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## Dose-dependent beneficial effects of citrulline supplementation in short bowel syndrome in rats

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1           **DOSE-EFFECT OF CITRULLINE SUPPLEMENTATION IN SHOR BOWEL**  
2                           **SYNDROME IN RATS : WHICH IMPACT ON MUSCLE ?**

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21

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23

24

## 25 **1. Abstract**

26 **Background:** Supplementing diet with citrulline has proved an efficient means of  
27 preserving nitrogen balance and improving nutritional status after massive intestinal  
28 resection. We aimed to model the action of citrulline in gut-resected rats using a dose-  
29 ranging study focused on skeletal muscle nitrogen homeostasis.

30 **Methods:** Forty-six rats were randomly assigned to one of the following groups: citrulline  
31 0.5g/kg/day (n=9), citrulline 1g/kg/day (n=7), citrulline 2.5g/kg/day (n=8), citrulline  
32 5g/kg/day (n=8), control (n=6), and sham (n=8). The sham group underwent transection and  
33 the other 6 groups underwent resection of 80% of the small intestine. All rats were then fed  
34 enteral nutrition (all diets were isocaloric and isonitrogenous). After 10 days, the rats were  
35 euthanized to measure and analyze animal weight, duodenum, jejunum and ileum weight,  
36 and muscle trophicity. Protein fractional synthesis rate (FSR) and mTORC1 activation were  
37 measured in the tibialis muscle.

38 **Results:** There was a significant dose-dependent association between rat weight and  
39 citrulline dose up to 2.5 g/kg/day (p=0.004). There was a significant improvement in tibialis  
40 weight correlated to plasma citrulline. Net protein FSR in the tibialis tended to be greater  
41 after resection and tended to return to baseline after citrulline supplementation. Citrulline  
42 supplementation significantly decreased the activated phosphorylated forms of S6K1  
43 (p=0.003) and S6RP (p=0.003), with a significant positive association between myofibrillar  
44 FSR and activation of S6K1 (r=0.614, p=0.02) and S6RP (r=0.601, p=0.023). Jejunum  
45 weight was significantly positively correlated with plasma citrulline (r=0.319, p=0.0345).

46 **Conclusion:** Citrulline promotes body weight gain, preserves muscle trophicity, and  
47 enhances intestinal adaptation in a dose-dependent way in a model of resected rats.

48

## 49 **1. Introduction**

50 Short bowel syndrome (SBS) occurs when there is insufficient length of small intestine to  
51 maintain adequate protein-energy, fluid, electrolyte, or micronutrient balance without  
52 parenteral support [1]. SBS most frequently occurs following extensive surgical resection of  
53 the intestine, congenital defect, or disease-associated loss of absorption. Current lines of  
54 research involve the development of pharmacological treatments and/or the use of specific  
55 nutrients with pharmacological properties to potentiate post-resectional gut adaptation and  
56 reduce parenteral nutrition requirements [2].

57 Citrulline is a non-protein amino acid synthesized for systemic use almost  
58 exclusively by the enterocytes of the proximal small bowel. It escapes splanchnic extraction.  
59 Citrulline is **mainly** metabolized by the kidney and converted into arginine [3-7]. As there  
60 are profound impairments of arginine metabolism in SBS [8,9], citrulline makes a good  
61 candidate to generate arginine and improve nutritional status in SBS. However, to date, there  
62 is only one clinical study of citrulline supplementation in SBS patients [10]. In 9 SBS  
63 patients with good nutritional status in the late phase of intestinal adaptation—which is  
64 likely too late—and with near-normal baseline citrulline homeostasis, a 7-day oral  
65 supplementation with citrulline ( $0.18 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ) enhanced citrulline and arginine  
66 bioavailability but had no anabolic effect on whole-body protein metabolism. However, the  
67 experimental data and this clinical study are not necessarily contradictory. Indeed, work  
68 carried out by Jourdan et al. [11] in healthy subjects found that citrulline intake significantly  
69 increases muscle protein synthesis but does not affect protein synthesis at whole-body level.  
70 Likewise, Kuçi et al [12] showed that in critically-ill rats, citrulline effect varies according to  
71 type of injury and to the skeletal muscle under study, with a limited effect at whole-body  
72 level. This result connects with the findings of Osowska et al. [13], who showed that old  
73 malnourished rats re-fed with a citrulline-enriched diet had higher muscle protein synthesis

74 and lower hepatic protein synthesis than control animals. These data may explain the lack of  
75 citrulline effect at whole-body level. Other hypotheses proposed by Jirka et al [10] to  
76 explain their disappointing results included the patients' good nutritional status, the late  
77 phase of intestinal adaptation, and the normal baseline citrulline level.

78 To date, there is no data available on the most appropriate therapeutic range and the  
79 dose-response relationship of CIT in the acute-phase SBS population. Here, to address this  
80 gap, we used a model of intestinal insufficiency mimicking SBS disease, i.e. gut-resected  
81 rats, to characterize the action of citrulline in a dose-ranging study design, focusing on  
82 muscle nitrogen homeostasis.

83

84

## 85 **2. Materials and Methods**

### 86 *2.1. Animals:*

87 Male Wistar rats (n=46) (230-250g, 2-month-old) were used. Before surgery, the rats were  
88 acclimatized for five days in metabolic cages with free access to standard laboratory chow  
89 (Safe, Epinay-sur-Orge, France) and water.

90

### 91 *2.2. Surgical procedures:*

92 As previously described [14], the rats were fasted for 12 hours before surgery, then  
93 anesthetized by isoflurane inhalation (3% isoflurane) using a regulated airflow apparatus  
94 (Minerve, Esternay, France). Enterectomy was performed on rats in the citrulline and control  
95 groups (see below for definition) by removing 80% of the small intestine, leaving 10% of the  
96 proximal jejunum and 10% of the distal ileum. Eight rats were sham-operated (transection).  
97 For both resected and transected animals, continuity was restored with an end-to-end  
98 anastomosis using a single running silk 6-0 suture. The gastrostomy tube was placed  
99 immediately following intestinal resection or transection. A silicone tube (Tygon, size 0.51  
100 mm; Fisher Bioblock Scientific, Illkirch, France) was introduced into the lumen of the  
101 stomach and the remaining end of the catheter was tunneled subcutaneously to the back of  
102 the neck and attached to a spring coil-swivel mechanism (Harvard Apparatus, Les Ulis,  
103 France) allowing the rat to move freely in the cage. Before the surgical procedure, all the  
104 rats received a painkiller (Temgesic®; Schering-Plough) subcutaneously at a dose of 0.05  
105 mg/kg of body weight.

106 One of the authors (CM) is authorized by the French government (No. 75522) to use this  
107 experimental model of surgery. Animal care complied with French regulations for the  
108 protection of animals used for experimental and other scientific purposes (D 2001-486) and

109 with European Community regulations (Official Journal of the European Community, L538  
110 12:18:1986)..

111

### 112 *2.3. Postoperative care:*

113 Recovery period (D-5 to D0): Rats were housed individually in metabolic cages and allowed  
114 a five-day recovery period. On the first two days they had free access to a 5% glucose  
115 solution. On day 3, glucose was withdrawn, and the rats had access to standard laboratory  
116 chow (UAR A04, Protein 16%, Arginine content: 9.8g.kg<sup>-1</sup> Dietex, France) and water. On  
117 day 4, enteral nutrition was introduced at a flowrate of 0.5 mL/h using a Harvard infusion  
118 pump (pump 11; Harvard Apparatus), and the rats also had free access to chow and water.  
119 Flowrate was increased gradually to a maximum on day 5 corresponding to an intake of 2 g  
120 N kg<sup>-1</sup>day<sup>-1</sup> [3]. From day 5 until the end of the experiment, rats received only enteral  
121 nutrition and had free access to water.

122

### 123 *2.4. Experimental groups:*

124 The rats were randomly assigned to one of the following groups (from D0 to D10):

- 125 • The citrulline groups (CITRULLINE) consisted of rats receiving standard enteral  
126 nutrition (Sondalis®; Nestlé Health Science, 1 kcal.mL<sup>-1</sup> and 4.5 mg Arg-bound  
127 protein .mL<sup>-1</sup>) supplemented with citrulline at different doses:
  - 128 ○ 0.5 g.kg<sup>-1</sup>day<sup>-1</sup> (n=9)
  - 129 ○ 1.0 g.kg<sup>-1</sup>day<sup>-1</sup> (n=7)
  - 130 ○ 2.5 g.kg<sup>-1</sup>day<sup>-1</sup> (n=8)
  - 131 ○ 5.0 g.kg<sup>-1</sup>day<sup>-1</sup> (n=8)
- 132 • The control group (n = 6) and the sham group (n = 6) consisted of rats receiving  
133 standard enteral nutrition.

134 To keep all groups isonitrogenous ( $2 \text{ g N}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ) and isocaloric ( $200 \text{ kcal kg}^{-1}\cdot\text{day}^{-1}$ ) to  
135 the CITRULLINE ( $5 \text{ g kg}^{-1}\cdot\text{day}^{-1}$ ) group, standard enteral nutrition was supplemented with a  
136 mixture of amino acids consisting of alanine, asparagine, glycine, serine, histidine, and  
137 proline in equimolar amounts (see Table 1, supplementary data).

138 Rats were weighed daily. Total enteral nutrition was administered for 10 days. Enteral  
139 nutrition was stopped two hours before decapitation.

140

#### 141 *2.5. Tissue removal:*

##### 142 Blood

143 Blood was sampled in heparinized tubes and then swiftly centrifuged.

144

##### 145 Liver

146 The abdominal cavity was opened. The liver was immediately removed and weighed, and a  
147 sample was cut off, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until analysis.

148

##### 149 Jejunum and ileum

150 The intestinal mucosa was washed with cold NaCl (0.9%), reverted and scraped, rapidly  
151 frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until analysis.

152

##### 153 Muscles

154 Five muscles of the hindlimbs (*extensor digitorum longus* (EDL), *gastrocnemius*, *soleus*,  
155 *plantaris*, and *tibialis anterior*) were rapidly removed, weighed, frozen in liquid nitrogen,  
156 and stored at  $-80^{\circ}\text{C}$  until analysis. These five muscles were selected because they differ  
157 widely in their function and fiber type and show different metabolic responses to stress [15].

158



159 2.6. *Parameters studied and analytical methods:*

160 2.6.1. Amino acid concentrations in plasma and EDL muscle

161 As previously described [14], plasma was deproteinized with a 30% (w/v) sulfosalicylic acid  
162 solution. Supernatants were stored at -80°C until analysis. Tissues were ground and  
163 deproteinized with a 10% trichloroacetic acid solution containing 0.5 mM EDTA.  
164 Supernatants were stored at -80°C until analysis. Amino acids were measured by ion  
165 exchange chromatography using an amino acid autoanalyzer. The results of our participation  
166 in the European Quality Control Scheme (ERNDIM) confirm the accuracy of our amino acid  
167 determinations.

168

169 2.6.2. Fractional synthesis rate of proteins

170 Just before euthanasia, each rat received a subcutaneous injection of a large dose of L-[<sup>13</sup>C]-  
171 valine (99 atom%, 300 μmol/100 g, Cambridge Isotope Laboratories, Andover, MA) to  
172 flood the protein synthesis precursor pool. The tracer incorporation time within the groups  
173 was different for each rat. In each group, a rat was killed at 20, 25, 30, 35, 40, 45 or 50 min  
174 after the tracer injection. Thus, the same kinetics of incorporation from 20 to 50 min was  
175 performed for each group of animals. We used the kinetics of tracer incorporation in the  
176 mitochondrial proteins, myosin and actin to calculate their synthesis rates (see the equation  
177 below). A 200 mg piece of *tibialis anterior* was used for isolation of mitochondrial proteins,  
178 myosin and actin, as previously described [16]. L-[<sup>13</sup>C]-valine enrichment in hydrolyzed  
179 proteins was measured using a GC-C-IRMS system (μGas System, Fisons Instruments, VG  
180 Isotech, Middlewich, UK). Amino acids in the tissue fluid were derivatized, and valine  
181 enrichments were used as precursor pool to calculate fractional synthesis rates (FSR) as  
182 previously described [13] ([Supplementary data, table 2](#)). Another set of four rats per regimen  
183 was used to determine basal isotopic abundance in mitochondrial proteins. The FSR of

184 mitochondrial proteins, actin and myosin was calculated using the following equation: FSR  
185 =  $(E_i \times 100) / (E_{prec} \times t)$ , where  $E_i$  is the enrichment as atom percent excess of  $^{13}\text{C}$  derived  
186 from combustion of valine from proteins at time  $t$  (minus basal enrichment),  $E_{prec}$  is the  
187 mean enrichment in the precursor pool (tissue fluid [ $^{13}\text{C}$ ] valine), and  $t$  is the incorporation  
188 time in hours [13]. Data are expressed as %/h.

189

### 190 2.6.3. Preparation of muscle lysates

191 Frozen muscles (*tibialis*) were ground in liquid nitrogen. Powdered muscles were weighed  
192 and homogenized in 10 volumes of buffer containing 150mM NaCl, 10mM Tris-HCl pH7.4,  
193 5mM EDTA, 1% NP40, phosphatase inhibitors (20mM sodium fluoride, 1mM sodium  
194 pyrophosphate, 25mM sodium glycerophosphate, 1mM sodium vanadate), and protease  
195 inhibitors (complete protease inhibitor cocktail, Roche, Meylan, France). The samples were  
196 continuously homogenized (1 hour, 4°C) and then centrifuged at 1000  $g$  for 30 min at 4°C.  
197 Supernatants were aliquoted and stored at -80°C until analysis. Protein concentrations were  
198 determined using the bicinchoninic acid method (BC Assay UP 40840A kit Uptima,  
199 Interchim, France)

200

### 201 2.6.4 Immunoblotting: determination of the phosphorylation state of the principal actors in 202 mTORC1

203 Total protein (50  $\mu\text{g}$ ) in SDS sample buffer (Laemmli Sample Buffer, Biorad) was heated to  
204 95°C, and the proteins were separated by SDS-PAGE using a denaturing gel. After  
205 electrophoresis, proteins were transferred to nitrocellulose (GE-Healthcare) and then  
206 blocked for 1 h with a blocking buffer (10mM Tris-HCl pH8.0, 150mM NaCl, 0.05%  
207 Tween20, 5% nonfat dry milk). Nitrocellulose blots were incubated overnight at 4°C with  
208 primary antibodies that recognize phosphorylated forms. After washing, membranes were

209 incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h in TBS/T  
210 under gentle shaking. The blots were developed using the enhanced chemiluminescence  
211 detection system (GE Healthcare) according to the manufacturer's protocol. Films were  
212 scanned and quantified using the Bio Imaging System (Syngene). After quantification of the  
213 relative intensity of the phosphorylated forms, membranes were stripped using a stripping  
214 buffer (0.5% acetic acid) and then probed with primary antibodies that recognize total forms.

215

## 216 **2.7. Statistical analysis**

217 Data are expressed as means  $\pm$  SEM. Comparisons between sets of data were performed  
218 using one-way analysis of variance (ANOVA) followed by the Duncan test (all analysis  
219 were performed on parameters measured at euthanasia and are statistically independent).

220 When statistical analysis **did** not show a dose-dependent effect of citrulline, we analyzed the  
221 global effect of citrulline supplementation versus no citrulline, results of all supplemented  
222 rats were pooled and compared to results of unsupplemented resected rats (controls).

223 Statistical analyses (ANOVA, Duncan test, correlation, quadratic analysis, linear regression  
224 etc.) were performed using Statview software (Cary, NC). Threshold of significance was set  
225 at  $p < 0.05$ .

226

## 227 **3. Results**

### 228 **3.1. Body weight**

229 There was no linear relationship between citrulline dose and body weight **gain** at day 10  
230 (Wd10). However, a polynomial regression model evidenced a significant relationship  
231 between citrulline supplementation dose and body weight (Figure 1):  $W(d10)$   
232  $=78.2+11.9(\text{dose})-2(\text{dose}^2) +0.6W(d0)$ ;  $p=0.02$ . Furthermore, a significant linear  
233 relationship was found when the citrulline dose of 5 g/kg/d was excluded:  $W(d10)$   
234  $=60.8+7.5(\text{dose})+0.7W(d0)$ ,  $p=0.004$ .

235

### 236 **3.2. Liver weight**

237 Liver weight was lower in the resected animals compared to the sham group (sham vs all  
238 resected rats: 8.95 g vs 7.65 g,  $p=0.038$ ). This difference was not corrected by citrulline  
239 supplementation at any dose (Table 1).

240

### 241 **3.3. Muscle weight**

242 Intestinal resection led to a significant **lowering** in soleus muscle mass and protein content  
243 (Table 1) which was partly restored after citrulline supplementation: soleus weight in  
244 unsupplemented resected rats (controls) vs all citrulline groups:  $p=0.029$ . However, we were  
245 unable to find a clear relationship between citrulline dose and muscle mass for any muscle  
246 (EDL, tibialis, soleus, plantaris anterior, gastrocnemius) or between citrulline  
247 supplementation and protein content of EDL and soleus muscles (in absolute weight or in  
248 percent of muscle mass). This was mainly due to major inter-individual variability in the  
249 group of rats receiving the highest dose of citrulline. A linear regression model using the  
250 plasma citrulline logarithm showed a 20% variation in tibialis anterior weight according to  
251 plasma citrulline:  $W= 0.05\log(\text{plasma citrulline})+0.033$ ;  $p=0.02$  (Figure 2).

252

### 253 **3.4. Intestinal adaptation**

254 The variation of jejunal and ileum weight according to citrulline load is presented in Table 1.

255 The variation of jejunal weight was significantly related to plasma citrulline:

256  $W=0.05\log(\text{plasma citrulline})+97.12$ ;  $p=0.035$ . However, there was no significant

257 association between ileum weight and plasma citrulline, and no significant association

258 between mucosal protein content of the intestines and citrulline dose or plasma

259 concentrations.

260

### 261 **3.5. Amino acid concentrations in plasma and muscles**

#### 262 3.5.1. Plasma amino acid concentrations:

263 Intestinal resection **resulted in a lowering** of plasma citrulline compared with the sham group

264 (control vs sham: 49 vs 75  $\mu\text{mol/L}$ ,  $p=0.002$ ) and did not have any significant influence on

265 any of the other AA concentrations. Plasma concentrations of citrulline, arginine and

266 ornithine **rose** dose-dependently with citrulline supplementation (Table 2).

#### 267 3.5.2. Muscles amino acid concentrations:

268 EDL levels of citrulline, arginine and ornithine tend **to be lowering** on average by 21–27%

269 following intestinal resection (control vs sham,  $p=0.07$ – $0.08$ ). Citrulline supplementation

270 significantly raised levels of citrulline, arginine and ornithine in the EDL muscle in a dose-

271 dependent manner ( $p<0.001$ , Table 2). Likewise, we found highly significant positive

272 associations between plasma citrulline and EDL levels of citrulline ( $r=0.96$ ,  $p<0.001$ ),

273 arginine ( $r=0.73$ ,  $p<0.001$ ) and ornithine ( $r=0.79$ ,  $p<0.001$ ) (Figure 3).

274

275

276

277 **3.6. Evaluation of muscle protein synthesis**

278 Protein FSR tended to **rise** after intestinal resection, but the difference was only significant  
279 for sarcoplasmic FSR (sham *versus* unsupplemented resected rats: 0.08%/h *vs* 0.12%/h,  
280  $p=0.014$ ) and myofibrillar FSR (0.1%/h *vs* 0.15%/h,  $p=0.028$ ). FSR levels tended to return to  
281 baseline levels with citrulline supplementation (myofibrillar FSR: control *versus* all  
282 citrulline-supplemented groups:  $p=0.018$ ;  $p>0.05$  for total FSR, mitochondrial FSR and  
283 sarcoplasmic FSR) (Figure 4). No dose-dependent effect of citrulline were observed.

284

285 **3.7. Evaluation of mTORC1 pathway activation**

286 Intestinal resection did not significantly modify the amounts of phosphorylated forms of  
287 AKT, 4E-BP1 and eIF-4E, S6K1 or S6 ribosomal protein (S6RP) in the tibialis. However,  
288 citrulline supplementation led to a significant **lowering** in the activated forms of S6K1  
289 ( $p=0.003$ ) and S6RP ( $p=0.003$ ), regardless of citrulline dose (Figure 5). Furthermore, we  
290 found a significant positive association between myofibrillar FSR and S6K1 ( $r=0.614$ ,  
291  $p=0.02$ ) and S6RP ( $r=0.601$ ,  $p=0.023$ ). No dose-dependent effect of citrulline were  
292 observed.

293

#### 294 **4. Discussion**

295 Citrulline is renowned for its homeostatic effect on nitrogen balance, and recent literature  
296 has consistently shown that this effect was directed towards skeletal muscles [11, 13, 17-20],  
297 by activation of the mTORC1/PI3K/MAPK pathway [21] and by modulation of muscle  
298 energy metabolism [22]. We thus focused on muscle nitrogen homeostasis in our animal  
299 model of SBS, and aimed to find a dose-ranging effect of citrulline supplementation.

300 In our study, citrulline supplementation generated dose-dependent **rises** in arginine and  
301 ornithine contents in both plasma and muscles, modulated weight, and enhanced intestinal  
302 adaptation. Furthermore, we demonstrated that this beneficial effect was dose-dependent,  
303 mainly from 0.5 to 2.5g kg<sup>-1</sup>day<sup>-1</sup> of citrulline supplementation.

304 Citrulline had a specific effect on muscles, i.e. on muscle weight, AA content, thus  
305 corroborating the results of previous studies [11,13,17-19]. However, the action of citrulline  
306 on muscle FSR in our resected animals was the opposite of what was expected. First,  
307 intestinal resection **resulted in a rise** in skeletal-muscle protein FSR despite a decrease in  
308 muscle mass. Among the possible explanations for this FSR **variation**, we posit that it could  
309 reflect the onset of an intestinal adaptation process. Second, this FSR **variation** tended to be  
310 normalized by citrulline supplementation, in particular for myofibrillar and sarcoplasmic  
311 FSR. These results were not artifacts as they correlated with the mTOR pathway response to  
312 citrulline, with a major reduction in the activated phosphorylated forms of S6k1 and S6R  
313 concomitant with the **lower values of** FSR in citrulline-supplemented rats. We advance two  
314 explanations to account for these unexpected results:

315 1) Our experimental model used a continuous enteral feeding mode. It has been shown that  
316 continuous enteral feeding had a much less stimulatory effect on muscle and visceral protein  
317 synthesis than intermittent bolus feeding [23,24]. Furthermore, Davis et al. (2015)  
318 demonstrated that only intermittent bolus feeding could elicit a pulsatile pattern of amino

319 acid-induced translation initiation, thereby promoting protein anabolism to a much greater  
320 extent than continuous enteral feeding [25]. Likewise, mTORC1 is mainly activated in the  
321 postprandial (bolus-style) state. 2) In patients with early-phase SBS, feeding fails to decrease  
322 proteolysis, in contrast to what is physiologically observed in healthy subjects. Note that the  
323 improvement in intestinal function and nutritional status achieved by rhGH treatment in SBS  
324 is mediated by the decrease in proteolysis in response to feeding [26]. We hypothesize that  
325 the same could occur in **work**. The **rise** in muscle FSR after resection could reflect an  
326 increased protein turnover due to excess proteolysis, and the stimulatory effect of citrulline  
327 on muscle protein synthesis could, at least in part, be mediated by a decrease in proteolysis.  
328 This potential effect of CIT on proteolysis was also hinted at in a proteomics study [27].  
329 Note that the systemic relation between protein synthesis and muscle mass is inconstant, as  
330 it was previously shown that CIT could increase protein synthesis without increasing muscle  
331 mass [18].

332

333 In conclusion, our results confirm that citrulline has potential benefit in SBS as it promotes  
334 body weight gain, preserves muscle trophicity, and enhances intestinal adaptation in our  
335 model of resected rats. Here we demonstrated that this beneficial effect was dose-dependent,  
336 with a maximal beneficial effect for a citrulline dose of 2.5 g/kg/day. Citrulline reversed the  
337 **rise** in protein synthesis in skeletal muscle induced by intestinal resection through the mTOR  
338 pathway, prompting the hypothesis that citrulline could reverse the post-resection  
339 **modification** in protein turnover. This new approach to the pharmacological properties of  
340 citrulline in SBS warrants further research, but we anticipate it could help clinicians develop  
341 valuable clinical studies in the early-adaptation phase of SBS.

342



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346 **Conflict of interest statement**

347 JF, CM, SLP and LC are Citrage shareholders. Other authors have no conflict of interest.

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353 **CRediT author statement**

354 **Jérôme Filippi:** Resources, Methodology, Investigation, Validation. **Amandine Rubio:**

355 Data curation, Writing- Original draft preparation. **Virginie Lasserre:** Methodology, Data

356 curation, Validation. **Jean Maccario:** Data curation, Validation. **Stéphane Walrand:**

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358 Validation. **Servane Le Plenier:** Methodology, Investigation, Validation. **Xavier**

359 **Hébuterne:** Methodology, Conceptualization, Validation. **Luc Cynober:** Conceptualization,

360 Methodology, Supervision, Project Administration, Funding Acquisition, Validation.

361 **Christophe Moinard:** Conceptualization, Methodology, Supervision, Project

362 Administration, Funding Acquisition, Writing- Reviewing and Editing, Validation.

363

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## Figure and table legends

**Table 1:** Organ weight and organ protein content in the sham group and in groups of resected and CIT-supplemented rats

**Table 2:** Plasma and EDL amino acid contents in the sham group and in groups of resected and CIT-supplemented rats

**Figure 1:** A polynomial regression model on rat weight at D10 (W10) and citrulline dose administered in sham and resected rats. X-Axis: Citrulline dose ( $\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ ), Y- Axis: Weight gain (g) (n=46)

$$W(\text{day}10) = 78.2 + 11.9(\text{dose}) - 2(\text{dose}^2) + 0.6W(\text{day}0)$$

**Figure 2:** Linear regression between log(citrullinemia) (X-Axis) and weight of *tibialis* (mg) (Y-Axis) in sham and resected rats under enteral nutrition (n=46)

**Figure 3:** Linear regression between plasma citrulline (Y-Axis) and levels of citrulline (X-Axis) (panel 1:  $r=0.96$ ,  $p<0.001$ ), arginine (panel 2:  $r=0.73$ ,  $p<0.001$ ) and ornithine (panel 3:  $r=0.79$ ,  $p<0.001$ ) in EDL muscle (n=46)

**Figure 4:** Protein fractional synthesis rate (FSR): total FSR, mitochondrial FSR, sarcoplasmic FSR, and myofibrillar FSR (expressed in %/h) in *tibialis* muscle in sham (transected rats fed standard enteral nutrition), control (resected rats fed standard enteral nutrition), and citrulline-supplemented rats (pooled results of resected rats fed enteral nutrition supplemented with either 0.5, 1, 2.5 or 5 mg/kg/day of citrulline) Results are presented as means  $\pm$  SEM

**Figure 5:** Phosphorylated forms of S6K1 and S6RP in *tibialis* muscle (expressed as phosphorylated to total form ratio) in sham (transected rats fed standard enteral nutrition), control (resected rats fed standard enteral nutrition), and citrulline-supplemented rats

(pooled results of resected rats fed enteral nutrition supplemented with either 0.5, 1, 2.5 or 5 mg/kg/day of citrulline). Results are presented as means  $\pm$  SEM.

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Figure 1

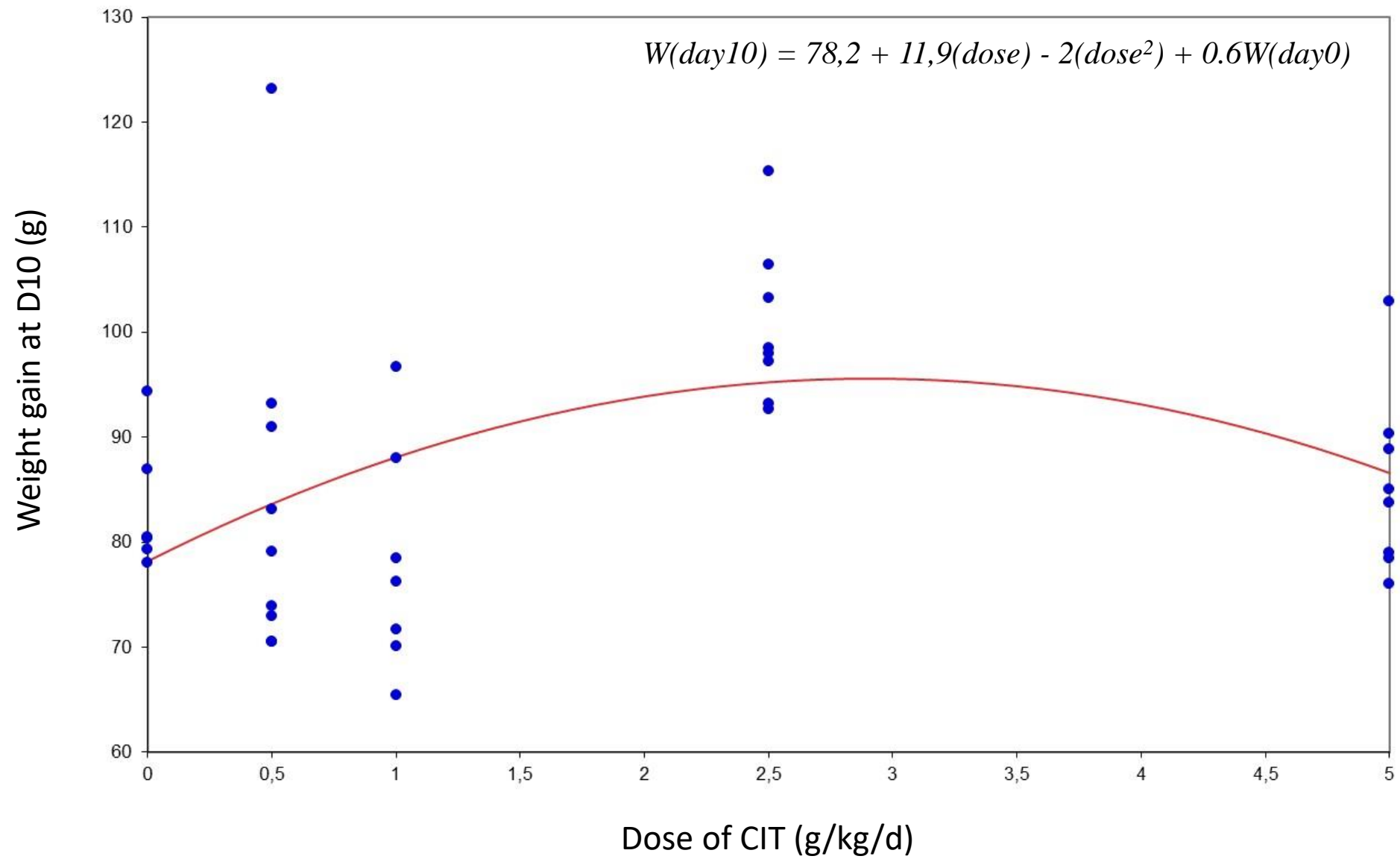


Figure 2

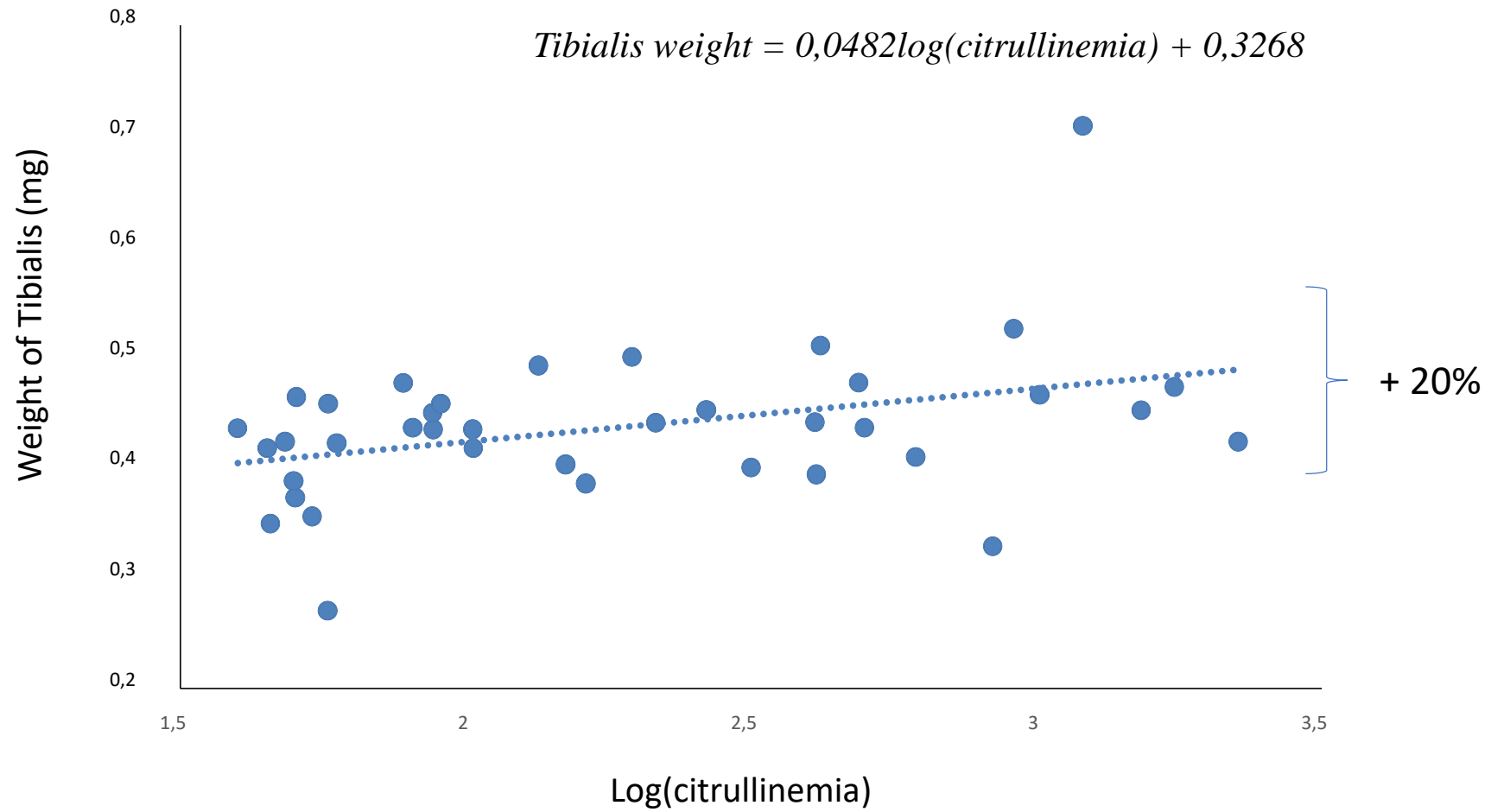


Figure 3

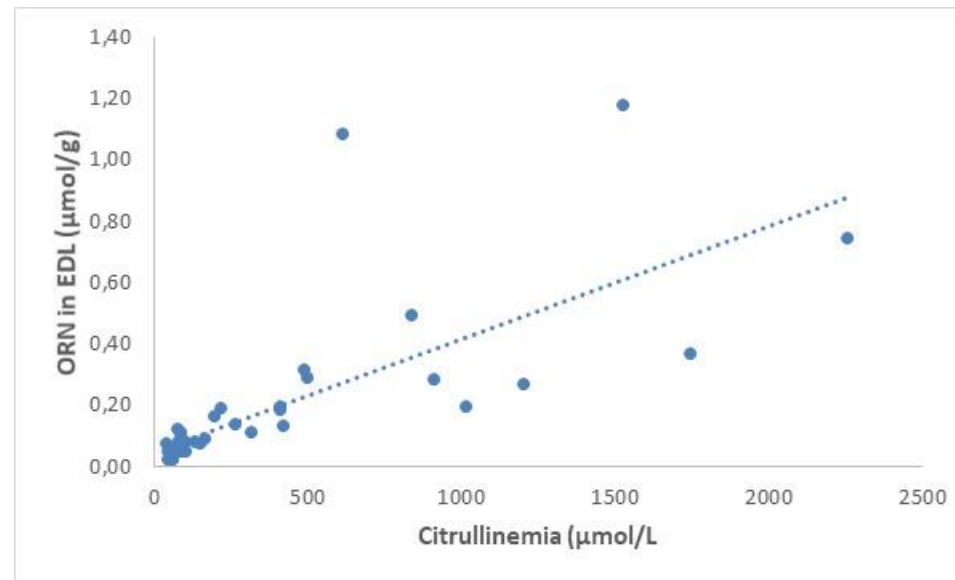
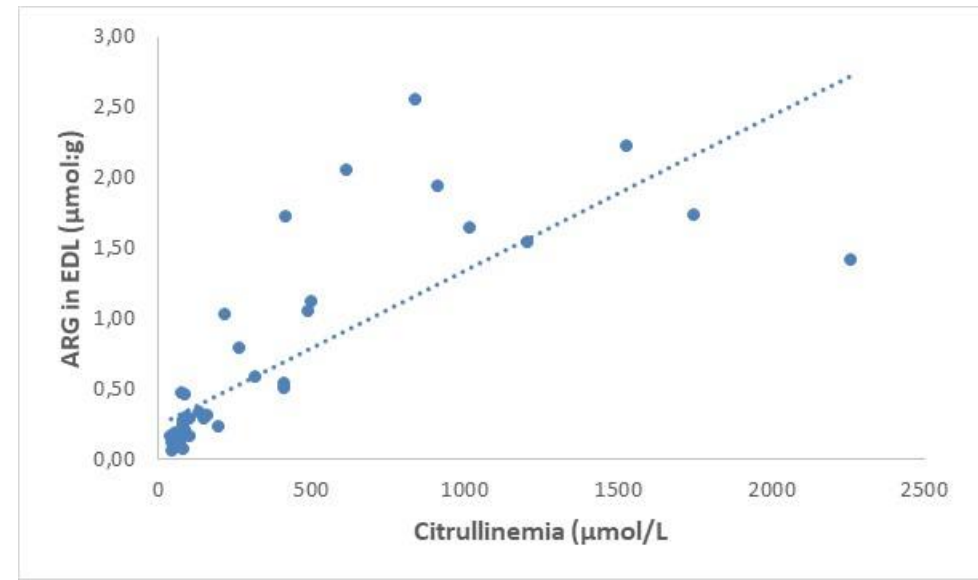
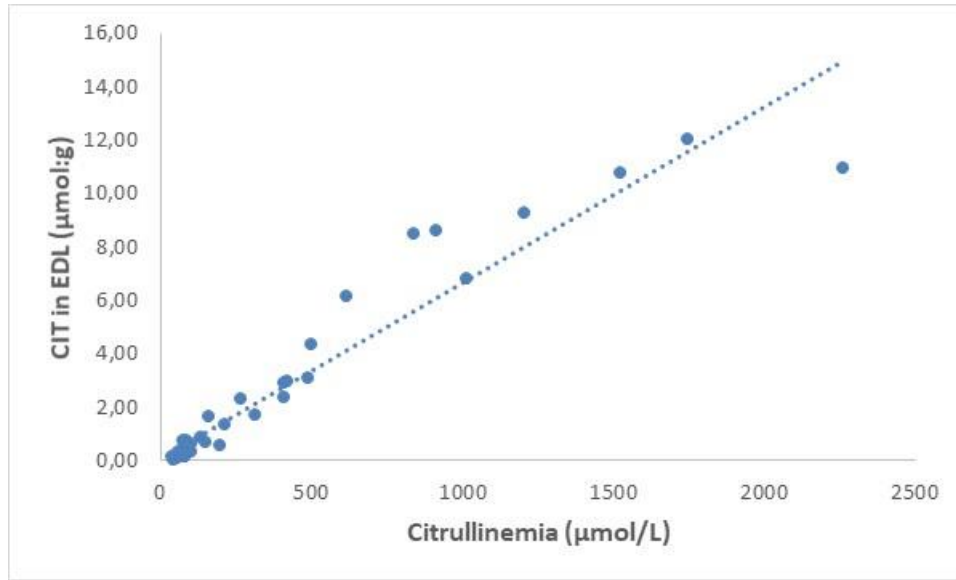




Figure 4

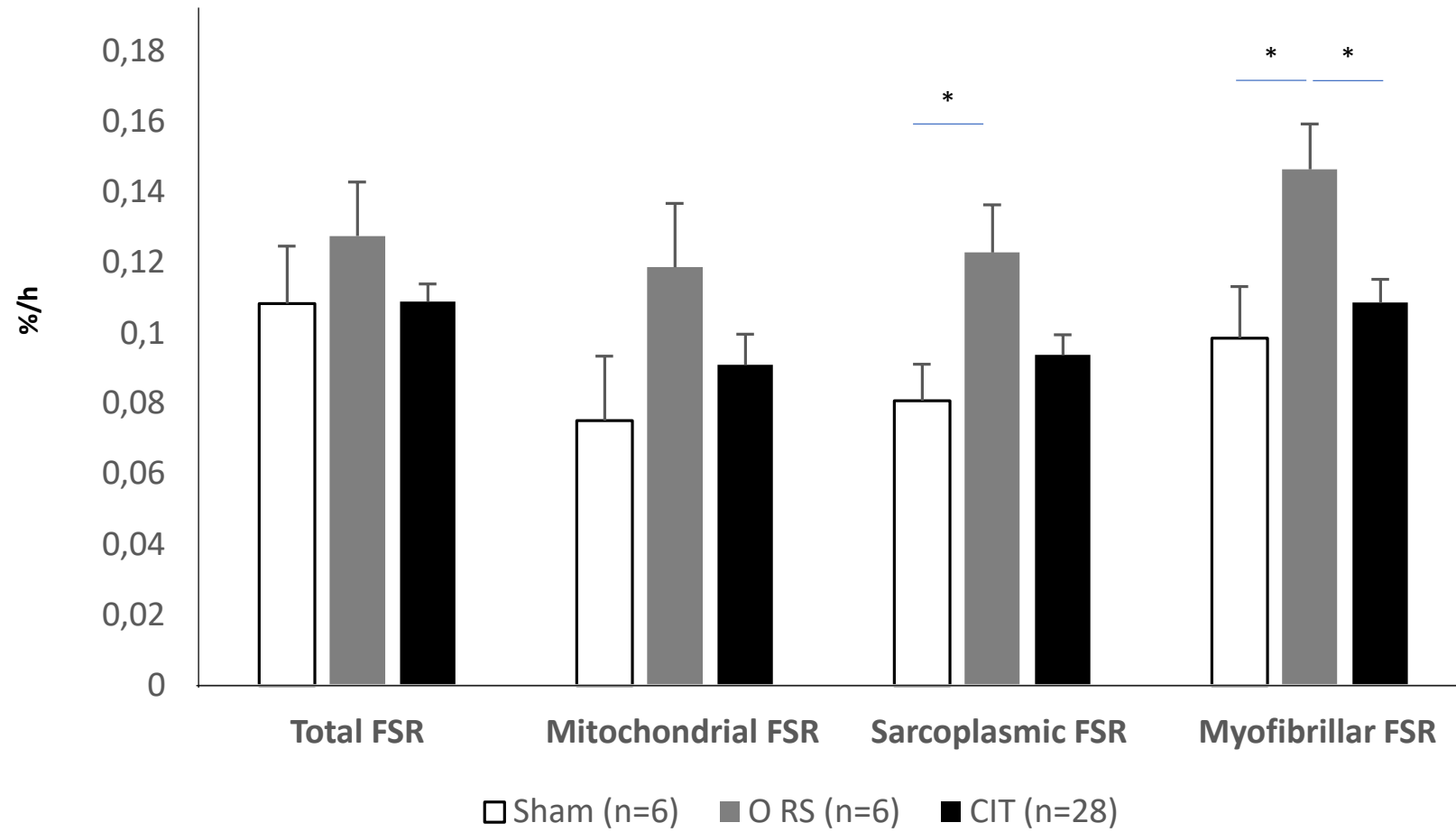
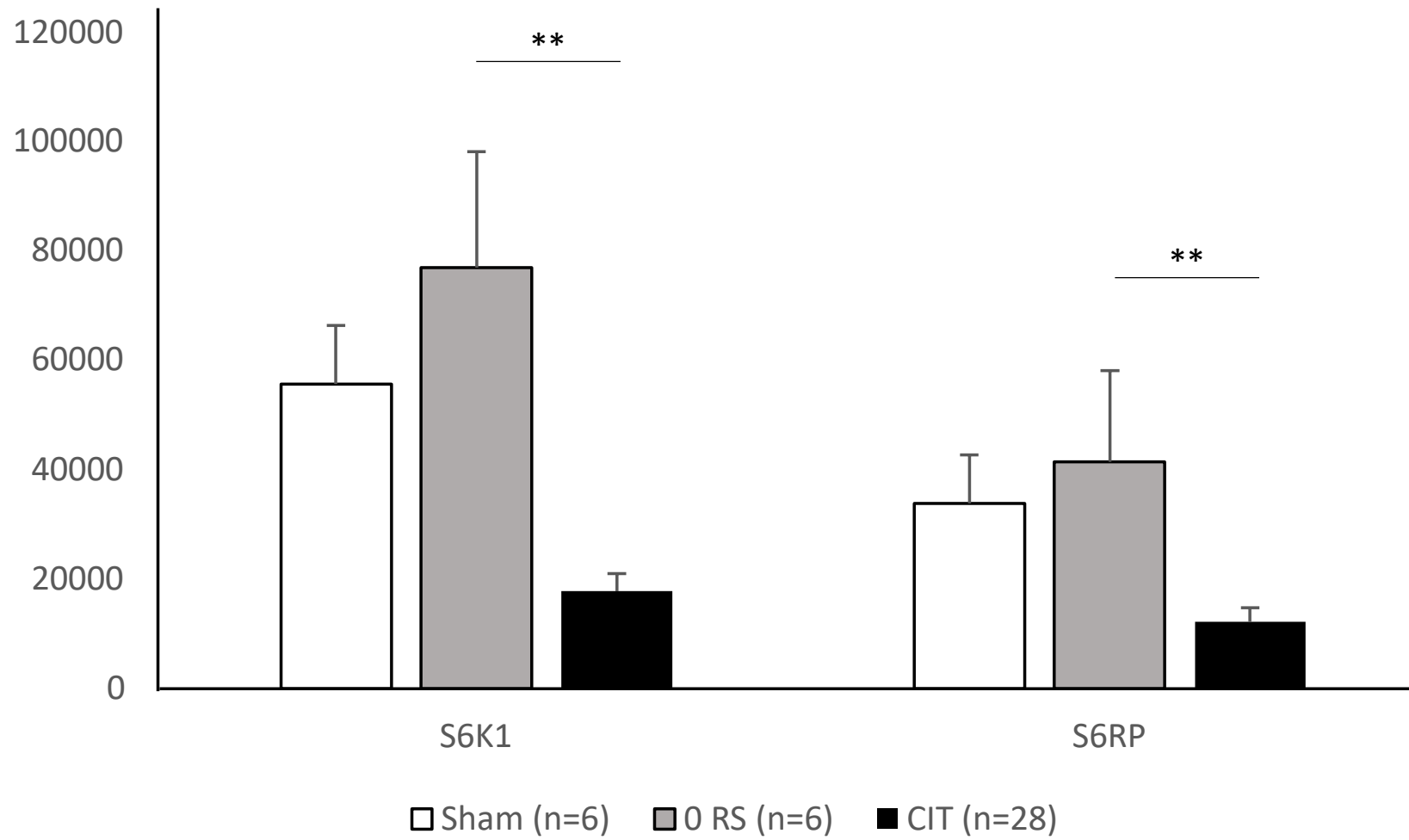


Figure 5



**Table 1:** Organ weight and organ protein content in the sham group and in groups of resected citrulline-supplemented rats (from 0 to 5g.kg<sup>-1</sup>.d<sup>-1</sup>) by enteral route during 10 days

		<i>Sham</i> (n=6)	<i>Control</i> (n=6)	<i>0.5 CIT</i> (n=9)	<i>1 CIT</i> (n=7)	<i>2.5 CIT</i> (n=8)	<i>5 CIT</i> (n=8)
<b>Soleus</b>	<i>Weight (mg)</i>	106±5 <sup>a</sup>	90±3 <sup>b</sup>	101±3 <sup>a,c</sup>	96±7 <sup>c</sup>	103±13 <sup>a,c</sup>	104±4 <sup>a,c</sup>
	<i>Protein (mg)</i>	11.7±0.8 <sup>a</sup>	9.0±1.0 <sup>b</sup>	11.7±1.2 <sup>a</sup>	11.8±1.5 <sup>b</sup>	11.7±1.0 <sup>b</sup>	10.6±0.8 <sup>b</sup>
<b>EDL</b>	<i>Weight (mg)</i>	110±4	106±4	104±3	104±6	103±3	109±6
	<i>Protein (mg)</i>	13.2±1.1	11.2±0.5	12.0±0.9	12.1±0.7	12.9±0.9	13.0±1.3
<b>Gastrocnemius</b>	<i>Weight (mg)</i>	1175±54	1087±67	1113±36	1090±84	1119±37	1133±78
<b>Tibialis</b>	<i>Weight (mg)</i>	450±20	417±19	426±13	422±28	437±15	473±39
<b>Plantaris</b>	<i>Weight (mg)</i>	194±9	181±9	175±9	194±13	180±14	192±13
<b>Liver</b>	<i>Weight (g)</i>	9.0±0.7 <sup>a</sup>	8.0±0.7 <sup>b</sup>	7.6±0.5 <sup>b</sup>	7.3±0.5 <sup>b</sup>	7.5±0.3 <sup>b</sup>	8.0±0.3 <sup>b</sup>
	<i>Protein (mg)</i>	NA	914±75	987±112	1095±212	955±86	1142±188
<b>Jejunum</b>	<i>Weight (mg/10 cm)</i>	118±16	108±21	94±14	93±11	107±17	161±44
	<i>Protein (mg/10 cm)</i>	4.3±0.3	4.6±1.1	4.0±0.4	5.8±0.5	4.5±0.9	6.0±1.1
<b>Ileum</b>	<i>Weight (mg)</i>	105±9	123±21	88±14	122±20	128±29	123±30
	<i>Protein (mg)</i>	63±10	53±10	54±9	74±9	58±8	54±14

Values are means ± SEM. ANOVA + Tukey–Kramer test.

Values with different superscripts are statistically different at p<0.05.

**Table 2:** Plasma and EDL amino acid contents (*respectively in  $\mu\text{mol/L}$  and  $\mu\text{mol/g}$* ) in the sham group and in groups of resected citrulline-supplemented rats (*from 0 to  $5\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$* ) by enteral route during 10 days

<b>Plasma</b> <b><math>\mu\text{mol/L}</math></b>	<i>Sham</i> <i>(n=6)</i>	<i>Control</i> <i>(n=6)</i>	<i>0.5 CIT</i> <i>(n=9)</i>	<i>1 CIT</i> <i>(n=7)</i>	<i>2.5 CIT</i> <i>(n=8)</i>	<i>5 CIT</i> <i>(n=8)</i>
<b>Citrulline</b>	75±3 <sup>a</sup>	49±2 <sup>b</sup>	91±15 <sup>a</sup>	110±22 <sup>c</sup>	372±40 <sup>d</sup>	1265±192 <sup>e</sup>
<b>Ornithine</b>	55±4 <sup>a</sup>	47±4 <sup>a</sup>	54±4 <sup>a</sup>	71±10 <sup>b</sup>	157±15 <sup>c</sup>	330±46 <sup>d</sup>
<b>Arginine</b>	94±8 <sup>a</sup>	79±8 <sup>a</sup>	114±10 <sup>b</sup>	147±21 <sup>c</sup>	299±24 <sup>d</sup>	528±35 <sup>e</sup>
<b>EDL</b> <b><math>\mu\text{mol/g}</math></b>						
<b>Citrulline</b>	0.2±0.02 <sup>a</sup>	0.16±0.04 <sup>a</sup>	0.4±0.08 <sup>b</sup>	0.66±0.16 <sup>c</sup>	2.67±0.31 <sup>d</sup>	9.14±0.73 <sup>e</sup>
<b>Ornithine</b>	0.15±0.03 <sup>a</sup>	0.11±0.02 <sup>a</sup>	0.21±0.04 <sup>b</sup>	0.39±0.11 <sup>c</sup>	0.83±0.17 <sup>d</sup>	1.89±0.13 <sup>e</sup>
<b>arginine</b>	0.06±0 <sup>a</sup>	0.05±0.01 <sup>a</sup>	0.07±0.02 <sup>a</sup>	0.09±0.02 <sup>a</sup>	0.18±0.03 <sup>b</sup>	0.58±0.14 <sup>c</sup>

Values are means ± SEM. ANOVA + Tukey–Kramer test.

Values with different superscripts are statistically different at  $p < 0.05$ .