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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Adam Jirka [1,3], Sabrina Layec[2], Denis Picot[2], Silvia Bernon-Ferreira[3], Nadège Grasset[3,5], Laurent Flet[4], Ronan Thibault[6,7], Dominique Darmaun[1,3]

[1]Nutrition Support Team, Division of Gastroenterology, IMAD, University Hospital, Nantes, France

[2] Nutrition Department, Clinique St-Yves, Rennes, France

[3]INRA UMR 1280 PHAN, University of Nantes, IMAD, CRNH-Ouest

[4]Pharmacy Department, University Hospital, Nantes, France

[5]Department of Dietetics, University Hospital, Nantes, France

[6]Unité de Nutrition, CHU Rennes, Université Rennes 1, Rennes, France

[7]Institut NuMeCan, INSERM, INRA, Université Rennes 1, Rennes, France

Address for correspondence: Dominique Darmaun, MD, PhD, INRA UMR 1280, CHU Hotel-Dieu, 44093 Nantes Cedex 1, France; Telephone: 33-2 40 08 42 75; e-mail: ddarmaun@chu-nantes.fr

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SUMMARY

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

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

Results: Plasma citrulline concentration rose 17-fold (25±9 *vs.* 384±95 µmol/L) and plasma arginine 3-fold after oral citrulline supplementation (both $p<4\times10^{-6}$). Supplementation did not alter leucine appearance rate (97±5 *vs.* 97±5 $\mu mol.kg^{-1}.h^{-1}$; p=0.88), leucine oxidation (14±1 *vs.* 12±1 $\mu mol.kg^{-1}.h^{-1}$; p=0.22), or non-oxidative leucine disposal (NOLD), an index of whole-body protein synthesis (83±4 *vs.* 85±5 $\mu mol.kg^{-1}.h^{-1}$; p=0.36), nor insulin or IGF-1 plasma concentrations. In each of the 3 patients with baseline citrulline<20 µmol/L, citrulline supplementation increased NOLD. Among the 7 patients with plasma citrulline <30µmol/L, the effect of supplementation on NOLD correlated inversely (r^2=0.81) with baseline plasma citrulline concentration.

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

intestinal adaptation, and with near-normal baseline citrulline homeostasis. 3) Whether oral citrulline would impact whole body protein anabolism in severely malnourished SBS patients in the early adaptive period, and with baseline plasma citrulline below 20 µmol/L, warrants further study. Registered under ClinicalTrials.gov Identifier no. NCT01386034

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

1. Introduction

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

Citrulline is a non-essential amino acid released by small intestine into portal blood [8], and is produced, in part, from glutamine metabolism in enterocytes [9,10]. Accordingly, glutamine supplementation increases citrulline availability in humans [11]. Crenn et al. were first to report a linear correlation between plasma citrulline concentration and residual small bowel length in SBS patients [12, and plasma citrulline was subsequently found to reflect functional 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

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

2. Patients and methods

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

2.1 Study population

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

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

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

To be enrolled, patients also had to :

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

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

2.2 Study design.(Fig 1)

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

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

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

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

At 08:00h, two catheters were inserted: the first catheter was placed in forearm vein for the stable isotope (L-[1-^{13}C]leucine) infusion. The second one was placed in a contralateral hand 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 μmol.kg^{-1} bolus injection at 08:00h (time 0), immediately
165 followed by a continuous, 5-hr infusion at the constant rate of 6 μmol.kg^{-1}.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 $^{13}CO_2$ 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 ($^2H_7$-

leucine and $^{2}H_{7}$-KIC). ^{13}C-enrichment in expired $^{13}CO_2$ was determined by isotope-ratio mass spectrometry coupled to gas chromatography (GC-IRMS) at the Biochemistry department of Medical Faculty at Lausanne, Switzerland.

2.4 Calculation of whole body leucine kinetics

Leucine appearance into plasma (Ra, $\mu mol.kg^{-1}.h^{-1}$), an index of whole body protein breakdown, was determined as Ra= i×[(Ei/Ep)-1], where Ei and Ep represent ^{13}C-enrichments (mole % excess) in infused leucine and plasma KIC at steady state, respectively. Leucine oxidation (Ox) was calculated as Ox = VCO_2 × ECO_2× [(1/EpKIC) - (1/EiLeu)] / 0.81; where ECO_2 is ^{13}C-enrichment in expired CO_2 at steady state, and VCO_2 is overall carbon dioxide measured using indirect calorimetry, and 0.81 corrects for incomplete ^{13}C recovery, as previously described [30].

Protein synthesis was estimated by non-oxidative leucine disposal (NOLD), using the equation: NOLD = Ra Leu – Ox.

2.5 Statistics

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

3. Results

3.1 Patient characteristics

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

Baseline serum albumin and prealbumin/transthyretin concentrations were 4.1±0.25 g/dL and 23±2 mg/dL, respectively, and plasma citrulline was 25 ± 9 µmol/L. (Table 1).

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

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

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

3.3 Effect of citrulline supplementation on protein metabolism.

There was no significant difference in plasma ^{13}C-KIC enrichment (6.03 ± 0.88 *vs.* 6.09 ± 0.92 mole % excess, MPE) nor in expired $^{13}CO_2$ (0.009 ± 0.001 *vs.* 0.009 ± 0.002 MPE) when comparing Citrulline *vs.* Placebo, and, consequently, no detectable effect on leucine oxidation, whether results were expressed per kg of body weight (Fig 3) or kg of FFM (data not shown).

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

Among the 9 patients enrolled, three (#2, 3, and 4) had a baseline citrulline of 13, 20, and 11 µmol/L, *i.e.*, at or below 20 µmol/L, a threshold found to associate with the persistent need for

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

Conversely, 2 patients (#7 and 9) had a baseline citrulline >30 µmol/L (39 and 35 µmol/L in subjects #7 and 9, respectively). If the latter 2 patients were excluded from analysis, a tight correlation would be apparent between the change in NOLD associated with citrulline supplementation and baseline plasma citrulline concentration (Fig. 4).

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

5. Conclusion

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

Authors' contribution

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

Acquisition, analysis, or interpretation of data: All authors

Drafting of the manuscript: AJ, DD

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

Statistical analysis: AJ, DD

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Conflict of interest statement

None for any of the authors.

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Table 1

Etiology and anatomic characteristics of short bowel syndrome (SBS), baseline anthropometric data, body composition from bioelectrical impedance analysis (BIA), and dietary intake in enrolled patients; WT: body weight; IBW: ideal body weight; MI: mesenterich ischemia; Radiation: post-radtion enteritis; Crohn: Crohn's disease; Type of SBS: type I: SBS with end jeunostomy; type II; SBS with jejuno-colic anastomosis; type III: ileo-jeuno-colic anastomosis.

Patient	Age yrs	Wt kg	BMI kg/m²	% of IBW	Fat free mass %	SBS etio-logy	SBS type	Rem-nant small bowel length cm	Rem-nant colon %	Time since surgery (months)	Plasma citrul-line µmol/ L	Plasma Creati-nine µmol/ 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	Volv-ulus	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	Radia-tion	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 ±SD	61 ±11	64 ±6	23 ±3.6	107 ±16	71 ±11)			90 ±48	70 ±30	63 ±63	25 ±9	82 ±19	42 ±14	1.9 ±0.8
median	64	66	23.3	104	75			70	71	39	27	79	40	1.8

Table 2

Effect of citrulline or placebo supplementation on plasma amino acid concentrations in short 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

439 **Fig 1. Study design.**

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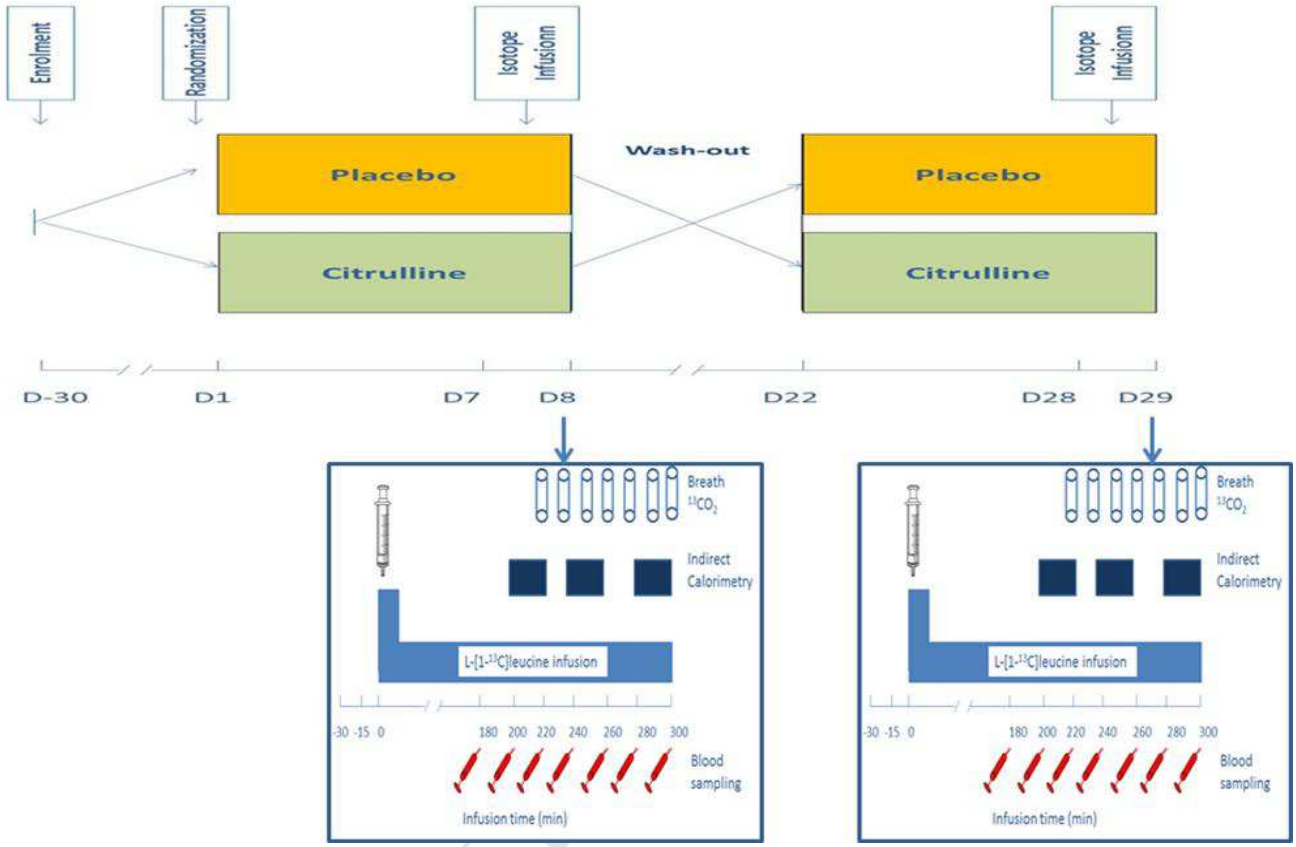

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444 **Fig. 2.** Flowchart of a double-blind, randomized, placebo-controlled, cross-over trial of oral
445 citrulline supplementation in short bowel patients.

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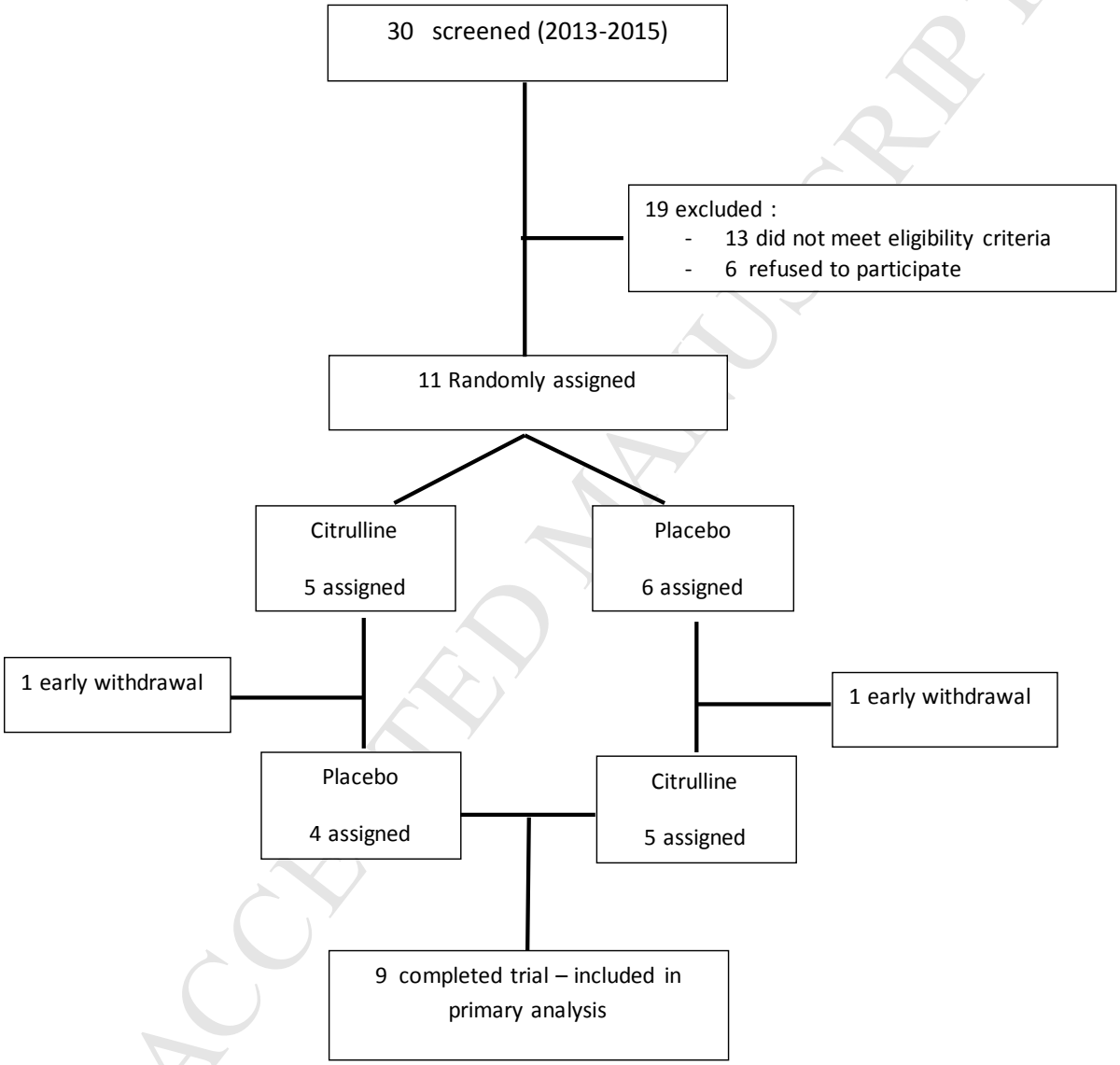

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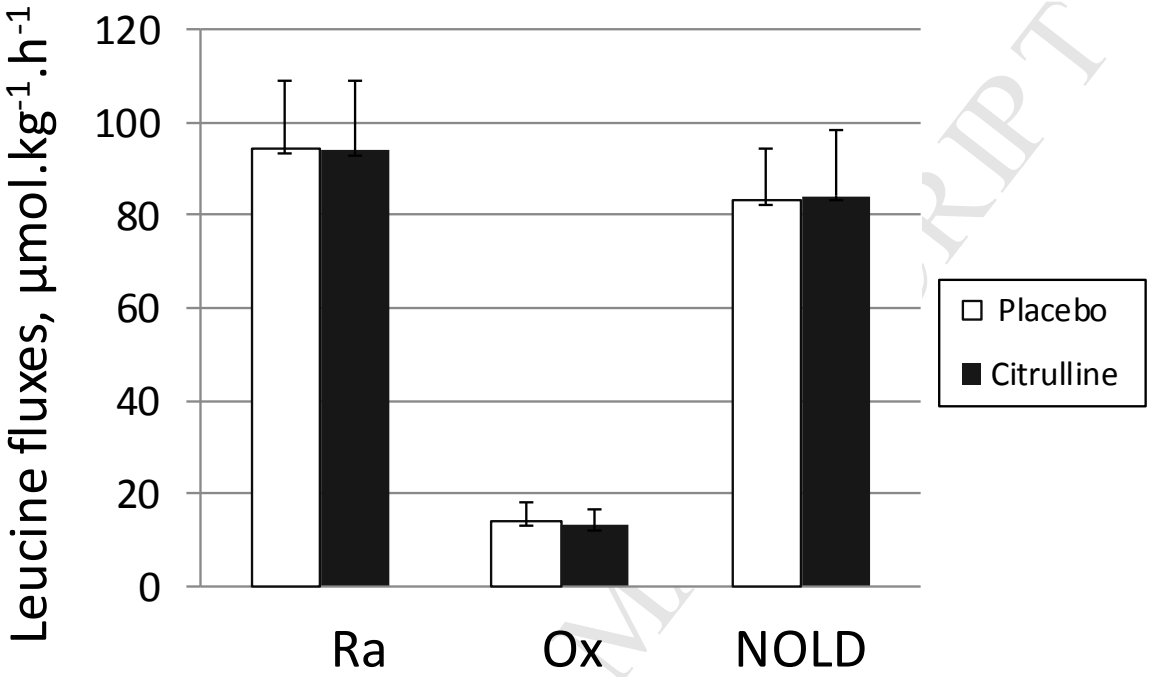
448 **Fig. 3.** Effect of citrulline or placebo supplementation on leucine release from protein
449 breakdown (Ra), leucine oxidation (Ox), and leucine utilization in protein synthesis (NOLD)
450 expressed per kg of body weight. Bars represent mean values ± SD of 9 patients.



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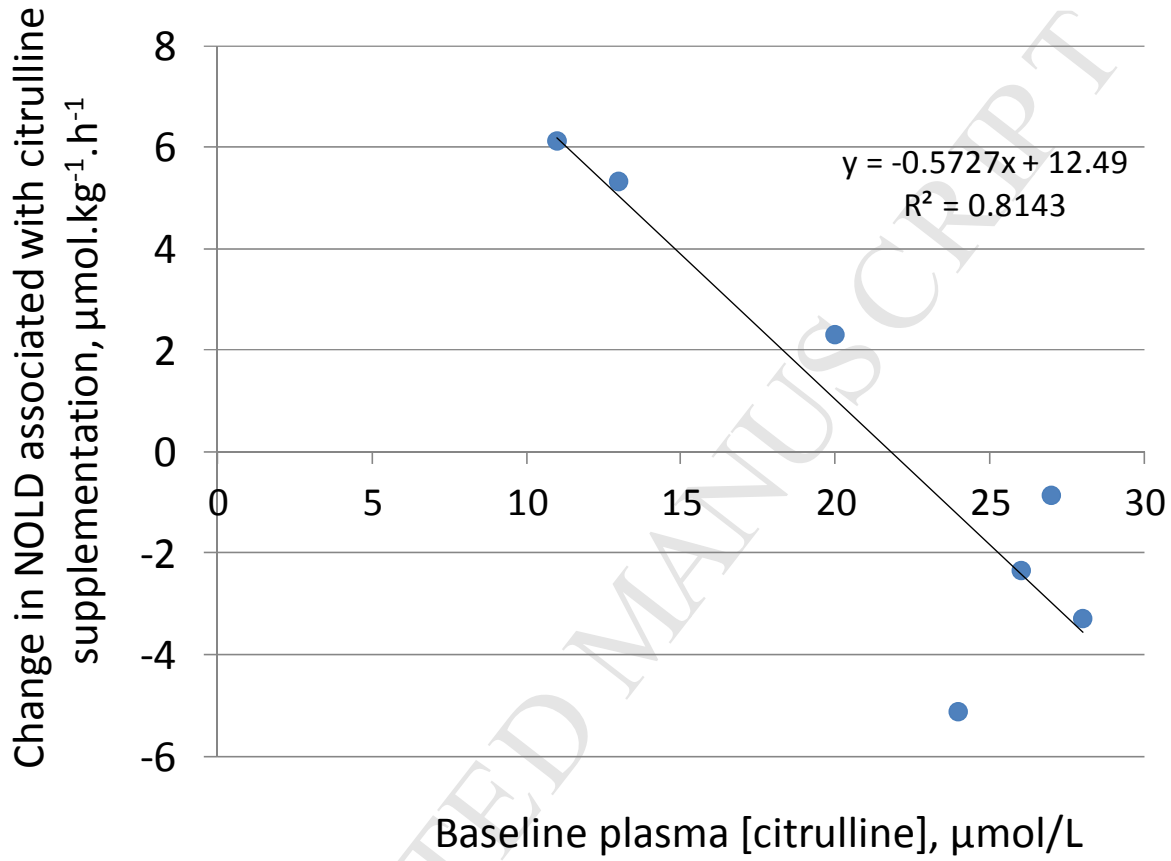
453 **Fig. 4**. Change in non-oxidative leucine disposal (NOLD, µmol.kg^{-1}.h^{-1}), an index of whole
454 body protein synthesis, between placebo and citrulline supplementation period, after
455 excluding the 2 patients (#7 and #9) with baseline plasma citrulline of 39 and 35 µmol/L.



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