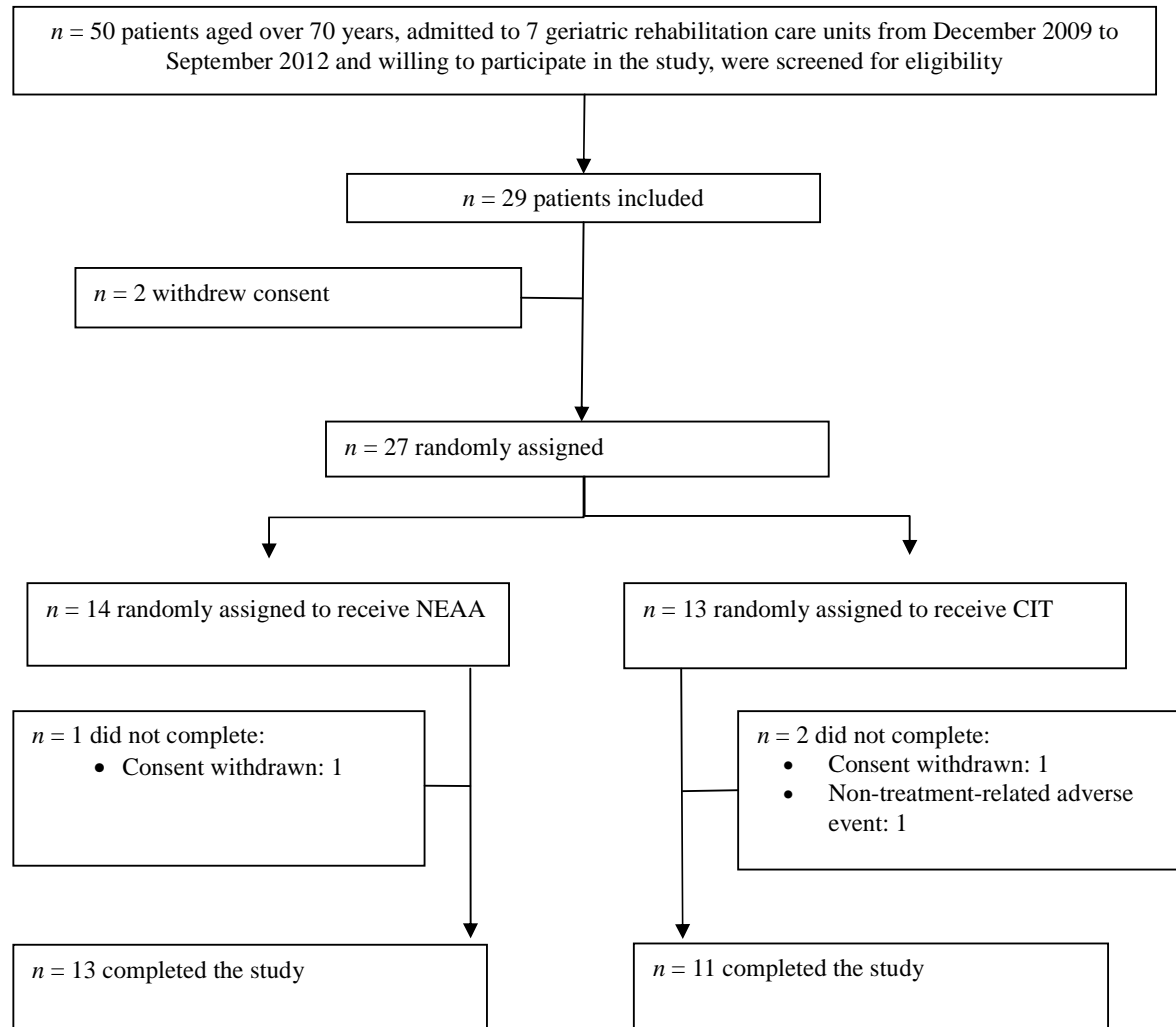
**Fig. 2.**

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		<i>p</i> ¹	Fed state		<i>p</i> ¹
	NEAA <i>n</i> = 13	CIT <i>n</i> = 11		NEAA <i>n</i> = 13	CIT <i>n</i> = 11	
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]

AAs: 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.