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1 **Dietary citrulline does not modify rat colon tumor response to chemotherapy, but failed**
2 **to improve nutritional status**

3

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25 **Abstract**

26 During cancer therapy many patients experience significant malnutrition, leading to decreased
27 tolerance to chemotherapy and decreased survival. Dietary citrulline supplementation
28 improves nutritional status in situations such as short bowel syndrome and aging, and is of
29 potential interest in oncology. However, a mandatory prerequisite is to test this amino acid for
30 interaction with tumor growth and chemotherapy response.

31 Dietary citrulline (Cit; 2 %), or an isonitrogenous mix of non-essential amino acids (control),
32 was given to Ward colon tumor-bearing rats the day before chemotherapy initiation.
33 Chemotherapy included 2 cycles, one week apart, each consisting of one injection of CPT-11
34 (50 mg/kg) and of 5-fluorouracil (50 mg/kg) the day after. Body weight, food intake and
35 tumor volume were measured daily. The day after the last injection, rats were killed, muscles
36 (*EDL, gastrocnemius*), intestinal mucosa, tumor, spleen and liver were weighed. Muscle and
37 intestinal mucosa protein content were measured. Phosphorylated 4E-BP1 was measured in
38 muscle and tumor as a surrogate for biosynthetic activation. FRAPS (Ferric Reducing Ability
39 of Plasma) and thiols in plasma, muscle and tumor were evaluated and plasma amino acids
40 and haptoglobin were measured.

41 Numerous parameters did not differ by diet overall: a) response of tumor mass to treatment, b)
42 tumor antioxidants and phosphorylated 4E-BP1 levels, c) relative body weight and relative
43 food intake, d) weight of *EDL, gastrocnemius*, intestinal mucosa, spleen and liver and e)
44 plasma haptoglobin concentrations. Moreover, plasma citrulline concentration was not
45 correlated to relative body weight, only cumulated food intake and plasma haptoglobin
46 concentrations were correlated to relative body weight.

47 Citrulline does not alter the tumor response to CPT-11/5FU based therapy but, has no effect
48 on nutritional status, which could be due to the anorexia and the low amount of citrulline and
49 protein ingested.

50 **Keywords:** Chemotherapy efficacy, Nutritional status, Protein synthesis, Inflammation,

51 Antioxidant

52

53 **Introduction**

54 Patients with cancer are at high risk for malnutrition due to the combined effect of cancer and
55 chemotherapy. Both cancer and chemotherapy are associated with inflammation and weight
56 loss, and more specifically muscle loss, which, in turn, leads to decreased tolerance to
57 chemotherapy and decreased survival rate (1-4). A vicious circle of malnutrition and toxicity
58 worsens patient outcome, a point underlined by the European Society for Enteral and
59 Parenteral Nutrition (ESPEN) expert group for action against cancer-related malnutrition (5).
60 Skeletal muscle loss is a specific focus of ESPEN recommendations, since sarcopenia is an
61 important prognostic factor for both mortality and treatment toxicity. Several strategies have
62 been proposed to manage these patients, in particular using anabolic and/or anti-catabolic
63 drugs and nutrients (6), however the amount and quality of evidence are insufficient and the
64 recommendation is to continue research (5). Specific nutrients include a variety of amino
65 acids and fatty acids and among these, citrulline could be a promising tool (7). Citrulline is an
66 amino acid which is not incorporated into protein, and it is now well known to be an activator
67 of muscle protein synthesis (MPS) and to increase muscle mass (8-10), particularly in
68 conditions of muscle mass loss (11-13). However, to date, there is no data concerning the use
69 of this amino acid in cancer patients, especially as plasma citrulline concentration (a marker
70 of functional intestinal mass (14)) is decreased in cancer patients (15).
71 But, before giving citrulline to cancer patients to thwart muscle mass loss, a prerequisite is to
72 check the safety of this amino acid and to determine whether there is any interaction between
73 citrulline and chemotherapy. Citrulline is able to activate mTORC1 pathway in muscle
74 (11;16), and could induce mTOR activation in tumor cells, and thus increase tumor size (17).
75 Citrulline has also important antioxidant properties (9;18) and this property could interfere
76 with chemotherapy treatment (19-20).

77 The purpose of this study was to evaluate the interaction of citrulline with tumor response to
78 chemotherapy in an animal model of colon cancer and chemotherapy. Secondly, we
79 assessed the ability of this amino acid to modify muscle mass loss.

80

81 **Material and methods**

82 Animals

83 Animal use was reviewed and approved by the Institutional Animal Care Committee and
84 conducted in accordance with the Guidelines of the Canadian Council on Animal Care
85 (Number ACC12200).

86 Thirty-four 11-week-old female Fischer 344 rats (110–130 g body weight) were obtained
87 from Charles River Laboratories (St. Constant, QC, Canada). Rats were housed two per cage
88 in a temperature ($22 \pm 2^\circ\text{C}$) and light (12 h light/12 h dark) controlled room with a positive air
89 pressure; water and food were available for *ad libitum* consumption.

90 After a 7-days period of acclimatization, the rats received a nutritionally complete semi-
91 synthetic diet (“basal diet”) : 80% “Basal Mix with Fat Source Omitted” (Teklad TD.84172;
92 Harlan Laboratories, Madison, WI, USA) and 20% fat (11.7% canola stearine, 5.2%
93 sunflower oil, 3.1% canola oil) (Table 1) (21;22). Rats were weighed and the food intake
94 recorded every other day.

95

96 Experimental design (Figure 1)

97 Tumor

98 After 3 days of semi-synthetic diet, 28 rats received a subcutaneous injection of Ward colon
99 tumor (~ 0.1 g), *via* trocar, on the back (22-24). The Ward colorectal carcinoma was provided
100 by Dr Y. Rustum, Roswell Park Institute (25). Subcutaneous injection was selected to
101 facilitate continuous evaluation of tumour dimensions. Tumors were measured in three
102 dimensions with a caliper: the length (L), width (W), and height (H). Then, tumor size was
103 calculated according to the following equation: tumor volume (cm^3) = $0.5 \times L$ (cm) $\times W$ (cm)
104 $\times H$ (cm) (23). Tumor volume was recorded every other day prior to initiation of
105 chemotherapy, and daily after the 1st dose of chemotherapy was administered. During

106 chemotherapy, relative tumor volume for each animal was compared to the baseline volume
107 (Day 0).

108

109 Diet

110 Rats were separated into individual cages 5 days before chemotherapy in order to measure
111 their food intake.

112 After two weeks of tumour growth, when it reached $\sim 2 \text{ cm}^3$, the 28 rats were divided into 2
113 groups (D-1): the Cit group (n=14) which received the citrulline diet which is the basal diet
114 complemented with citrulline (gift from Citrage[®] Company) at 2% of diet weight
115 (corresponding to around 1 g/kg/day; 12;16) and the Control group (Ctrl; n=14) which
116 received the diet containing an isonitrogenous mix of amino acids (alanine, glycine, histidine
117 and serine in equimolar ratio) instead of citrulline (Table 1). From the day of the start of
118 citrulline or control diets (D-1), weight of the rats, food intake, and tumor growth were
119 recorded daily. Relative food intake for each animal was compared to the baseline food intake
120 (mean of the food intake of the 4 days before the beginning of the chemotherapy; i.e. Cit:
121 8.1 ± 0.6 and Ctrl: 7.9 ± 0.8 g/day). Relative cumulative food intake at the end of the study was
122 calculated as the summed daily food intake from D0 (beginning of the chemotherapy) to D9
123 (euthanasia), and relative to baseline food intake (see above).

124

125 Chemotherapy

126 The day after beginning of the citrulline or control diets (D0), rats received one intra-
127 peritoneal injection of irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-
128 camptothecin; CPT-11; Camptosar[®]; 50 mg/kg) and the day after (D1), one injection of 5-
129 fluorouracil (5-FU; 50 mg/kg). This corresponds to one cycle of chemotherapy. Atropine
130 (1 mg/kg body weight, *subcutaneous*) was administered immediately prior to each CPT-11

131 injection to alleviate early onset cholinergic symptoms (26). One week after, they received a
132 second cycle of chemotherapy (CPT-11 on D7 and 5-FU on D8).

133

134 Another set of rats (Reference group; n=6), of the same age as the tumour-bearing rats at the
135 beginning of the chemotherapy, but without cancer or chemotherapy and fed the basal diet,
136 were used as control for plasma amino acid and haptoglobin concentrations.

137

138 Euthanasia

139 The day after the end of the second cycle of chemotherapy (D9), in order to study the animals
140 in a catabolic state. Animals were killed by CO₂ asphyxia and cervical dislocation. This was
141 done at least 2 h after lights on in the animal room, at which time animals would be in the
142 postabsorptive state. Blood was collected by cardiac puncture on EDTA and centrifuged to
143 collect plasma.

144 *Tibialis* and proximal half of the colon were rapidly removed, weighed and frozen in liquid
145 nitrogen. The entire weight of the dissected tumor was recorded, and that a sample was taken
146 of the tissue at the tumor margin, avoiding any necrotic central portion, and frozen in liquid
147 nitrogen for biochemical assays. The proximal part of the jejunum, the distal part of the ileum
148 and the distal part of the colon were scraped and mucosa were collected, weighed and frozen
149 in liquid nitrogen. *Gastrocnemius*, spleen and liver were removed and weighed.

150 Plasma, muscle, tumour, and intestinal samples were stored at –80°C until analysis.

151

152 Plasma amino acid measurements

153 Plasma was deproteinized with 10% (w/v) sulfosalicylic acid and centrifuged for 10 min.
154 Individual free amino acids (Alanine, Arginine, Asparagine, Aspartate, Citrulline, Cysteine,
155 Glutamate, Glutamine, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine,

156 Ornithine, Phenylalanine, Proline, Serine, Taurine, Threonine, Tyrosine, and Valine) were
157 measured in the supernatant by cation exchange chromatography with ninhydrin post-column
158 derivatization and spectrophotometric detection on an Aminotac-JLC-500/V analyzer (Jeol,
159 Croissy-sur-Seine, France) (27). Only plasma amino acids were analysed because citrulline
160 and related amino acids in muscle are well correlated to plasma amino acids after citrulline
161 administration (9;13;16), and the tumour is too heterogeneous (with necrotic and non necrotic
162 parts) to be measured and a global value does not reflect the complexity of the tissues.

163

164 Haptoglobin measurements

165 Commercial ELISA kits for rat haptoglobin was purchased from Life Diagnostics (West
166 Chester, Pennsylvania, USA) and used according to the manufacturer's instructions on rat
167 plasma.

168

169 Antioxidant measurements

170 Ferric reducing antioxidant power (FRAP), and thiol groups were determined as described
171 previously (28).

172

173 Tissue protein content

174 Frozen *tibialis*, jejunum mucosa and colon mucosa were ground and homogenised in 10
175 volumes of ice-cold 10% trichloroacetic acid, 0.5 mmol/l EDTA. After delipidation with
176 ethanol/ether (v/v), the pellets were solubilised in NaOH 1N and total protein content was
177 determined by a method based on bicinchoninic acid (Pierce™ BCA Protein Assay Kit;
178 ThermoScientific, Rockford, IL, USA).

179

180

181 mTORC1 pathway activation

182 The most pertinent way to measure mTORC1 pathway activation is to determine the ratio
183 between the phosphorylated form of eukaryotic initiation factor 4E-binding protein 1 (4E-
184 BP1) on serine 65 and the total form of 4E-BP1, the downstream target of mTORC1 (11).

185 *Tibialis* muscles were homogenized in extraction buffer (Mammalian buffer -GE Healthcare
186 Buckinghamshire, UK-, DTT 1mM, protease inhibitor 1X, phosphatase inhibitor 1X, EDTA 1
187 mM, EGTA 1 mM) using a ball extractor at 4°C. After centrifugation, the supernatant was
188 collected and the soluble proteins were measured by BCA method. Samples were then
189 standardized to 2 mg/ml by dilution with 3X Laemmli SDS sample buffer containing 30%
190 glycerol, 1 M Tris (pH 6.8), 20% (wt/vol) SDS, 0.1% (wt/vol) bromophenol blue, dH₂O, and
191 2mM β-mercaptoethanol and heated at 95°C for 10 min.

192 Proteins at 30 µg/lane were loaded onto sodium dodecyl sulfate polyacrylamide gel (15%)
193 and transferred on a nitrocellulose membrane (AmershamTM ProtranTM; GE Healthcare).
194 Proteins were revealed on the membrane with Ponceau Red (Sigma- Aldrich). After
195 incubation in blocking buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20, 5%
196 nonfat skimmed milk powder), the membranes were incubated overnight at 4°C with the
197 phosphorylated form of 4E-BP1 on serine 65 (Cell Signaling Technology, Ozyme, France).
198 After washing, the primary antibody was removed and a 1 hr-incubation was done with
199 horseradish peroxidase-conjugated secondary antibodies (1:10 000 dilution; Jackson
200 ImmunoResearch Laboratories, Baltimore, Maryland, United States).

201 Proteins were then visualized using enhanced chemiluminescence (ECL SelectTM Western
202 Blotting Detection Reagents; GE Healthcare) on ImageQuant Las 4000 system (GE-
203 Healthcare) using a CCD camera. Band density was quantified using ImageJ software. For
204 normalization, blots were stripped using antibody stripping buffer (Gene Bio-Application,

205 Paris, France) and then reprobod for total 4E-BP1 proteins (Cell Signaling Technology) to
206 verify the relative amount analyzed.

207

208 Statistics

209 Data are expressed as means \pm SEM. The effect of citrulline on body weight, food intake and
210 tumor growth following chemotherapy was tested using 2-way repeated-measures analysis of
211 variance (ANOVA) (dietary treatment x time) followed by Holm Sidak post-hoc tests.
212 Treatment differences on tumor, oxidative stress and anthropometric data were analysed using
213 the t test for independent samples, or Mann-Whitney rank sum test when necessary. Plasma
214 parameters were analyzed using Kruskal-Wallis one way ANOVA on ranks (SigmaPlot).
215 $p < 0.05$ was considered significant.

216 To establish the effects of plasma citrulline concentration, food intake, plasma haptoglobin
217 concentration, relative tumor volume and relative protein content of both jejunum and colon
218 on relative body weight, multiple linear regression analysis was carried out. All required
219 assumptions of homogeneity of variance and linearity, and the residuals distribution were
220 assessed to validate the relevance of the model. Independence of observations was assessed
221 using Durbin-Watson statistic (1.920). The included variables met both the assumptions of
222 homogeneity of variance and linearity, and the residuals were approximately normally
223 distributed.

224

225 **Results**

226 ***Mortality***

227 No animal died during the study, whatever the group.

228

229 ***Chemotherapy efficacy***

230 Tumor response to chemotherapy

231 *Tumor volume and weight*

232 Mean initial tumor volume was $1.7 \pm 0.2 \text{ cm}^3$ and its decrease in response to the chemotherapy
233 is illustrated in Fig. 2. Tumor response to treatment over time did not differ between diets.

234 Concerning tumor weight obtained at the end of the study, there was no significant difference
235 between the two groups (Table 2).

236

237 *mTORC1 activation in the tumor*

238 mTORC1 activation showed no difference attributable to diet (Table 2).

239

240 Antioxidant measurements

241 Antioxidant capacity has been measured through FRAP (Ferric Reducing Ability of Plasma)
242 and thiols amount in plasma, muscle, and colon. FRAP and thiols quantities were not altered
243 by citrulline whatever the tissues considered (Table 2).

244

245 ***Nutritional status***

246 Rat body weight

247 Body weight decreased (-6%) after the first cycle of chemotherapy, then increased from day 6
248 to day 7 and decreased again after the second cycle of chemotherapy to 94% of initial body
249 weight, whatever the diet (Figure 3A).

250 Relative body weight was positively correlated to cumulated food intake from D0 to D9 and
251 negatively correlated to plasma haptoglobin concentration. On the other hand, citrullinemia,
252 relative tumor volume, and protein content of the intestine were not significantly explaining
253 the relative bodyweight variation (Table 3).

254

255 Food intake

256 Animals presented an anorexia due to chemotherapy administration: food intake dropped from
257 the first day after the chemotherapy to correspond to 20% of the initial food intake of the rats,
258 and increased from day 4 to reach 100% of the initial food intake, and even 115% at days 5,
259 and 6, whatever the diet. Food intake decreased again after the second cycle of chemotherapy
260 without any difference between the 2 groups (Figure 3B). The cumulative food intake at the
261 end of the study was also the same in the 2 groups (Cit: 76.9 ± 5.1 vs Ctrl: $85.0 \pm 5.0\%$ of the
262 pre-chemotherapy value)

263 Owing to individual variations in overall food intake, citrulline intake varied from 0.34 to
264 1.34 g/kg/day between days after start of chemotherapy, with a mean citrulline intake of
265 0.89 ± 0.03 g/kg/day over 9 days. Amino acid intake from the control amino acid mixture
266 varied from 0.39 to 2.06 g/kg/day (Figure 3C).

267 Nitrogen intake from citrulline or control amino acid mix was the same in the two groups
268 (Figure 3D).

269

270 Organ weight

271 Liver, spleen and muscle (*Tibialis* and *gastrocnemius*) weights were similar in the 2 groups
272 (Table 4). The size of the small intestine (from duodenum to ileum) (*data not shown*) and the
273 weight of the small intestine mucosa (jejunum and ileum) and this of the large intestine
274 mucosa (colon) were not different between the 2 groups (Table 4).

275

276 Tissue protein content

277 Total protein content of the *Tibialis*, jejunum mucosa and colon mucosa were similar between
278 the groups (Table 4).

279

280 Plasma amino acid levels

281 As expected, plasma citrulline, arginine and ornithine concentrations were higher in rats of Cit
282 group compared to rats of control group. Plasma citrulline was lower in the control group
283 compared to the pre-chemotherapy values in the Reference group (Table 5).

284 Concerning alanine, glycine, histidine and serine (non-essential amino acids contained in the
285 control diet), plasma glycine and serine concentrations were increased in the control group
286 (Serine: Cit: 307 ± 11 $\mu\text{mol/l}$ vs Control: 352 ± 23 ; Glycine: Cit: 258 ± 12 vs Control: $316 \pm$
287 15) and alanine and histidine were not modified (Alanine: Cit: 465 ± 32 $\mu\text{mol/l}$ vs Control:
288 468 ± 26 ; Histidine: Cit: 86 ± 3 vs Control: 85 ± 2).

289 Plasma phenylalanine and glutamine concentrations were not modified by diet, or the cancer
290 (Table 5).

291 Citrulline supplementation had no effects on the concentration of branched amino acids but
292 they were decreased in plasma of cancer and chemotherapy rats compared to the Reference
293 group (Table 5).

294

295 Haptoglobin measurements

296 Plasma haptoglobin was the same whatever the diet (Table 5). As expected, it was higher in
297 cancer and chemotherapy rats compared to the Reference group.

298

299 **Discussion**

300 In this study, we evaluated the interaction of citrulline with chemotherapy in an animal model
301 of colon cancer and chemotherapy, and its potential beneficial effect on nutritional status. We
302 show, in our model, that citrulline had no effect on CPT-11/5-FU-based chemotherapy
303 toxicity, with no modification of Ward colon tumor response to therapy. Concerning
304 nutritional status, citrulline supplementation had no effect.

305

306 *Citrulline interaction with chemotherapy*

307 Chemotherapy in cancer patients is a situation with an important challenge for nutrition. It is
308 important to nourish the person, but it is also fundamental to not nourish the tumour or to not
309 interfere with the action of the chemotherapy (5). In our study, as well known in this rat
310 model of cancer and chemotherapy, the initiation of the chemotherapy led to a large decrease
311 in the tumor size (22-23) and, in this animal model, citrulline supply did not modify the size
312 and the weight of the tumor. This point deserves to be clarified because citrulline increased
313 tumor growth in two different cancer models (29-30). In the first study, citrulline (1g/kg/day)
314 was injected subcutaneously to C26 cells-injected mice, and in the second study, Ward colon
315 tumor bearing-Fisher 344 rats (same animal model than ours) received citrulline by parenteral
316 nutrition. This discrepancy could be related to the fact that, in our work, our animals received
317 chemotherapy, which is more relevant to the clinic situation. In fact, citrulline action observed
318 in their studies, i.e. tumor growth activation by citrulline, could be too weak compared to
319 chemotherapy action, explaining the fact that citrulline had no effect on tumor size evolution
320 in our study.

321 To the best of our knowledge, the mechanism of action of citrulline at the tumor site has never
322 been studied, but some hypothesis can be proposed. First, citrulline is known to be a potent
323 activator of mTORC1 (11;16), and mTORC1 pathway in the tumor is thought to be implicated

324 in tumor growth (17), but, in our study, citrulline did not activate mTORC1 pathway in the
325 tumor. This can explain the fact that citrulline did not increase tumor size. But, a very recent
326 set of data allowed us to make progress in the mechanism of action of citrulline on the
327 mTORC1 pathway and the regulation of MPS. Citrulline, unlike leucine, is not an activator of
328 the mTORC1 pathway but a normalizer of its activity (31). Indeed, in healthy conditions,
329 citrulline is ineffective but under certain stress conditions, citrulline increases MPS by
330 specifically reallocating mitochondrial fuel to the protein synthesis machinery (and to restore
331 mTORC1 activity). Secondly, some studies showed that antioxidant could impair the
332 chemotherapy used in our model (CPT-11 and 5-FU) (19;20), even if the mechanisms of
333 CPT-11 and 5-FU are not based on oxidative stress (32;33). So it was important to verify if
334 the antioxidant properties of citrulline did not interact with the chemotherapy (9;34). In the
335 present study, citrulline supplementation had no effect on antioxidant parameters in plasma,
336 muscle or colon. Thirdly, at the tumor site, citrulline, due to its ability to generate nitric oxide
337 ($^{\circ}\text{NO}$) by endothelial cells (35), could activate angiogenesis. In our study, an absence of effect
338 of citrulline on tumor size could be due to the action of chemotherapy which thwart citrulline
339 effect at this level. So citrulline effect on tumor growth could be multifactorial (figure 4), but,
340 in any case, in our study, these potential effects have been thwarted by the CPT-11/5-FU-
341 based chemotherapy.

342 On the contrary, citrulline could have potentiate this treatment, due to its ability to generate
343 $^{\circ}\text{NO}$ by macrophages (36;37) and to activate immunity at the tumour site (figure 4), but, in
344 our study, citrulline has no beneficial effects on tumour size.

345

346 *Citrulline effects on nutritional status*

347 As it has been well demonstrated in this model (22;23), the animals suffered an important
348 degree anorexia, associated with a significant body weight loss (6%) due to chemotherapy.

349 As citrulline did not interact negatively with chemotherapy, it seemed of interest to evaluate
350 its capacities to maintain the nutritional status in this model. Citrulline could act either
351 directly on muscle mass (8-10;13), or indirectly by decreasing inflammation (36;37) or by
352 improving intestinal integrity (38). Citrulline supply had no effect on muscle weight or
353 muscle protein content. It is important to note that, in our model, citrulline supplementation at
354 0.89 g/kg BW/day (from 0.34 to 1.34, depending on food intake), leads to double plasma
355 citrulline concentration compared to healthy rat values, and to multiply the values of control
356 rats by four (confirming that despite anorexia, citrulline is well ingested). But this increase in
357 plasma citrulline concentration was not sufficient to have beneficial effect on muscle. The
358 lack of effect of citrulline on muscle mass could be due to anorexia and the reduced amount of
359 citrulline ingested but also the reduced amount of protein ingested. Hence, citrulline is known
360 to activate MPS but, as limiting amino acids availability strongly affects MPS, the presence of
361 enough available amino acids is needed (16;39). These results are consistent with the use of
362 enteral nutrition to go over the anorexia, especially since the body weight of the animals was
363 correlated to the cumulated food intake throughout the study.

364 Finally, in our study, citrulline did not improve inflammation or intestine integrity as already
365 observed in other situations (36-38).

366

367 *Limitation of the study*

368 Even if our data support the idea of an absence of effect of citrulline on CPT-11/5-FU-based
369 chemotherapy, it is impossible in the current state of things to generalize the absence of
370 effects of citrulline on all the chemotherapies. In fact, available data are only on Ward colon
371 tumour and CPT-11/5-FU-based chemotherapy. Some additional studies on other types of
372 tumours and assessment of effects citrulline on side effects due to different chemotherapeutic
373 agents are needed.

374

375 *Conclusion*

376 In this cancer and chemotherapy animal model, citrulline does alter the tumour response to
377 CPT-11/5FU based therapy but, failed to improve nutritional status. This could be due to the
378 anorexia which leads to reduced citrulline and energy ingestion. In conclusion, this study is
379 not favour for the use of citrulline during cancer, but further studies are required to clarify
380 whether higher citrulline doses associated to higher food intake, maintained by enteral
381 nutrition, would render additional nutritional benefit.

382

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386

387 **Conflict of Interest**

388 CB, LC and CM are shareholders of Citrage company; AG, NN, AdR, VM and VB: no
389 conflict to declare.

390

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396

397 **Author contribution**

398 **Charlotte Breuillard:** Conceptualization; Data curation; Formal analysis; Funding
399 acquisition; Investigation; Methodology; Project administration; Validation; Visualization;
400 Writing - original draft. **Christophe Moinard:** Conceptualization; Funding acquisition;
401 Methodology; Resources; Validation; Writing - review & editing. **Arthur Goron:** Data
402 curation; Formal analysis; Investigation; Writing - review & editing. **Nathalie Neveux:** Data
403 curation; Investigation; Resources; Validation; Writing - review & editing. **Antoine De**
404 **Reviere:** Formal analysis. **Vera Mazurak:** Funding acquisition; Resources; Writing - review
405 & editing. **Luc Cynober:** Conceptualization; Funding acquisition; Methodology; Resources;
406 Writing - review & editing. **Vickie E. Baracos:** Conceptualization; Data curation; Funding

407 acquisition; Investigation; Methodology; Resources; Supervision; Validation; Writing -
408 review & editing.
409

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533

534 **Figure legend**

535

536 **Figure 1: Experimental design of the study**

537 After a 1-week acclimation period, 28 11-week old Fisher female rats received the control
538 diet, and tumour implantation 1 week after. Then, after 13 days of tumor growth (D-1), the
539 rats received either citrulline (n=14) or control (n=14) diet. The day after, the first
540 chemotherapy cycle was initiated: irinotecan (CPT-11; 50 mg/kg) at D0, and 5-fluorouracil
541 (5-FU; 50 mg/kg) at D1. One week after, the rats received the second cycle of chemotherapy
542 (CPT-11 at D7 and 5-FU at D8). They are euthanized at D9.

543

544 **Figure 2: Relative tumor size of the tumor-bearing rats under chemotherapy**

545 Relative tumor size compared to the first day of chemotherapy for each animal. The rats
546 received a first chemotherapy cycle: irinotecan (CPT-11; 50 mg/kg; $\hat{\uparrow}$) at D0, and 5-
547 fluorouracil (5-FU; 50 mg/kg; $\hat{\uparrow}$) at D1, and a second cycle of chemotherapy (CPT-11 $\hat{\uparrow}$ at D7
548 and 5-FU $\hat{\uparrow}$ at D8).

549 Results are expressed in Mean \pm SEM. a,b,c: Mean values with unlike letters were
550 significantly different

551

552 **Figure 3: Relative body weight, relative food intake, citrulline or NEAA ingestion and**
553 **nitrogen from Citrulline or NEAA ingestion of tumor-bearing rats under chemotherapy**

554 Relative body weight (A) and relative food intake (B) compared to the first day of
555 chemotherapy for each animal. Citrulline and NEAA (from control diet) ingestion (C) and
556 nitrogen from Citrulline and NEAA ingestion (D). The rats received the first chemotherapy

557 cycle: irinotecan (CPT-11; 50 mg/kg;↑↑) at D0, and 5-fluorouracil (5-FU; 50 mg/kg;↑) at D1,
558 and a second cycle of chemotherapy (CPT-11 ↑↑ at D7 and 5-FU ↑ at D8).

559 Results are expressed in Mean ± SEM. a,b,c,d: Mean values with unlike letters were
560 significantly different.

561

562 **Figure 4: Role and mechanism of action of citrulline at the tumor site and on nutritional**
563 **status**

Fig 1

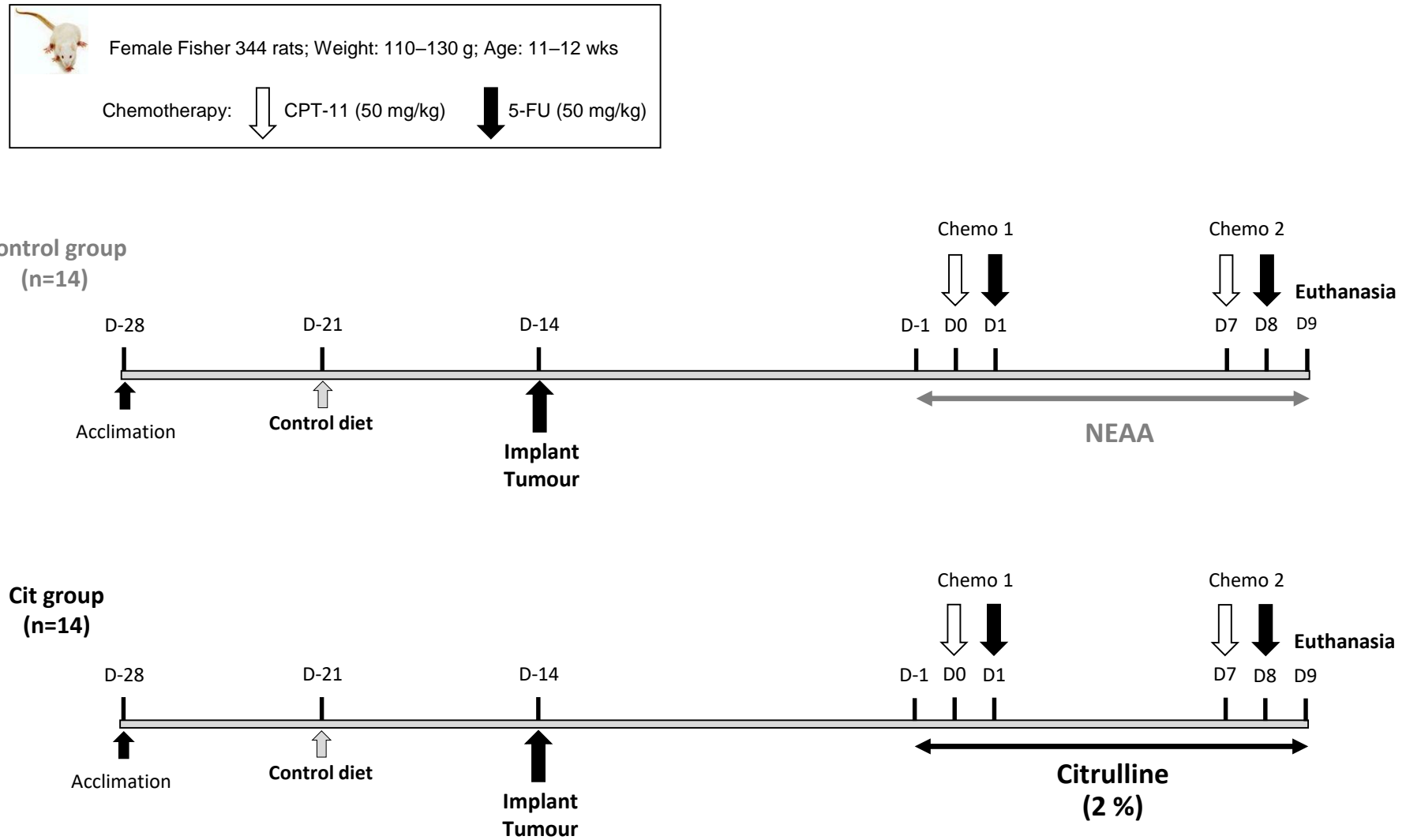


Fig 2

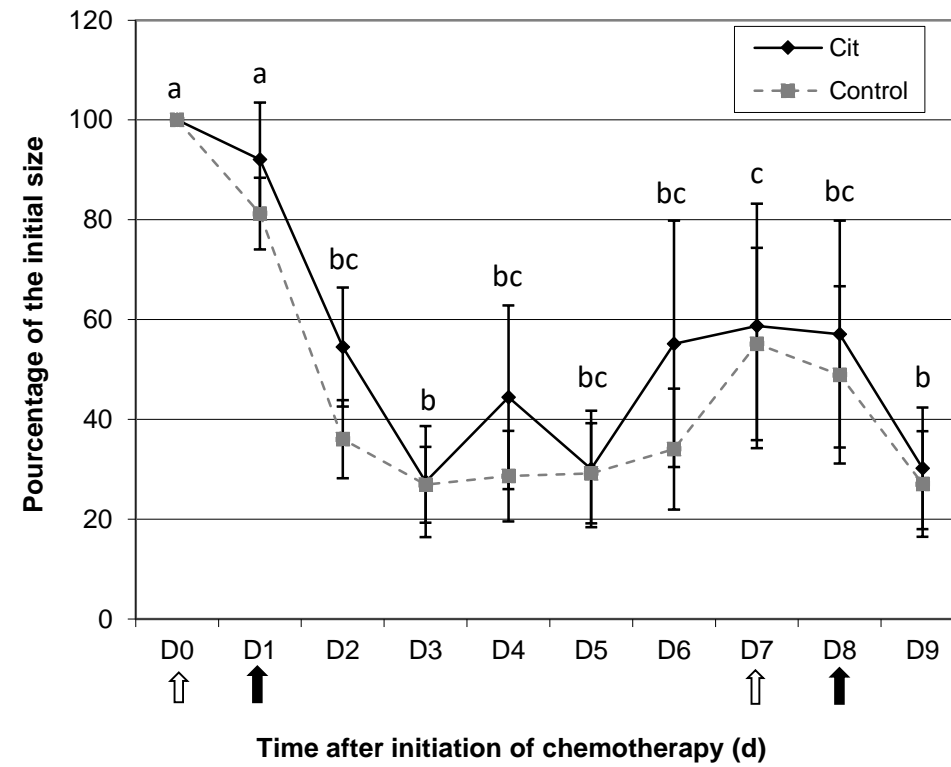


Fig 3A

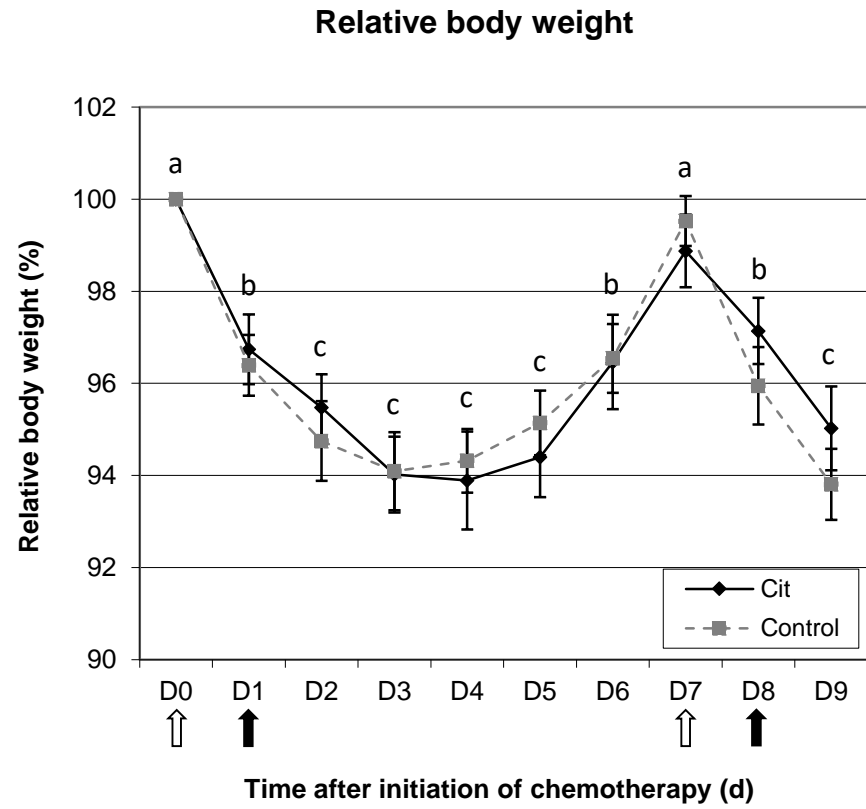


Fig 3B

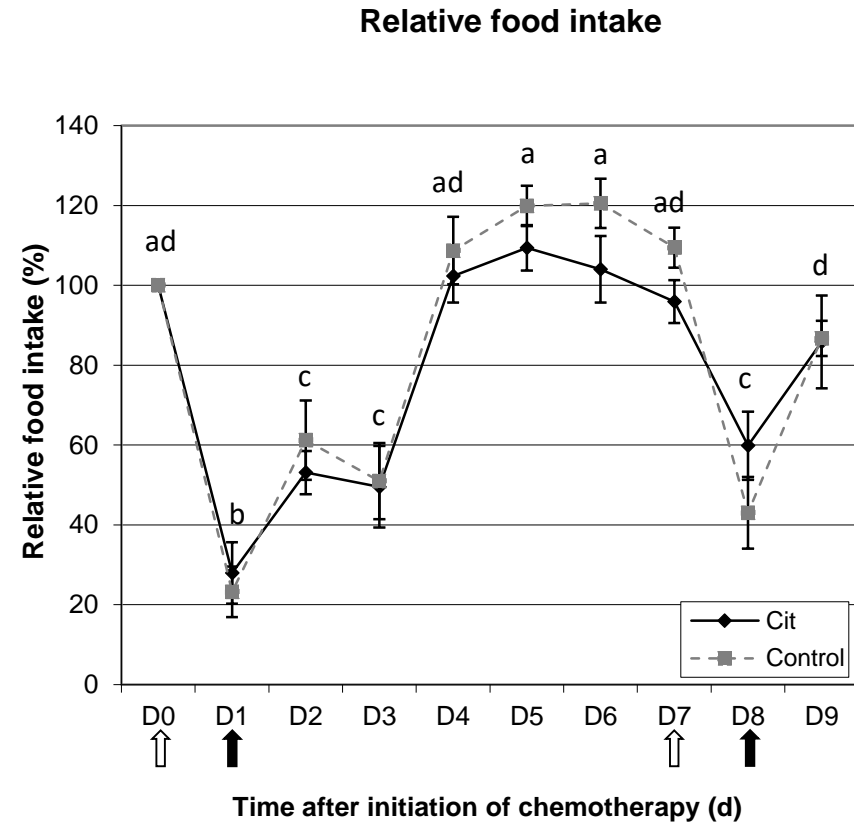


Fig 3C

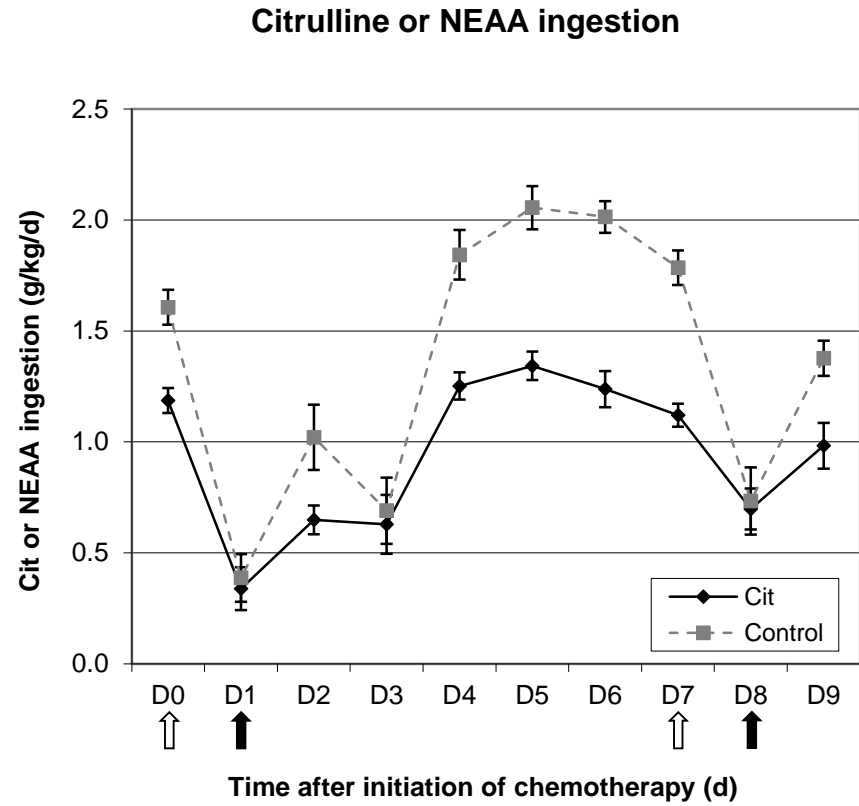


Fig 3D

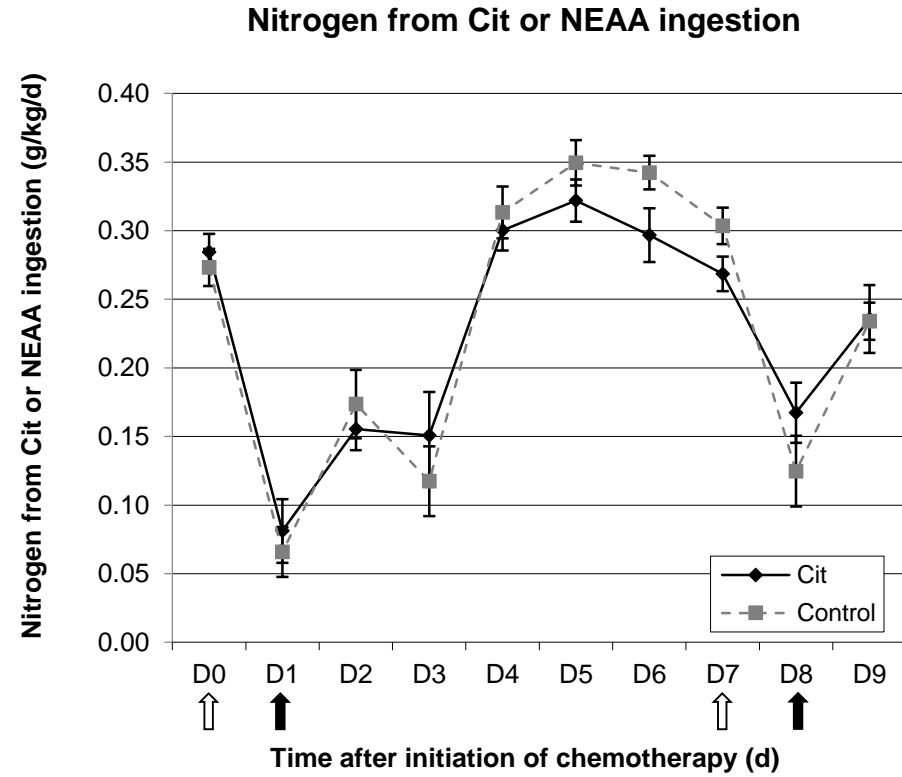


FIG 4

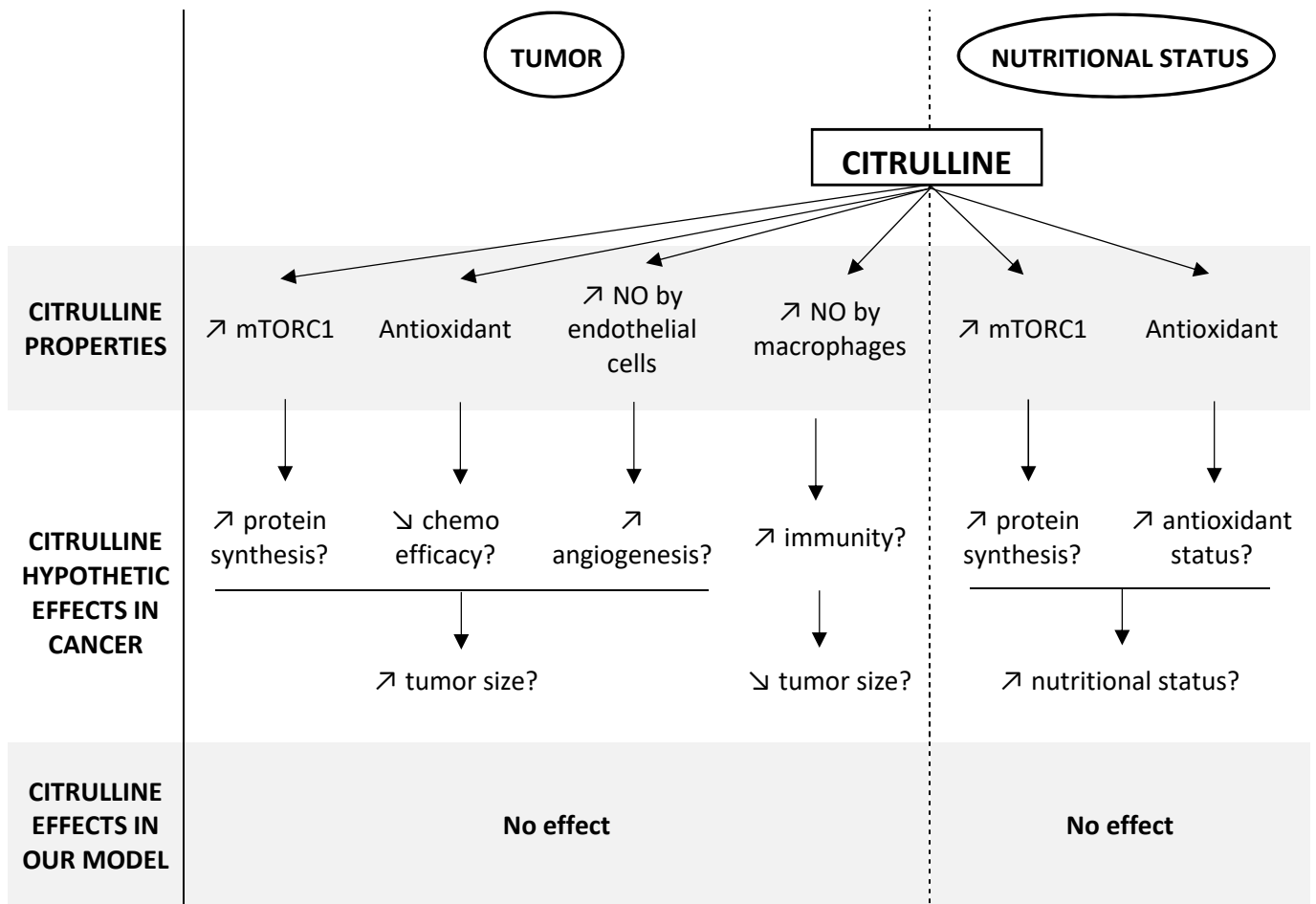


Table 1: Diets composition

	Basal diet	Citrulline diet	Control diet
	(g/100g)	(g/100g)	(g/100g)
Basal Mix	80	77.7	77.7
Casein	27	26.22	26.22
L-Méthionine	0.25	0.24	0.24
Dextrose	20.85	20.25	20.25
Corn starch	20	19.43	19.43
Cellulose	5	4.86	4.86
Mineral mix	5.09	4.94	4.94
Sodium selenite	0.03	0.03	0.03
Manganese sulfate	0.02	0.02	0.02
Vitamin mix	1	0.97	0.97
Inositol	0.63	0.61	0.61
Choline chloride	0.14	0.13	0.13
Fat	20	19.42	19.42
Canola stearine	11.7	11.36	11.36
Sunflower oil	5.2	5.05	5.05
Canola oil	3.1	3.01	3.01
Amino Acids	0	2.9	2.9
Citrulline	0	2	0
Alanine	0	0	0.75
Glycine	0	0	0.65
Histidine	0	0	0.6
Serine	0	0	0.9
Corn starch	0	0.9	0

Three days before tumor injection, rats of cancer and chemotherapy groups received the basal diet which is a nutritionally complete semi-synthetic diet with 80% “Basal Mix with Fat Source Omitted” (Teklad TD.84172; Harlan Laboratories, Madison, WI, USA) and 20% fat (11.7% canola stearine, 5.2% sunflower oil, 3.1% canola oil). The day before the start of the chemotherapy, rats were divided into 2 groups, and received the associated diet (i.e. Citrulline or control diet). Rats of reference group received the basal diet only.

Table 2: Chemotherapy efficacy according to the diet in cancer rats under chemotherapy

	<i>Control group</i>	<i>Citrulline group</i>	<i>Citrulline effect</i>
Tumor			
<i>Weight (g)</i>	0.92 ± 0.24	1.22 ± 0.32	<i>p=0.566</i>
<i>mTORC1 pathway (Relative 4EBP1 phosphorylated values (AU))</i>	0.72 ± 0.05	0.82 ± 0.09	<i>p=0.782</i>
Oxidative stress			
Plasma			
<i>FRAPS (μmol/l)</i>	370 ± 33	399 ± 45	<i>p=0.685</i>
<i>Thiols (μmol/l)</i>	256 ± 19	272 ± 14	<i>p=0.583</i>
Muscle (Tibialis)			
<i>FRAPS (μmol/g prot)</i>	28.7 ± 0.6	27.0 ± 0.6	<i>p=0.070</i>
<i>Thiols (μmol/g prot)</i>	50.6 ± 0.8	50.3 ± 0.5	<i>p=0.773</i>
Colon			
<i>FRAPS(μmol/g prot)</i>	159 ± 14	167 ± 18	<i>p=0.726</i>
<i>Thiols (μmol/g prot)</i>	34.7 ± 3.3	36.1 ± 4.6	<i>p=0.807</i>

Tumor-bearing rats have received, as soon as the day before the initiation of the first chemotherapy cycle, the control diet (Control group -n=14-, isonitrogenous to citrulline group) or the citrulline diet (Cit group -n=14-, 2% of the diet). Then, the animals received the

2 cycles of chemotherapy: irinotecan (CPT-11; 50 mg/kg) at D0 and D7, and 5-fluorouracil (5-FU; 50 mg/kg) at D1 and D8. They are euthanized at D9.

Data are presented as mean \pm SEM.

Table 3: Multiple regression analysis results, with the coefficients of each parameters explaining the relative bodyweight variation.

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B		Collinearity Statistics	
	B	Std. Error	Beta			Lower bound	Upper bound	Tolerance	VIF
(Constant)	73.751	4.828		15.276	.000	63.680	83.822		
Citrullinemia	.001	.004	.042	.262	.796	-.006	.008	.504	1.983
Relative tumor volume	.012	.009	.151	1.263	.221	-.008	.031	.921	1.085
Cumulative food intake	.248	.079	.454	3.126	.005	.083	.414	.626	1.597
Food intake at D9	.215	.281	.145	.764	.454	-.372	.801	.365	2.737
Relative protein content in colon	.311	.408	.095	.762	.455	-.540	1.161	.858	1.165
Relative protein content in jejunum	1.357	.868	.243	1.563	.134	-.454	3.168	.548	1.826
Haptoglobin	-1.127	.451	-.405	-2.497	.021	-2.068	-.185	.502	1.991

Adjusted R² = 0.643. Dependent Variable: Relative body weight. VIF: Variable Inflation Factor.

Table 4: Organ weight, muscles and intestine mucosa data in cancer rats under chemotherapy fed control diet or a citrulline enriched diet

	<i>Control group</i>	<i>Citrulline group</i>	<i>Citrulline effect</i>
Organ weight			
Liver			
g	5.2 ± 0.1	5.1 ± 0.1	NS
g/100g final BW	4.0 ± 0.1	3.9 ± 0.1	NS
Spleen			
mg	392 ± 21	360 ± 19	NS
g/100g final BW	0.30 ± 0.02	0.27 ± 0.01	NS
Muscles			
<i>Gastrocnemius</i>			
Weight			
mg	780 ± 20	798 ± 17	NS
g/100g final BW	0.59 ± 0.01	0.61 ± 0.01	NS
<i>Tibialis</i>			
Weight			
mg	236 ± 9	237 ± 6	NS
g/100g final BW	0.18 ± 0.006	0.18 ± 0.003	NS
Protein content			
mg	21.5 ± 1.1	23.2 ± 1.1	NS
g/100g muscle	9.1 ± 0.38	9.8 ± 0.26	NS

mTOR pathway			
<i>Relative 4EBP1 phosphorylated values (AU)</i>	0.84 ± 0.05	1.06 ± 0.16	<i>NS</i>
Intestine mucosa			
Jejunum			
Weight			
<i>mg/10cm jej.</i>	377 ± 28	371 ± 29	<i>NS</i>
<i>g/100g final BW</i>	0.72 ± 0.05	0.71 ± 0.06	<i>NS</i>
Protein content			
<i>mg/10cm</i>	16.4 ± 1.3	17.4 ± 1.6	<i>NS</i>
<i>g/100g mucosa</i>	4.4 ± 0.1	4.6 ± 0.2	<i>NS</i>
<i>g/100g final BW</i>	0.031 ± 0.002	0.033 ± 0.003	<i>NS</i>
Ileum			
Weight			
<i>mg/10cm ileum</i>	285 ± 15	268 ± 14	<i>NS</i>
<i>g/100g final BW</i>	0.55 ± 0.04	0.51 ± 0.03	<i>NS</i>
Colon			
Weight			
<i>g</i>	0.39 ± 0.04	0.33 ± 0.05	<i>NS</i>
<i>g/100g final BW</i>	0.30 ± 0.03	0.25 ± 0.04	<i>NS</i>
Protein content			
<i>g/100g mucosa</i>	3.8 ± 0.3	4.1 ± 0.3	<i>NS</i>

Tumor-bearing rats have received, as soon as the day before the initiation of the first chemotherapy cycle, the control diet (Control group -n=14-, isonitrogenous to citrulline group) or the citrulline diet (Cit group -n=14-, 2% of the diet). Then, the animals received the 2 cycles of chemotherapy: irinotecan (CPT-11; 50 mg/kg) at D0 and D7, and 5-fluorouracil (5-FU; 50 mg/kg) at D1 and D8. They are euthanized at D9.

Data are presented as mean \pm SEM.

NS: Non significant

Table 5: Plasma parameters in cancer rats under chemotherapy (CC), fed the control diet or a citrulline enriched diet compared to reference group

	<i>Reference group</i>	<i>Control group</i>	<i>Citrulline group</i>	<i>CC effect</i>	<i>Citrulline effect</i>
Selected Amino Acids ($\mu\text{mol/l}$)					
Citrulline	129 \pm 9	47 \pm 5	213 \pm 47	$p < 0.05$	$p < 0.05$
Arginine	175 \pm 13	135 \pm 5	260 \pm 24	NS	$p < 0.05$
Ornithine	49 \pm 7	44 \pm 4	67 \pm 7	NS	$p < 0.05$
Glutamine	651 \pm 16	757 \pm 20	727 \pm 36	NS	NS
Phenylalanine	74 \pm 2	73 \pm 3	71 \pm 3	NS	NS
Leucine	249 \pm 11	206 \pm 6	206 \pm 10	$p < 0.05$	NS
Isoleucine	150 \pm 5	122 \pm 4	120 \pm 6	$p < 0.05$	NS
Valine	350 \pm 14	264 \pm 7	268 \pm 13	$p < 0.05$	NS
Inflammatory marker					
Haptoglobin (g/l)	0.36 \pm 0.04	2.10 \pm 0.38	1.59 \pm 0.22	$p < 0.05$	NS

Tumor-bearing rats have received, as soon as the day before the initiation of the first chemotherapy cycle, the control diet (Control group -n=14-, isonitrogenous to citrulline group) or the citrulline diet (Citrulline group -n=14-, 2% of the diet). Then, the animals received the 2 cycles of chemotherapy: irinotecan (CPT-11; 50 mg/kg) at D0 and D7, and 5-fluorouracil (5-FU; 50 mg/kg) at D1 and D8. They are euthanized at D9. The “reference group” correspond to healthy control rats of the same strain.

Data are presented as mean \pm SEM.

NS: Non significant