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Effect of oral citrulline supplementation on whole body protein metabolism in adult patients with short bowel syndrome: a pilot, randomized, double-blind, cross-over study

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Clinical Trial Registry number: NCT01386034 in the www.clinicaltrials.gov website.

1 SUMMARY

2 *Background & aims:* As citrulline is produced by small intestine, plasma citrulline
3 concentration is decreased and may become essential in patients with short bowel syndrome
4 (SBS). In a rat model of SBS, citrulline supplementation enhanced muscle protein synthesis.
5 The aim of the study was to determine whether citrulline impacts whole body protein
6 metabolism in patients with SBS.

7 *Methods:* Nine adults with non-malignant SBS (residual small bowel 90±48 cm; mean±SD)
8 who were in near-normal nutritional status without any artificial nutrition, were recruited long
9 after surgery. They received 7-day oral supplementation with citrulline (0.18 g/ kg/day), or an
10 iso-nitrogenous placebo in a randomized, double-blind, cross-over design with a 13-day wash-
11 out between regimens, and an intravenous 5-h infusion of L-[1-¹³C]-leucine in the
12 postabsorptive state to assess protein metabolism after each regimen.

13 *Results:* Plasma citrulline concentration rose 17-fold (25±9 vs. 384±95 µmol/L) and plasma
14 arginine 3-fold after oral citrulline supplementation (both $p < 4 \times 10^{-6}$). Supplementation did not
15 alter leucine appearance rate (97±5 vs. 97±5 µmol.kg⁻¹.h⁻¹; $p=0.88$), leucine oxidation (14±1
16 vs. 12±1 µmol.kg⁻¹.h⁻¹; $p=0.22$), or non-oxidative leucine disposal (NOLD), an index of
17 whole-body protein synthesis (83±4 vs. 85±5 µmol.kg⁻¹.h⁻¹; $p=0.36$), nor insulin or IGF-1
18 plasma concentrations. In each of the 3 patients with baseline citrulline <20 µmol/L, citrulline
19 supplementation increased NOLD. Among the 7 patients with plasma citrulline <30µmol/L,
20 the effect of supplementation on NOLD correlated inversely ($r^2=0.81$) with baseline plasma
21 citrulline concentration.

22 *Conclusion:* 1) Oral citrulline supplementation enhances citrulline and arginine bioavailability
23 in SBS patients. 2) Oral citrulline supplementation does not have any anabolic effect on whole
24 body protein metabolism in patients with SBS in good nutritional status, in the late phase of

25 intestinal adaptation, and with near-normal baseline citrulline homeostasis. 3) Whether oral
26 citrulline would impact whole body protein anabolism in severely malnourished SBS patients
27 in the early adaptive period, and with baseline plasma citrulline below 20 $\mu\text{mol/L}$, warrants
28 further study. Registered under ClinicalTrials.gov Identifier no. NCT01386034

29 **Keywords:** Intestinal deficiency, Amino acids, Stable isotopes, Arginine

30

31

32 1. Introduction

33 Short bowel syndrome (SBS) is defined by a residual small bowel length below 200 cm
34 following intestinal resection. SBS initially results in intestinal failure [1] requiring
35 intravenous delivery of nutrients and electrolytes. Weaning SBS patients off parenteral
36 nutrition, however, often becomes feasible after several weeks or months, as oral food intake
37 enhances intestinal adaptation [2]. In recent years, treatment with glucagon-like peptide 2
38 (GLP2) was proven to enhance intestinal mucosa growth [3], but GLP2 remains expensive,
39 and is covered by health insurance only in the US and a few European countries. Moreover, as
40 GLP-2 promotes crypt cell proliferation, treatment with GLP-2 analogs may, in theory,
41 increase the risk of cancer. Even though GLP-2 was shown to promote tumor growth in
42 animals with pre-existing cancer, treatment with GLP-2(1-33) up to 30 months was not found
43 to increase the risk of cancer in humans without any known pre-existing neoplasia; the
44 relatively small number of patients treated with GLP-2 analogs, however, precludes any final
45 conclusion [4]. Although recombinant human growth hormone (rhGH) improved intestinal
46 adaptation, no consensus has been reached regarding its use in SBS patients [5]. Even though
47 glutamine is known to be the main fuel of enterocytes, glutamine supplementation failed to
48 improve intestinal absorption or nutritional status in short bowel patients [6,7]. Whether other
49 specific nutrients promote intestinal adaptation or lean body mass accretion remains to be
50 explored [6,7]

51 Citrulline is a non-essential amino acid released by small intestine into portal blood [8], and is
52 produced, in part, from glutamine metabolism in enterocytes [9,10]. Accordingly, glutamine
53 supplementation increases citrulline availability in humans [11]. Crenn et al. were first to
54 report a linear correlation between plasma citrulline concentration and residual small bowel
55 length in SBS patients [12, and plasma citrulline was subsequently found to reflect functional
56 intestinal enterocyte mass in several conditions associated with altered intestinal function [13-

15]. Although citrulline is not incorporated into protein, it is taken up by kidney, where it is converted to arginine. Arginine, a semi-essential amino acid for protein synthesis, is known to exert anabolic effects through a stimulation of GH and insulin secretion in healthy humans [16]. Extensive small bowel resection depletes plasma arginine in rats, and is associated with negative nitrogen balance and slower growth, implying arginine may become conditionally essential in SBS [17]. Orally administered arginine, however, undergoes extensive first pass extraction in the splanchnic bed; oral arginine supplementation therefore often is relatively ineffective to increase systemic arginine availability. In contrast, citrulline, whether exogenous or endogenous, escapes splanchnic uptake, so oral citrulline supplementation is an attractive alternative to raise plasma arginine levels [18]. Osowska *et al.* were first to demonstrate that supplementation of enteral feeding with citrulline improved nitrogen balance and increased muscle protein synthesis in rats that had undergone extensive small intestinal resection [19]. An anabolic effect of citrulline supplementation on muscle protein synthesis was documented in other experimental models of undernutrition in aged, malnourished rats [20], or rat fetuses with intrauterine growth restriction due to maternal undernutrition [21]. Little is known about the putative effect of citrulline supplementation on protein metabolism *in vivo* in humans. Jourdan *et al.* found that orally supplemented citrulline increased skeletal muscle protein synthesis in healthy young men fed a low protein diet [22], with no detectable effect on whole body protein metabolism. In healthy young adults fed an adequate protein diet, we failed to observe any effect of oral citrulline supplementation on nitrogen balance nor on whole body protein kinetics [23].

The best of our knowledge, the effect of oral citrulline supplementation has yet to be explored in patients with SBS. We hypothesized that citrulline supplementation would enhance whole body protein synthesis in short bowel patients since (a) undernutrition and impaired protein absorption due to severe short bowel syndrome (extensive small bowel resection) would make

82 arginine an essential amino acid. and (b) literature suggests citrulline had a protein anabolic
83 effect in several conditions associated with malnutrition in animals.

84

85

ACCEPTED MANUSCRIPT

86 2. Patients and methods

87 The study was designed as a bi-centric, prospective, randomized, double blind, cross-over
88 trial, registered as NCT01386034 in the www.clinicaltrials.gov database
89 (<https://clinicaltrials.gov/ct2/show/NCT01386034>). The primary objective was to determine whether
90 oral citrulline supplementation would have an anabolic effect *via* a stimulation of whole body
91 protein synthesis or a decrease in proteolysis—assessed using infusion of stable isotope labeled
92 leucine (L-[1-¹³C]leucine)—in short bowel patients.

93 2.1 Study population

94 Subjects were recruited among out-patients followed for short bowel syndrome by the
95 Nutrition Support Team in the gastroenterology unit of University Hospital at Nantes, and
96 Clinique St Yves at Rennes, specialized in intestinal rehabilitation. Prior to enrolment, each
97 subject signed an informed consent form, according to protocols approved by the ethical
98 committee of the Pays de La Loire region (CPP agreement # 2011-00255-16 dated April 19,
99 2011).

100 Short bowel syndrome was defined as bowel length < 200 cm [24], or evidence for
101 malabsorption from intestinal insufficiency. Though balance studies using fecal calorimetric
102 analysis clearly are the 'gold standard' [2,25], many tests can be used to assess malabsorption
103 such as a wet weight absorption <1.41 kg/d, or an energy absorption < 84% of the calculated
104 basal metabolic rate in 48-h metabolic balance studies [25]. Performance of balance studies,
105 however, is cumbersome as it requires several days of stool collection and the availability of
106 suitable equipment [26]. Similarly, the multiple probe sugar test can be used to assess both
107 intestinal permeability (mostly in inflammatory bowel disease) [27] but is not routinely
108 available in our hospital so we have no experience with its use. We accepted as evidence of
109 malabsorption the presence of at least one of the following criteria:

- 110 - output stoma > 1.2 L/24h for an ileostomy; although high output stoma was defined
111 as an output >2 L/24h by Baker et al [28], there is no clear-cut definition of high
112 output as multiple factors influence stoma output [29]; or >300 g/24h for a colostomy
113 - steatorrhea $>15\%$ of ingested fat, nitrogen loss $> 20\%$ of ingested protein/24hs,
114 determined using a 3-day stool collection when data were available.

115 To be enrolled, patients also had to :

- 116 - Be between 18 and 75 years of age,
117 - Be in a stable condition, at least 6 months after the last gastrointestinal surgery,
118 - Be weaned from parenteral or enteral nutrition and on full oral nutrition,
119 - Receive no citrulline supplementation (commercially available in France as Stimol®),
120 - Be free of end stage kidney, pulmonary, or cardiac insufficiency,
121 - Be free of diabetes, and have a fasting blood glucose < 6 mmol/L,
122 - Receive no glucocorticosteroid treatment ,
123 - Be free of any active inflammatory disease (IBD, intercurrent infection...), chronic or
124 acute pancreatitis, or cancer.

125 A sample of 12 patients was calculated to be sufficient to detect a 24% change in leucine
126 oxidation, with an α risk of 10%, and a power of 80%.

127

128 2.2 Study design.(Fig 1)

129 A dietary history was obtained by an experienced dietician upon enrolment and before each
130 tracer study. Patients were encouraged to maintain a constant oral intake particularly
131 regarding protein intake (around 20% of total caloric intake), and to keep the same physical
132 activity level during the study period.

133 Each patient was admitted twice to the Clinical Research Center (CRC) of Nantes University
134 Hospital for a half-day, within a 4-week interval. Before each admission, each patient
135 received a one-week supplementation with oral natural L-citrulline (0.18g/kg/day), or with an
136 iso-nitrogenous placebo mixture constituted of an equimolar mix of 6 free amino acids: L-
137 alanine, L-aspartic acid, L-glycine, L-histidine, L-proline and L-serine supplied by INRESA
138 Pharma (France). The supplements were given in a blinded fashion as a powder in single dose
139 vials that the patients had to dissolve in water at home. The list of randomization was
140 established by the Pharmacy at Nantes University Hospital. The supplements were
141 administered in randomized order, and separated by a 13-day wash-out period. A stable
142 isotope infusion took place the day after the week of supplementation (citrulline or placebo).
143 Each patient was used as his/her own control in a cross-over design. Patients and investigators
144 were blinded as to the nature of the supplement administered.

145 A 5-hr, stable isotope infusion study was carried out on the 8th and 29th day of the study. In
146 the morning of each tracer study, subjects reported to the CRC in the post-absorptive state
147 after a 12-h overnight fast.

148 Weight, height, and body mass index (BMI) were measured at inclusion visit and before each
149 of the tracer infusion studies. Body composition was evaluated by multi-frequency body
150 impedance analysis (QuadScan 4000 ®, Bodystat Ltd., UK). Additional blood samples were
151 obtained for determination of plasma citrulline and arginine concentrations, serum
152 electrolytes, albumin, thansthyretin, insulin, and insulin-like growth factor 1 (IGF-1). A urine
153 sample was taken before and after the tracer infusion to determine urinary nitrogen, nitrite,
154 and nitrate concentration.

155 At 08:00h, two catheters were inserted: the first catheter was placed in forearm vein for the
156 stable isotope (L-[1-¹³C]leucine) infusion. The second one was placed in a contralateral hand
157 vein and used for blood sampling; the hand was placed in a heating pad to obtain arterialized

158 venous blood at baseline, and 180, 200, 220, 240, 260, and 280 min after the start of isotope
159 infusion to determine ^{13}C -enrichment in plasma keto-isocaproate (KIC), the keto-acid of
160 leucine. Throughout the tracer infusion, subjects were taking an oral dose of citrulline or
161 placebo dissolved in water every 20 min but remained fasting otherwise through the end of
162 tracer infusion. Plasma amino acid concentrations were measured after 180 min of isotope
163 infusion while patients received q.20 min aliquots of either citrulline or 'placebo'.

164 Tracer infusion was started with a $6 \mu\text{mol.kg}^{-1}$ bolus injection at 08:00h (time 0), immediately
165 followed by a continuous, 5-hr infusion at the constant rate of $6 \mu\text{mol.kg}^{-1}.\text{h}^{-1}$ until 13:00h.

166 Simultaneously, overall rates of oxygen consumption (VO_2), carbon oxide production
167 (VCO_2), and resting energy expenditure were measured for three 20-min periods over the
168 course of isotope infusion *via* indirect calorimetry (Quark RMR®, Cosmed, Italy), and
169 aliquots of expired air were collected at timed intervals over the last 2 hrs of isotope infusion
170 to define $^{13}\text{CO}_2$ enrichments.

171 At 13:00h, intravenous lines were removed, patients were fed, and discharged home to resume
172 their routine treatment.

173 *2.3 Analytical methods*

174 Plasma citrulline was determined by liquid chromatography-tandem mass spectrometry (LC-
175 MSMS).

176 The ^{13}C -enrichment in plasma KIC was measured by gas chromatography-mass spectrometry
177 (GCMS) using a Hewlett-Packard-MSD 5971® instrument (Agilent Technologies Inc., Santa
178 Clara, California, USA) in the Mass Spectrometry Platform of the Human Nutrition Research
179 Center (CRNH-Ouest) at Nantes as described [30]. Plasma concentrations of leucine and KIC
180 were determined by GCMS by reverse isotope dilution using labeled internal standards ($^2\text{H}_7$ -

181 leucine and $^2\text{H}_7\text{-KIC}$). ^{13}C -enrichment in expired $^{13}\text{CO}_2$ was determined by isotope-ratio mass
182 spectrometry coupled to gas chromatography (GC-IRMS) at the Biochemistry department of
183 Medical Faculty at Lausanne, Switzerland.

184

185 *2.4 Calculation of whole body leucine kinetics*

186 Leucine appearance into plasma (R_a , $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$), an index of whole body protein
187 breakdown, was determined as $R_a = i \times [(E_i/E_p) - 1]$, where E_i and E_p represent ^{13}C -enrichments
188 (mole % excess) in infused leucine and plasma KIC at steady state, respectively. Leucine
189 oxidation (Ox) was calculated as $\text{Ox} = \text{VCO}_2 \times \text{ECO}_2 \times [(1/E_p\text{KIC}) - (1/E_i\text{Leu})] / 0.81$; where
190 ECO_2 is ^{13}C -enrichment in expired CO_2 at steady state, and VCO_2 is overall carbon dioxide
191 measured using indirect calorimetry, and 0.81 corrects for incomplete ^{13}C recovery, as
192 previously described [30].

193 Protein synthesis was estimated by non-oxidative leucine disposal (NOLD), using the
194 equation: $\text{NOLD} = R_a \text{ Leu} - \text{Ox}$.

195

196 *2.5 Statistics*

197 Statistical analysis was performed using the BiostaTGV:
198 (<http://marne.u707.jussieu.fr/biostatgv>) and The R project for statistical computing software
199 (<https://www.R-project.org>). Continuous variables are expressed as mean and standard
200 deviation ($\pm\text{SD}$). Wilcoxon test was used to compare the two treatments (Placebo and
201 Citrulline). P-value < 0.05 was considered statistically significant.

202

203 3. Results

204 3.1 Patient characteristics

205 Among the 11 patients enrolled in the study, 1 patient in each group withdrew his/her
206 participation after the first regimen. A total of 9 patients (4 men and 5 women) completed the
207 study between 2013 and 2015, and were included in analysis (Fig 2). The mean age was $64 \pm$
208 11 years. The mean small intestine length was 93 cm, ranging between 40 and 200 cm. The
209 etiology of short bowel syndrome, the remaining small and large bowel length and short
210 bowel type as well as the time elapsed since last surgery. Anthropometric parameters
211 confirmed all patients were in a normal nutrition status. The results of bioelectrical impedance
212 analysis showed a fat mass and fat free mass of $24 \pm 3.94\%$ and $76.1 \pm 3.32\%$, respectively, in
213 men, and $37.5 \pm 9.3\%$ and $62.5 \pm 9.3\%$ in women. All patients likely had developed
214 hyperphagia with a mean energy intake of $45.4 \pm 16.2 \text{ kcal.kg}^{-1}.\text{d}^{-1}$ and a protein intake of
215 $1.97 \pm 0.92 \text{ g.kg}^{-1}.\text{d}^{-1}$.

216 Baseline serum albumin and prealbumin/transthyretin concentrations were $4.1 \pm 0.25 \text{ g/dL}$ and
217 $23 \pm 2 \text{ mg/dL}$, respectively, and plasma citrulline was $25 \pm 9 \text{ } \mu\text{mol/L}$. (Table 1).

218 In theory, citrulline may have a long-lasting effect, and impact protein metabolism even after
219 cessation of citrulline supplementation. Such putative, long-lasting effect, would, however, be
220 unlikely to last more than a week, as is the case for alterations in leucine kinetics upon acute
221 alterations of dietary protein intake. This was the rationale for the 2-week washout period
222 inbetween the citrulline and placebo supplementation periods. According to the cross-over
223 design (Fig 1), 5 patients received citrulline supplementation first, followed by placebo,
224 whereas were assigned to the opposite sequence. We did not detect any difference between
225 patients who received placebo first and those who received citrulline first. No side effect was
226 observed in either group.

227

228 ***3.2 Effect of citrulline supplementation on plasma amino acid, insulin and IGF-1*** 229 ***concentrations***

230 Plasma citrulline rose 17-fold with supplementation (Citrulline vs. Placebo : 384 ± 95 vs. $25 \pm$
231 $9 \mu\text{mol/L}$, $p < 0.05$) (Table 2). Plasma arginine concentration rose 3-fold (Citrulline vs.
232 Placebo: 146 ± 28 vs. $47 \pm 19 \mu\text{mol/L}$, $p < 0.05$), and plasma ornithine 2-fold (Citrulline vs.
233 Placebo: 135 ± 18 vs. $54 \pm 14 \mu\text{mol/L}$, $p < 0.05$). Plasma glutamine and leucine
234 concentrations were not affected by citrulline supplementation (Table 2). As expected, the
235 plasma concentrations of the non-essential amino acids supplied as part of the placebo
236 regimen were higher on the placebo day (Table 2). There was no statistically significant
237 difference in the plasma concentrations of IGF-1 and insulin (data not shown).

238

239 ***3.3 Effect of citrulline supplementation on protein metabolism.***

240 There was no significant difference in plasma ^{13}C -KIC enrichment (6.03 ± 0.88 vs. $6.09 \pm$
241 0.92 mole % excess, MPE) nor in expired $^{13}\text{CO}_2$ (0.009 ± 0.001 vs. 0.009 ± 0.002 MPE) when
242 comparing Citrulline vs. Placebo, and, consequently, no detectable effect on leucine
243 oxidation, whether results were expressed per kg of body weight (Fig 3) or kg of FFM (data
244 not shown).

245 Similarly, plasma ^{13}C -KIC enrichment did not differ between study days, so oral
246 supplementation with citrulline did not affect leucine Ra, oxidation, or estimates of leucine
247 incorporation into protein (NOLD). This held true whether results were expressed per kg of
248 body weight or per of kg of fat free mass (data not shown).

249 Among the 9 patients enrolled, three (#2, 3, and 4) had a baseline citrulline of 13, 20, and 11
250 $\mu\text{mol/L}$, *i.e.*, at or below $20 \mu\text{mol/L}$, a threshold found to associate with the persistent need for

251 parenteral nutrition in earlier studies [12]. In the latter 3 patients, citrulline supplementation
252 was consistently associated with a rise in NOLD (from 85.9 to 91.3; 70.3 to 72.6; and 77.4 to
253 83.5 $\mu\text{mol.kg}^{-1}.\text{h}^{-1}$ in subjects #2,3 and 4, respectively).

254 Conversely, 2 patients (#7 and 9) had a baseline citrulline $>30 \mu\text{mol/L}$ (39 and 35 $\mu\text{mol/L}$ in
255 subjects #7 and 9, respectively). If the latter 2 patients were excluded from analysis, a tight
256 correlation would be apparent between the change in NOLD associated with citrulline
257 supplementation and baseline plasma citrulline concentration (Fig. 4).

258

259 4. Discussion

260 To the best of our knowledge, the current study is first to explore the effect of oral citrulline
261 supplementation in patients with short bowel syndrome. We demonstrate that oral citrulline
262 supplementation can efficiently raise the concentration of plasma citrulline and arginine in
263 humans after extensive small intestinal resection. Citrulline supplementation, however, did
264 not enhance whole body protein accretion under the conditions tested.

265 Our study documents the bioavailability of citrulline during oral supplementation of citrulline
266 in short bowel patients. The 17- and 3-fold rise in the plasma concentration of citrulline and
267 arginine, respectively, suggests that 1) citrulline may be predominantly absorbed in the
268 proximal small bowel (duodenum, jejunum) since most of our patients had a very short
269 residual ileum; 2) splanchnic citrulline uptake is likely low, and 3) citrulline conversion to
270 arginine is unaltered in short bowel patients in the absence of renal insufficiency.

271 Since arginine has long been known to stimulate GH and insulin secretion, we measured the
272 concentrations of IGF1 and insulin with or without citrulline supplementation, and did not
273 find any significant difference between the two periods (Citrulline vs. Placebo), consistent
274 with our earlier findings using the same dose of citrulline in healthy subjects [23].

275 The lack of any protein anabolic effect of citrulline supplementation in the current study
276 contrasts with the dramatic anabolic effect of citrulline documented in enterectomized rats
277 [20]. Besides obvious differences between species, several factors could account for such
278 discrepancy, including 1) a type 2 statistical error; 2) the characteristics of the patient
279 population sample; and 3) a true lack of any anabolic effect of citrulline either on whole body
280 or muscle protein metabolism. These potential causes are addressed below.

281 The first potential explanation is the small number of patients enrolled in the study. We
282 encountered difficulties in recruiting the number of 12 patients suggested by our initial

283 calculation of statistical power. In theory, we may have missed a true anabolic effect of
284 citrulline on whole body leucine kinetics because only 9 patients completed the 2 arms of the
285 study. Yet no consistent pattern was observed: with citrulline supplementation, NOLD
286 increased in 4 patients, but decreased in 5, and the mean change in NOLD was less than 1
287 $\mu\text{mol.kg}^{-1}.\text{h}^{-1}$ (i.e., <1% of NOLD), between regimens. The number of 12 patients was
288 calculated based on the assumption that it would be large enough to detect a 24% change in
289 leucine oxidation, with an α risk of 10%, and a power of 80%. In the 9 patients enrolled, we
290 observed a change in leucine oxidation of only $\approx 10\%$, from $13.8 \pm 3.6 \mu\text{mol.kg}^{-1}.\text{h}^{-1}$ with
291 placebo to $12.3 \pm 3.6 \mu\text{mol.kg}^{-1}.\text{h}^{-1}$ with citrulline supplementation. The putative number of
292 patients required to reach statistical significance with such change can be re-calculated as $n=2$
293 $+ C (\sigma/\delta)^2$ where $C=7.85$ for an α risk of <0.05, and a power $(1-\beta)$ of 80%, and σ^2 is the
294 variance of the measured parameter, and δ is the expected difference between the two studies
295 on the measured parameter [31]. In our case, the measured parameter is leucine oxidation, δ is
296 $13.8-12.3=1.5$, and $\sigma=3.6$, so $(\sigma/\delta)=3.6/1.5=2.4$; therefore $n=2 + 7.85 \times (2.4)^2 = 2 + (7.85 \times$
297 $5.8)= 47$. As short bowel syndrome is a relatively rare disease, recruiting 47 patients from our
298 unit seemed unrealistic to us, and we therefore elected to report our observation as a
299 preliminary, 'pilot' study instead.

300 A second factor may be the characteristics of the sample population. Whereas the
301 enterectomized rats that benefited from citrulline supplementation showed evidence of severe
302 malnutrition in the study by Oszowska et al [19], patients enrolled in the current study did not
303 show any evidence of undernutrition. The normal body mass index ($23.97 \pm 3.69 \text{ kg.m}^{-2}$), fat
304 free mass ($71 \pm 11\%$), and normal serum albumin ($4.1 \pm 0.25 \text{ g.dL}^{-1}$) and prealbumin (23 ± 2
305 mg/dL) together suggest that our patients were not significantly undernourished. Moreover, in
306 the current study, the time elapsed since last surgery was, on average, 23 months; such time
307 laps was likely long enough for intestinal adaptation to have taken place between intestinal

308 resection and enrolment in the current study. All patients indeed presented with high dietary
309 intakes, suggesting compensatory hyperphagia, which is an important determinant of
310 intestinal adaptation, and all had been successfully weaned off home parenteral nutrition
311 (HPN). HPN independence was one of the inclusion criteria, to avoid any potential effect of
312 parenteral amino acid infusion on protein metabolism. In contrast, in the study by Osowska et
313 al., enterectomized rats received citrulline supplementation only 4 days after surgery [17].
314 Among our patients, no effect of citrulline was observed even in the subgroup of patients who
315 had undergone surgery less than 1 year prior to study and who would meet the criteria for type
316 II intestinal failure (prolonged acute condition for weeks to months) [1].

317 The baseline plasma citrulline concentration of $25 \pm 9 \mu\text{mol/L}$ (mean \pm SD) observed in our
318 patients, was similar to the $27 \pm 5 \mu\text{mol/L}$ measured in our earlier study in healthy volunteers
319 [23], and consistent with the fact that our patients had achieved full intestinal adaptation
320 before the study. Crenn et al. found a concentration of citrulline $>20 \mu\text{mol/L}$ to predict
321 successful HPN weaning [9]. Even though subgroup analysis cannot be statistically valid on
322 small groups, we attempted to better characterize responders versus non-responders. In our
323 study, citrulline supplementation was consistently associated with a protein anabolic response
324 in the 3 patients at or below that threshold (data not shown). The reason for the lower baseline
325 plasma citrulline concentration in that subset of patients is unclear. Postabsorptive plasma
326 citrulline concentration was shown to be decreased, not only in short bowel syndrome but in
327 many other conditions such as radiation enteritis [14], intestinal graft rejection [15], active
328 coeliac disease with villous atrophy [13], or transient enterostomy—for instance plasma
329 citrulline was decreased in patients with enterostomy, and rose after chime reinfusion [32].
330 This is why citrulline has emerged as a biomarker of functional intestinal mass [34]. Whether
331 a short transit time *per se*, regardless of its cause, impacts plasma citrulline, has not, to our
332 knowledge, been investigated. In theory, a rapid transit time may decrease amino acid

333 absorption from intestinal lumen by decreasing the time of exposure of nutrients to intestinal
334 mucosa, and reduce the absorption of amino acid precursors of citrulline, such as glutamine or
335 proline [9-11]. In the current study, in the small sample population studied, we did not
336 observe any obvious relationship between the etiology of short bowel syndrome, the number
337 of stools per day, and plasma citrulline.

338 These 3 patients had the shorter residual small bowel length; they tended to have low
339 transthyretin, 2 had a distal ileum and an entire colon in continuity, 2 had a delay after surgery
340 of <1 yr (Table 1); they did not appear to differ from the rest regarding age, energy or protein
341 intake, weight, BMI, and lean body mass, nor regarding the effect of citrulline
342 supplementation on IGF-1. Yet animal studies suggest citrulline may exert its putative
343 protein anabolic effect directly through the PI3K/MAPK/4E-BP1 pathway, a signaling
344 pathway involved in the regulation of protein synthesis [34]. The response of NOLD to
345 citrulline supplementation may indeed depend on baseline citrulline concentration: when the 2
346 patients with the higher baseline plasma citrulline concentration ($>30 \mu\text{mol/L}$) were excluded
347 from analysis, a tight correlation was observed between the response of NOLD, an index of
348 whole body protein synthesis, and baseline plasma citrulline (Fig 3); in other words, the lower
349 the baseline plasma citrulline concentration, the more anabolic effect was observed upon
350 citrulline supplementation. Although such small sample size precludes any firm conclusion,
351 those preliminary results strongly suggest that citrulline supplementation may have a protein
352 anabolic role in patients who are truly citrulline deficient.

353 A limitation of our study is the fact that we did not quantitate intestinal absorption nor
354 intestinal permeability, so our patients likely had various degrees of fat and protein
355 malabsorption.

356 Another limitation of our study is the fact we explored the effect of citrulline solely on whole
357 body protein metabolism. In the study by Jourdan *et al*, even though citrulline enhanced

358 fractional synthesis rate in skeletal muscle in healthy volunteers fed a low protein diet, no
359 effect of citrulline was detected at the whole body level [22]. Similarly, in previous studies,
360 we failed to observe any effect of oral citrulline supplementation on whole body protein
361 metabolism in healthy volunteers [23]. A recent study showed a significant rise in
362 appendicular skeletal muscle mass evaluated by DEXA scan, in malnourished, elderly women
363 after 3 weeks of oral citrulline supplementation [35]. Taken together, literature thus suggests
364 that the protein anabolic effect of citrulline may be specific to skeletal muscle [36].

365 The main strength of the current study nevertheless stems from the fact that this is the first
366 pilot trial of oral citrulline in a carefully selected, relatively homogenous group of patients
367 with short bowel syndrome explored with sensitive methods to detect any change in whole
368 body protein metabolism.

369

370 **5. Conclusion**

371 The results of our study demonstrate that oral citrulline supplementation can increase
372 citrulline and arginine bioavailability in short bowel patients. They further suggest that oral
373 citrulline supplementation does not have any anabolic effect on whole body protein
374 metabolism in patients with short bowel syndrome in good nutritional status, in the late phase
375 of intestinal adaptation, and with near-normal baseline citrulline homeostasis. Whether oral
376 citrulline would impact whole body or skeletal muscle protein anabolism in short-bowel
377 patients in the early adaptive period, who are severely malnourished, and/or have plasma
378 citrulline below 20 $\mu\text{mol/L}$, would clearly warrant further study.

379

380 **Authors' contribution**

381 Author Contributions: AJ and DD had full access to all of the data in the study and take
382 responsibility for the integrity of the data and the accuracy of the data analysis. Study concept
383 and design: AJ, DP, RT, DD

384 Acquisition, analysis, or interpretation of data: All authors

385 Drafting of the manuscript: AJ, DD

386 Critical revision of the manuscript for important intellectual content: All authors

387 Statistical analysis: AJ, DD

388 Study supervision: AJ, DD

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392 study; collection, management, analysis, and interpretation of the data; preparation, review, or
393 approval of the manuscript; or decision to submit the manuscript for publication.

394

395 **Conflict of interest statement**

396 None for any of the authors.

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403

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ACCEPTED MANUSCRIPT

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Table 1

423 Etiology and anatomic characteristics of short bowel syndrome (SBS), baseline
 424 anthropometric data, body composition from bioelectrical impedance analysis (BIA), and
 425 dietary intake in enrolled patients; WT: body weight; IBW: ideal body weight; MI:
 426 mesenteric ischemia; Radiation: post-radiation enteritis; Crohn: Crohn's disease; Type of SBS:
 427 type I: SBS with end jejunostomy; type II; SBS with jejunocolic anastomosis; type III: ileo-
 428 jejunocolic anastomosis.

429

Patient	Age yrs	Wt kg	BMI kg/m ²	% of IBW	Fat free mass %	SBS etiology	SBS type	Remnant small bowel length cm	Remnant colon %	Time since surgery (months)	Plasma citrulline $\mu\text{mol/L}$	Plasma Creatinine $\mu\text{mol/L}$	Dietary energy intake kcal/kg/d	Dietary protein intake g/kg/d
1	64	61.5	23.7	106	75	MI	II	120	71	169	27	91	40	1.9
2	75	61	23.5	155	73	MI	II	50	57	88	13	66	55	2.5
3	64	67	23.3	104	76	Volvulus	III	65	100	11	20	96	36	1.4
4	41	72	29.2	133	62	MI	III	40	100	10	11	56	22	1.0
5	55	60	20.0	94	76	Radiation	II	100	71	55	24	94	41	1.8
6	71	52	20.6	94	55	MI	I	200	0	23	28	79	73	3.9
7	69	66.5	21.1	94	79	MI	II	100	85	138	39	69	46	2.3
8	66	68	29.9	135	56	MI	II	70	71	8	28	73	31	1.1
9	49	70	21.5	96	87	Crohn	II	200	71	378	35	119	38	1.5
mean \pm SD	61 \pm 11	64 \pm 6	23 \pm 3.6	107 \pm 16	71 \pm 11)			90 \pm 48	70 \pm 30	63 \pm 63	25 \pm 9	82 \pm 19	42 \pm 14	1.9 \pm 0.8
median	64	66	23.3	104	75			70	71	39	27	79	40	1.8

430

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432

433

434 **Table 2**

435 Effect of citrulline or placebo supplementation on plasma amino acid concentrations in short
 436 bowel patients.

	Placebo	Citrulline	p
Aminoisobutyric acid	17±4	14±4	0.22
Alanine	342±165	283±188	0.25
Arginine	54±31	164±41	3.5x10 ⁻⁶
Asparagine	45±10	39±6	0.10
Aspartic acid	5±4	2±1	0.03
Citrulline	26±10	384±95	3.5x10 ⁻⁶
Cysteine	38±22	47±33	0.25
Glutamine	578±165	567±112	0.88
Glutamic acid	43±18	36±12	0.09
Glycine	369±96	230±44	0.006
Histidine	156±77	64±17	0.003
Isoleucine	51±10	53±10	0.83
Leucine	96±11	102±13	0.63
Lysine	158±48	139±30	0.31
Methionine	18±4	16±3	0.49
Ornithine	61±23	163±48	3.4x10 ⁻⁵
Phenylalanine	40±11	41±10	0.5
Proline	239±78	176±31	0.14
Serine	143±45	73±15	0.002
Taurine	50±21	49±14	0.65
Threonine	88±32	71±19	0.14
Tryptophan	34±11	29±9	0.08
Tyrosine	37±11	37±9	0.96
Valine	158±17	157±25	0.99

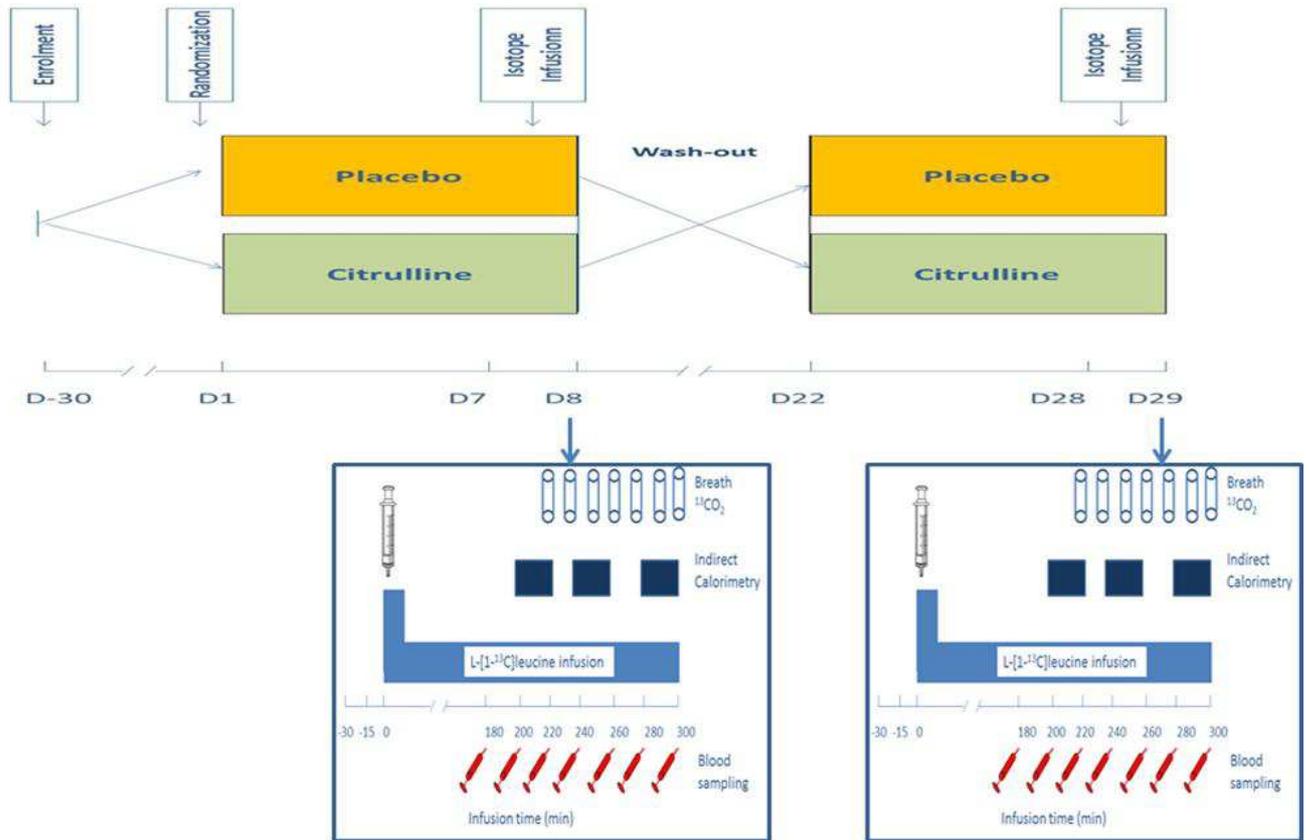
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439 **Fig 1. Study design.**

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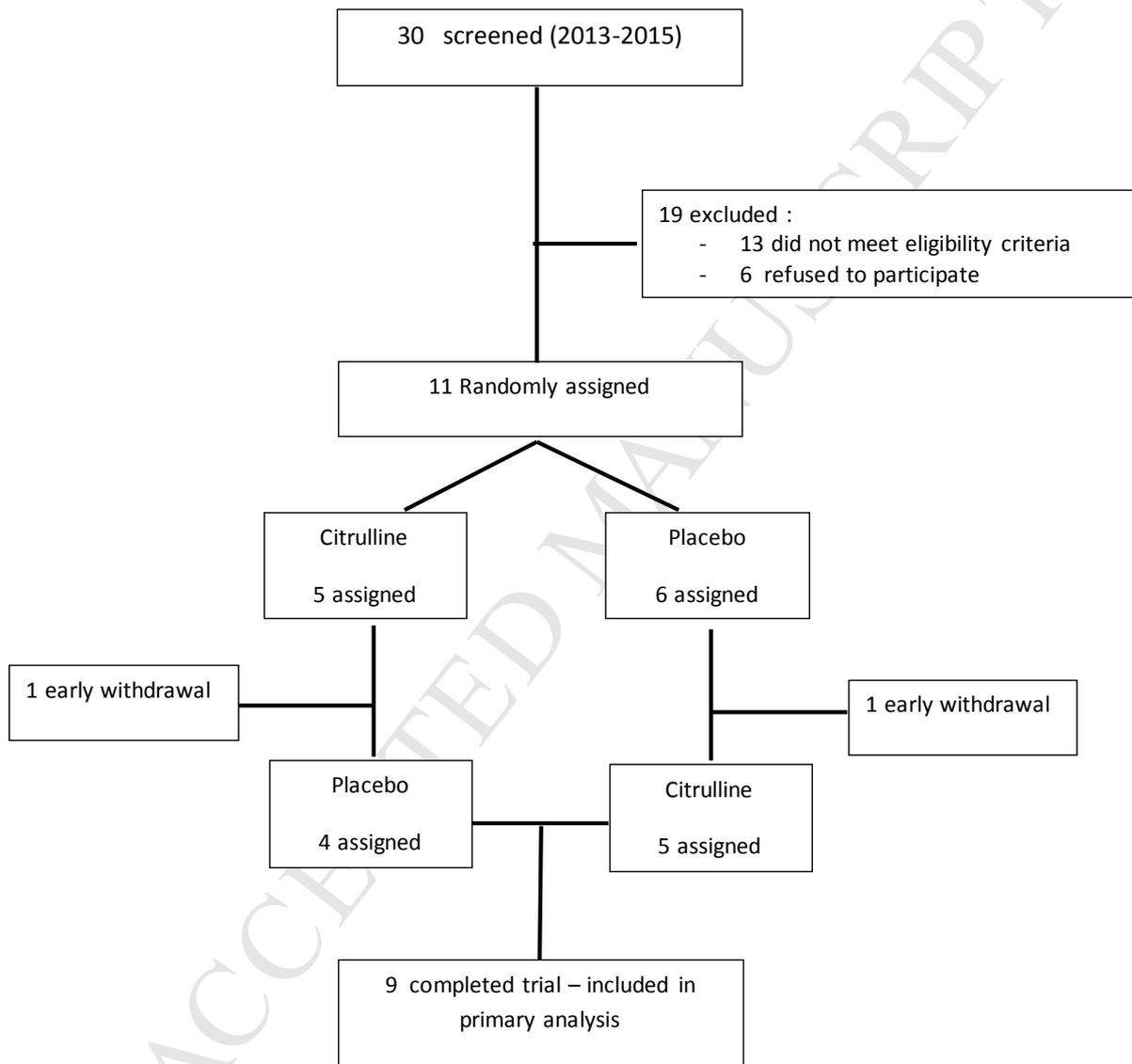
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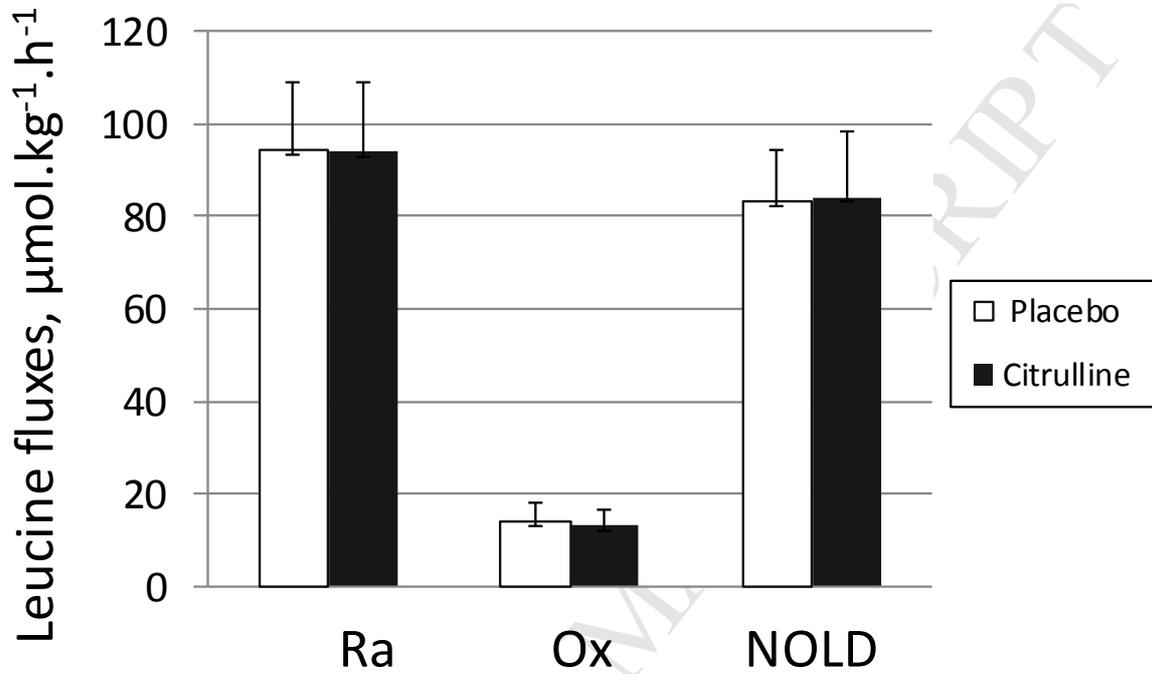
444 **Fig. 2.** Flowchart of a double-blind, randomized, placebo-controlled, cross-over trial of oral
445 citrulline supplementation in short bowel patients.



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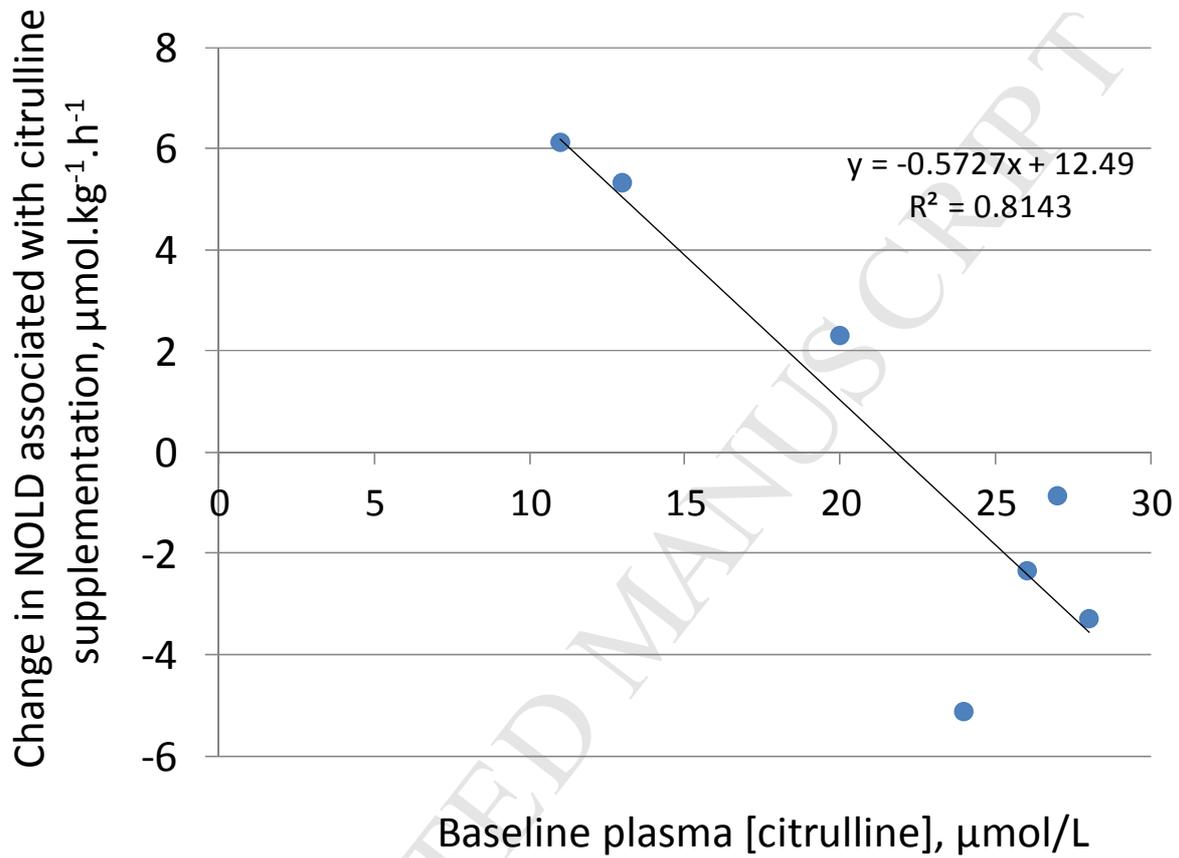
448 **Fig. 3.** Effect of citrulline or placebo supplementation on leucine release from protein
449 breakdown (Ra), leucine oxidation (Ox), and leucine utilization in protein synthesis (NOLD)
450 expressed per kg of body weight. Bars represent mean values \pm SD of 9 patients.



451

452

453 **Fig. 4.** Change in non-oxidative leucine disposal (NOLD, $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$), an index of whole
454 body protein synthesis, between placebo and citrulline supplementation period, after
455 excluding the 2 patients (#7 and #9) with baseline plasma citrulline of 39 and 35 $\mu\text{mol/L}$.



456