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

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Abstract

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

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

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

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

Keywords: Chemotherapy efficacy, Nutritional status, Protein synthesis, Inflammation, Antioxidant

Introduction

Patients with cancer are at high risk for malnutrition due to the combined effect of cancer and chemotherapy. Both cancer and chemotherapy are associated with inflammation and weight loss, and more specifically muscle loss, which, in turn, leads to decreased tolerance to chemotherapy and decreased survival rate (1-4). A vicious circle of malnutrition and toxicity worsens patient outcome, a point underlined by the European Society for Enteral and Parenteral Nutrition (ESPEN) expert group for action against cancer-related malnutrition (5). Skeletal muscle loss is a specific focus of ESPEN recommendations, since sarcopenia is an important prognostic factor for both mortality and treatment toxicity. Several strategies have been proposed to manage these patients, in particular using anabolic and/or anti-catabolic drugs and nutrients (6), however the amount and quality of evidence are insufficient and the recommendation is to continue research (5). Specific nutrients include a variety of amino acids and fatty acids and among these, citrulline could be a promising tool (7). Citrulline is an amino acid which is not incorporated into protein, and it is now well known to be an activator of muscle protein synthesis (MPS) and to increase muscle mass (8-10), particularly in conditions of muscle mass loss (11-13). However, to date, there is no data concerning the use of this amino acid in cancer patients, especially as plasma citrulline concentration (a marker of functional intestinal mass (14)) is decreased in cancer patients (15).

But, before giving citrulline to cancer patients to thwart muscle mass loss, a prerequisite is to check the safety of this amino acid and to determine whether there is any interaction between citrulline and chemotherapy. Citrulline is able to activate mTORC1 pathway in muscle (11;16), and could induce mTOR activation in tumor cells, and thus increase tumor size (17). Citrulline has also important antioxidant properties (9;18) and this property could interfere 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. Secondarily, we
79 assessed the ability of this amino acid to modify muscle mass loss.
80

Material and methods

Animals

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

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

After a 7-days period of acclimatization, the rats received a nutritionally complete semi-synthetic diet ("basal diet") : 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) (Table 1) *(*21;22*)*. Rats were weighed and the food intake recorded every other day.

Experimental design (Figure 1)

Tumor

After 3 days of semi-synthetic diet, 28 rats received a subcutaneous injection of Ward colon tumor (~ 0.1 g), *via* trocar, on the back *(*22-24*)*. The Ward colorectal carcinoma was provided by Dr Y. Rustum, Roswell Park Institute (25). Subcutaneous injection was selected to facilitate continuous evaluation of tumour dimensions. Tumors were measured in three dimensions with a caliper: the length (L), width (W), and height (H). Then, tumor size was calculated according to the following equation: tumor volume (cm^3) = 0.5 x L (cm) x W (cm) x H (cm) (23). Tumor volume was recorded every other day prior to initiation of chemotherapy, and daily after the 1^{st} 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 ~ 2 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

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

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

Euthanasia

The day after the end of the second cycle of chemotherapy (D9), in order to study the animals in a catabolic state. Animals were killed by CO_2 asphyxia and cervical dislocation. This was done at least 2 h after lights on in the animal room, at which time animals would be in the postabsorptive state. Blood was collected by cardiac puncture on EDTA and centrifuged to collect plasma.

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

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

Plasma amino acid measurements

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

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

Haptoglobin measurements

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

Antioxidant measurements

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

Tissue protein content

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

mTORC1 *pathway activation*

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

Tibialis muscles were homogenized in extraction buffer (Mammalian buffer -GE Healthcare Bukinghamshire, UK-, DTT 1mM, protease inhibitor 1X, phosphatase inhibitor 1X, EDTA 1 mM, EGTA 1 mM) using a ball extractor at 4°C. After centrifugation, the supernatant was collected and the soluble proteins were measured by BCA method. Samples were then standardized to 2 mg/ml by dilution with 3X Laemmli SDS sample buffer containing 30% glycerol, 1 M Tris (pH 6.8), 20% (wt/vol) SDS, 0.1% (wt/vol) bromophenol blue, dH_2O, and 2mM β-mercaptoethanol and heated at 95°C for 10 min.

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

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

205 Paris, France) and then reprobed for total 4E-BP1 proteins (Cell Signaling Technology) to
206 verify the relative amount analyzed.
207
208 *Statistics*
209 Data are expressed as means ± 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

Results

Mortality

No animal died during the study, whatever the group.

Chemotherapy efficacy

Tumor response to chemotherapy

Tumor volume and weight

Mean initial tumor volume was 1.7±0.2 cm^3 and its decrease in response to the chemotherapy is illustrated in Fig. 2. Tumor response to treatment over time did not differ between diets. Concerning tumor weight obtained at the end of the study, there was no significant difference between the two groups (Table 2).

mTORC1 activation in the tumor

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

Antioxidant measurements

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

Nutritional status

Rat body weight

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

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

Food intake

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

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

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

Organ weight

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

Tissue protein content

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

Plasma amino acid levels

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

Concerning alanine, glycine, histidine and serine (non-essential amino acids contained in the control diet), plasma glycine and serine concentrations were increased in the control group (Serine: Cit: 307 ± 11 µmol/l *vs* Control: 352 ± 23; Glycine: Cit: 258 ± 12 *vs* Control: 316 ± 15) and alanine and histidine were not modified (Alanine: Cit: 465 ± 32 µmol/l *vs* Control: 468 ± 26; Histidine: Cit: 86 ± 3 *vs* Control: 85 ± 2).

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

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

Haptoglobin measurements

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

Discussion

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

Citrulline interaction with chemotherapy

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

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

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

On the contrary, citrulline could have potentiate this treatment, due to its ability to generate °NO by macrophages (36;37) and to activate immunity at the tumour site (figure 4), but, in our study, citrulline has no beneficial effects on tumour size.

Citrulline effects on nutritional status

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

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

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

Limitation of the study

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

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

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Author contribution

Charlotte Breuillard: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Validation; Visualization; Writing - original draft. **Christophe Moinard**: Conceptualization; Funding acquisition; Methodology; Resources; Validation; Writing - review & editing. **Arthur Goron**: Data curation; Formal analysis; Investigation; Writing - review & editing. **Nathalie Neveux**: Data curation; Investigation; Resources; Validation; Writing - review & editing. **Antoine De Reviers**: Formal analysis. **Vera Mazurak**: Funding acquisition; Resources; Writing - review & editing. **Luc Cynober**: Conceptualization; Funding acquisition; Methodology; Resources; Writing - review & editing. **Vickie E. Baracos**: Conceptualization; Data curation; Funding

407 acquisition; Investigation; Methodology; Resources; Supervision; Validation; Writing -
408 review & editing.
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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;⇧) at D0, and 5-
547 fluorouracil (5-FU; 50 mg/kg;⬆) at D1, and a second cycle of chemotherapy (CPT-11 ⇧ at D7
548 and 5-FU ⬆ at D8).
549 Results are expressed in Mean ± 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

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

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

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

Fig 1

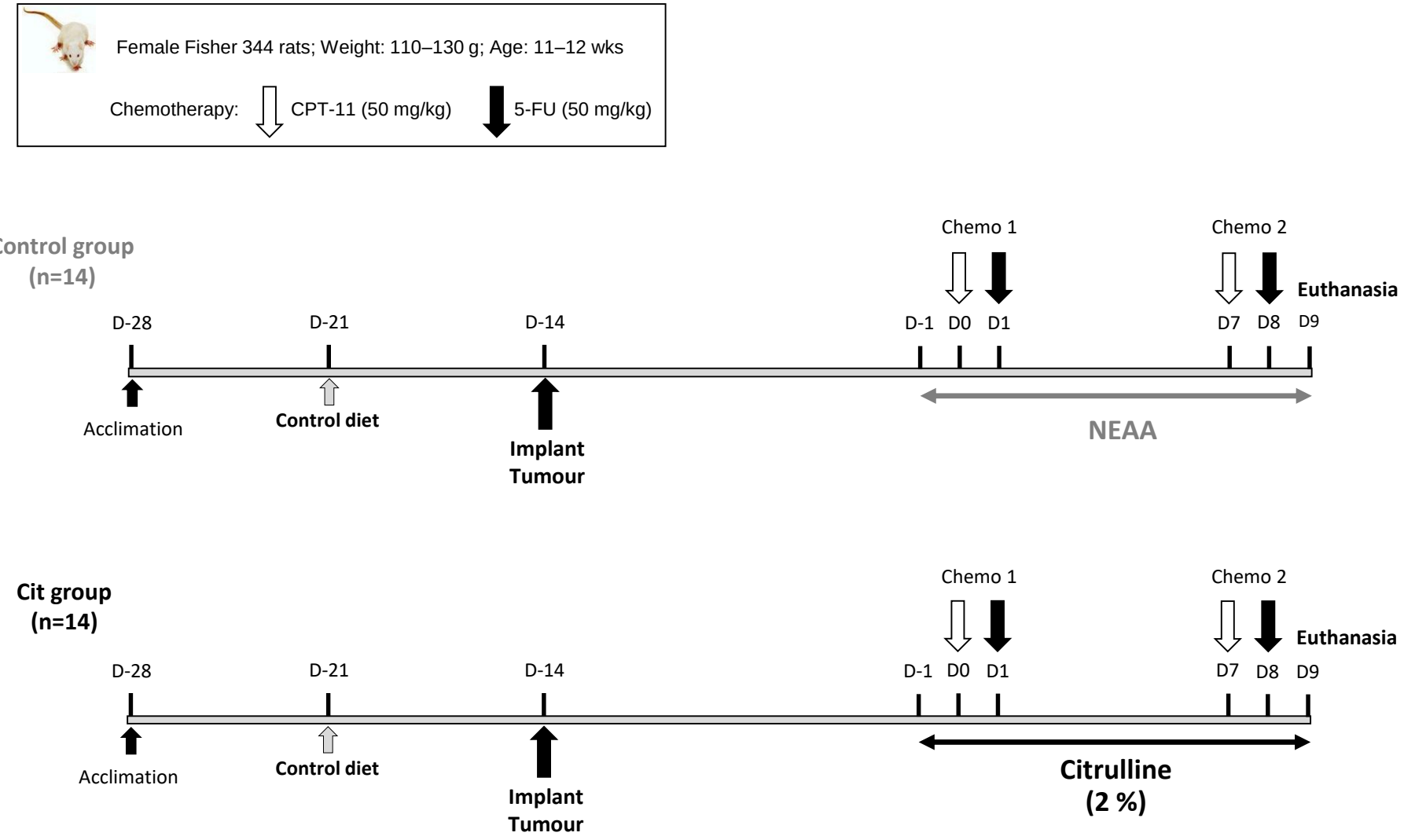
Female Fisher 344 rats; Weight: 110–130 g; Age: 11–12 wks
Chemotherapy:
CPT-11 (50 mg/kg)
5-FU (50 mg/kg)
Control group
(n=14)
Chemo 1
Chemo 2
Euthanasia
D-28
D-21
D-14
D-1
D0
D1
D7
D8
D9
Acclimation
Control diet
Implant
Tumour
NEAA
Cit group
(n=14)
Chemo 1
Chemo 2
Euthanasia
D-28
D-21
D-14
D-1
D0
D1
D7
D8
D9
Acclimation
Control diet
Implant
Tumour
Citrulline
(2 %)

Fig 2

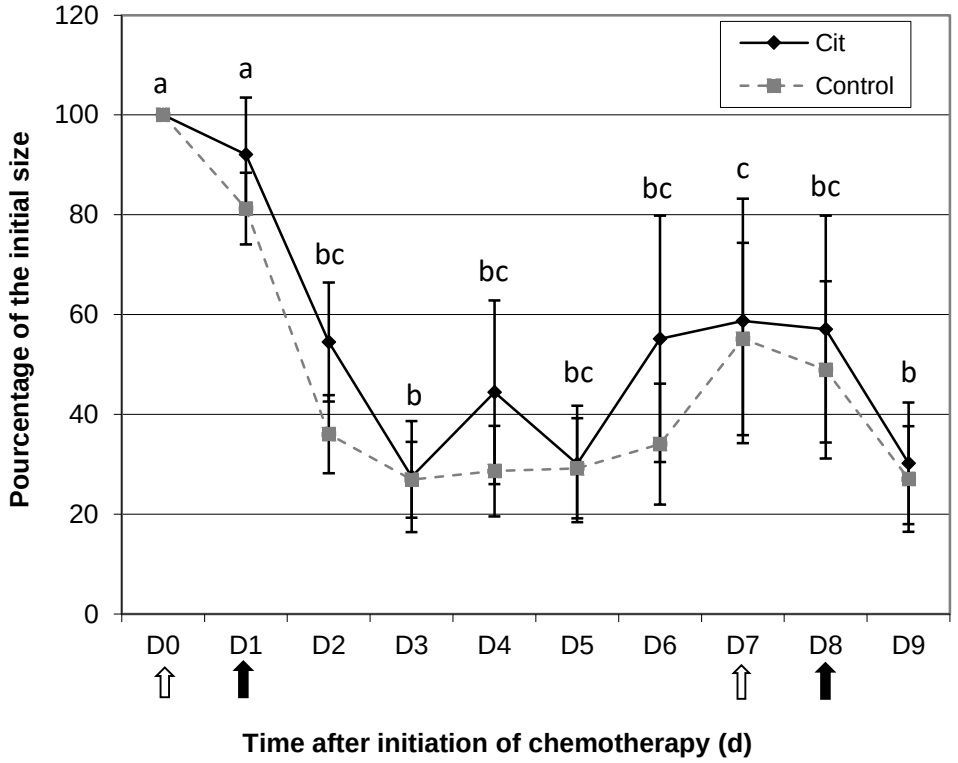
Cit
Control
Pourcentage of the initial size
120
100
80
60
40
20
0
a
a
bc
b
bc
bc
bc
c
bc
b
D0
D1
D2
D3
D4
D5
D6
D7
D8
D9
Time after initiation of chemotherapy (d)

Fig 3A

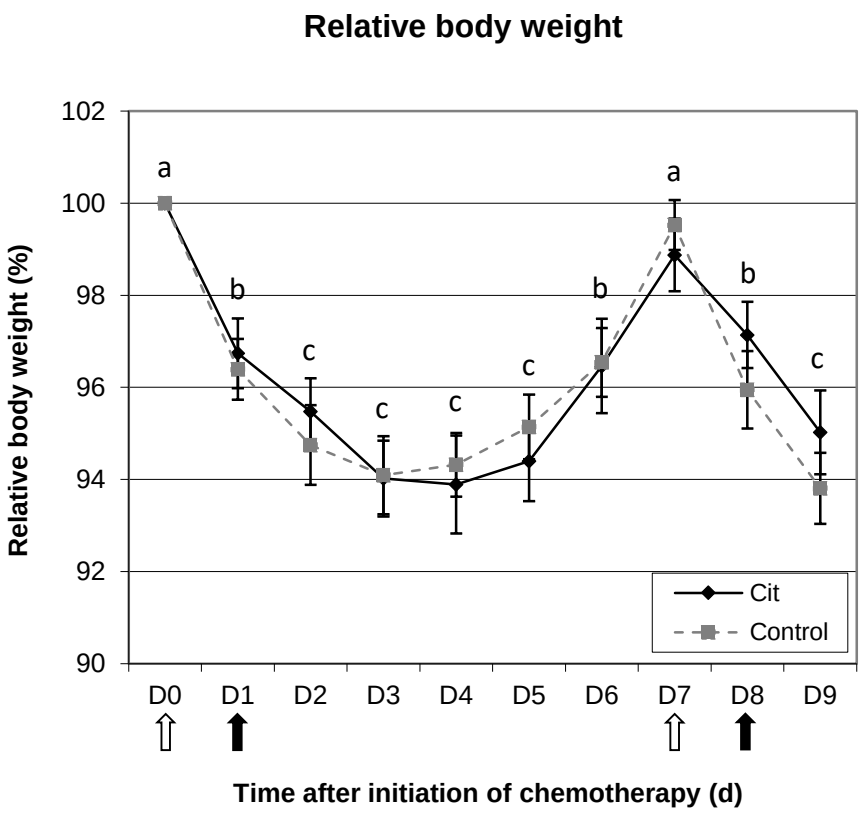
Relative body weight
Relative body weight (%)
102
100
98
96
94
92
90
a
b
c
c
c
c
b
a
b
c
D0
D1
D2
D3
D4
D5
D6
D7
D8
D9
Cit
Control
Time after initiation of chemotherapy (d)

Fig 3B

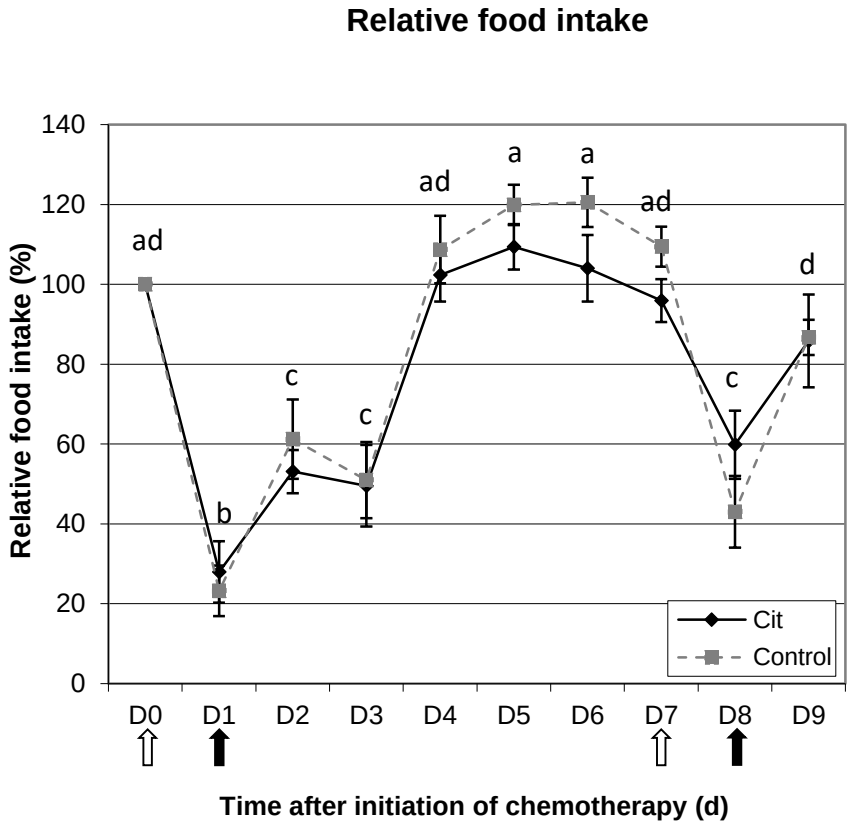
Relative food intake
Relative food intake (%)
140
120
100
80
60
40
20
0
ad
b
c
c
ad
a
a
ad
c
d
D0
D1
D2
D3
D4
D5
D6
D7
D8
D9
Cit
Control
Time after initiation of chemotherapy (d)

Fig 3C

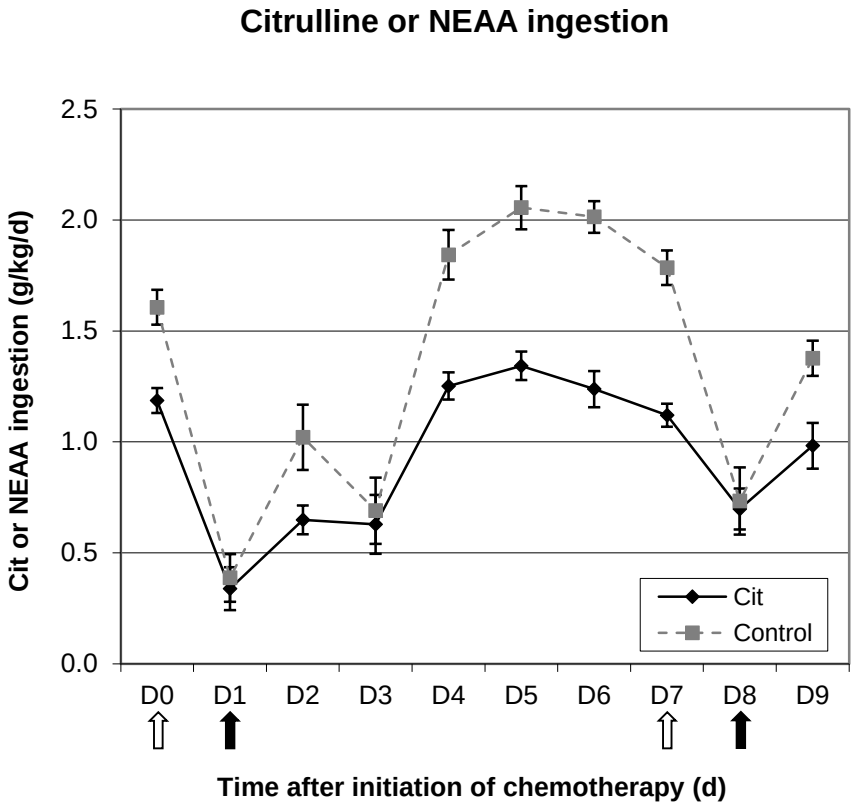
Citrulline or NEAA ingestion
Cit or NEAA ingestion (g/kg/d)
2.5
2.0
1.5
1.0
0.5
0.0
D0
D1
D2
D3
D4
D5
D6
D7
D8
D9
Cit
Control
Time after initiation of chemotherapy (d)

Fig 3D

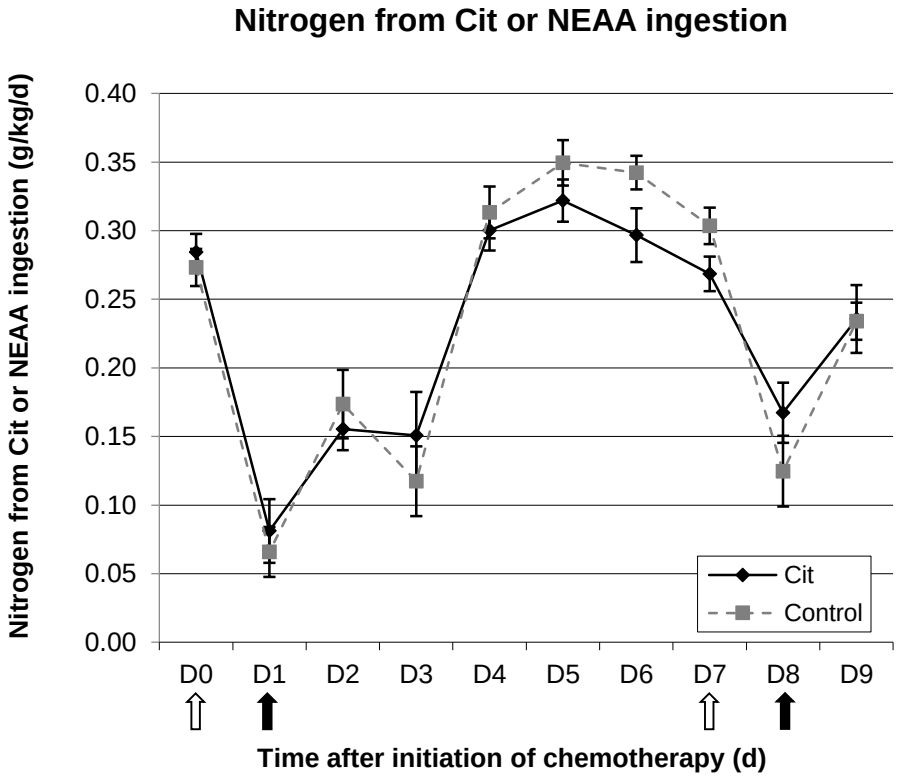
Nitrogen from Cit or NEAA ingestion
Nitrogen from Cit or NEAA ingestion (g/kg/d)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
D0
D1
D2
D3
D4
D5
D6
D7
D8
D9
Cit
Control
Time after initiation of chemotherapy (d)

FIG 4

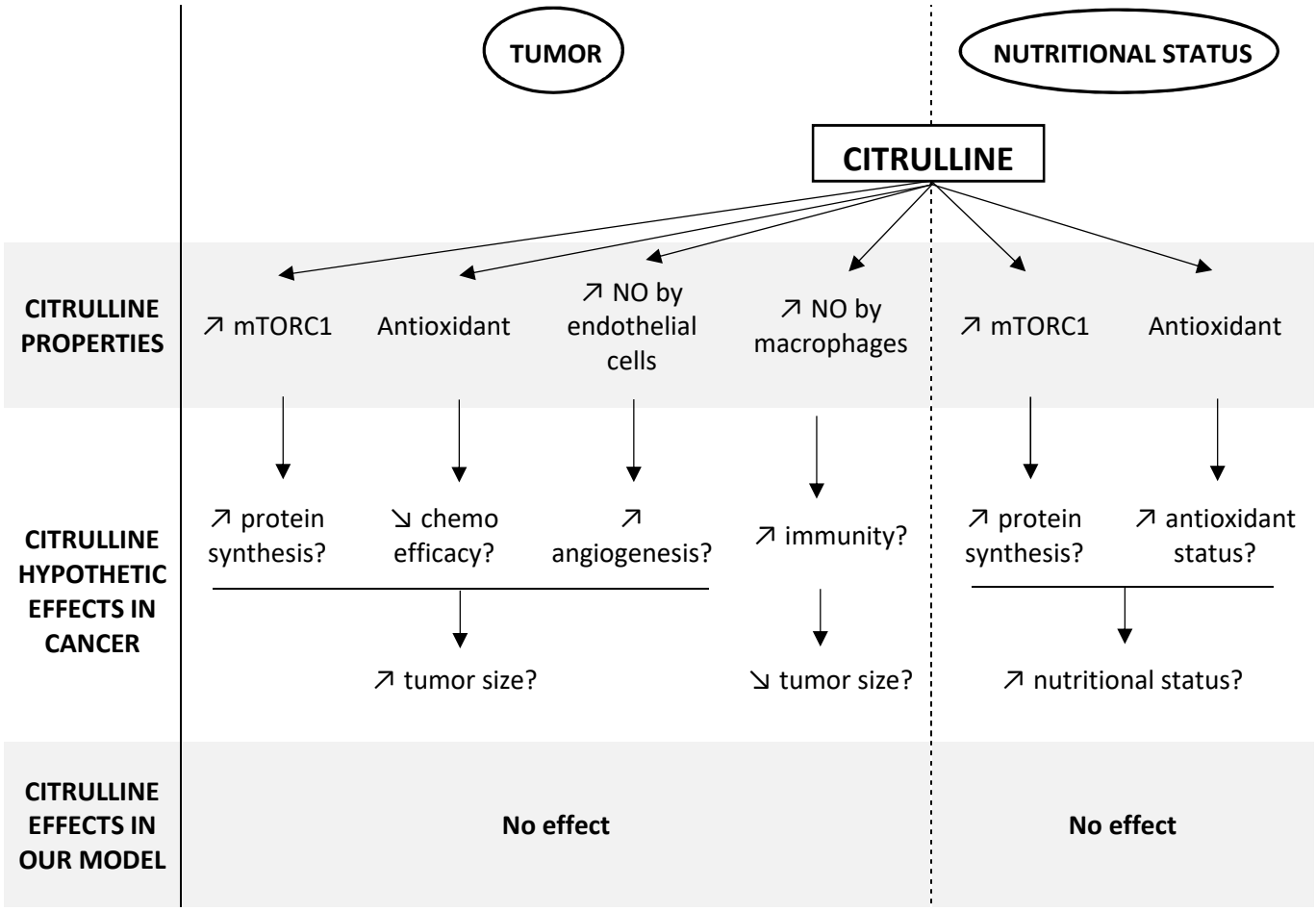
TUMOR
NUTRITIONAL STATUS
CITRULLINE
CITRULLINE PROPERTIES
↗ mTORC1
Antioxidant
↗ NO by endothelial cells
↗ NO by macrophages
↗ mTORC1
Antioxidant
CITRULLINE HYPOTHETIC EFFECTS IN CANCER
↗ protein synthesis?
↘ chemo efficacy?
↗ angiogenesis?
↗ immunity?
↗ protein synthesis?
↗ antioxidant status?
↗ tumor size?
↘ tumor size?
↗ nutritional status?
CITRULLINE EFFECTS IN OUR MODEL
No effect
No effect

Table 1: Diets composition

	Basal diet (g/100g)	**Citrulline diet** (g/100g)	**Control diet** (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

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

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 ± SEM.

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

	Unstandardized Coefficients		Standardized Coefficients			95.0% Confidence Interval for B		Collinearity Statistics	
Model	B	Std. Error	Beta	t	Sig.	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 $R^2 = 0.643$. 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

	Control group	***Citrulline group***	***Citrulline effect***
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			
Gastrocnemius			
Weight			
mg	780 ± 20	798 ± 17	*NS*
g/100g final BW	0.59 ± 0.01	0.61 ± 0.01	*NS*
Tibialis			
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			
Relative 4EBP1 phosphorylated values (AU)	0.84 ± 0.05	1.06 ± 0.16	*NS*
Intestine mucosa			
Jejunum			
Weight			
mg/10cm jej.	377 ± 28	371 ± 29	*NS*
g/100g final BW	0.72 ± 0.05	0.71 ± 0.06	*NS*
Protein content			
mg/10cm	16.4 ± 1.3	17.4 ± 1.6	*NS*
g/100g mucosa	4.4 ± 0.1	4.6 ± 0.2	*NS*
g/100g final BW	0.031 ± 0.002	0.033 ± 0.003	*NS*
Ileum			
Weight			
mg/10cm ileum	285 ± 15	268 ± 14	*NS*
g/100g final BW	0.55 ± 0.04	0.51 ± 0.03	*NS*
Colon			
Weight			
g	0.39 ± 0.04	0.33 ± 0.05	*NS*
g/100g final BW	0.30 ± 0.03	0.25 ± 0.04	*NS*
Protein content			
g/100g mucosa	3.8 ± 0.3	4.1 ± 0.3	*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 (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 ± 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

	Reference group	*Control group*	*Citrulline group*	*CC effect*	*Citrulline effect*
Selected Amino Acids *(µmol/l)*					
Citrulline	129 ± 9	47 ± 5	213 ± 47	*p<0.05*	*p<0.05*
Arginine	175 ± 13	135 ± 5	260 ± 24	*NS*	*p<0.05*
Ornithine	49 ± 7	44 ± 4	67 ± 7	*NS*	*p<0.05*
Glutamine	651 ± 16	757 ± 20	727 ± 36	*NS*	*NS*
Phenylalanine	74 ± 2	73 ± 3	71 ± 3	*NS*	*NS*
Leucine	249 ± 11	206 ± 6	206 ± 10	*p<0.05*	*NS*
Isoleucine	150 ± 5	122 ± 4	120 ± 6	*p<0.05*	*NS*
Valine	350 ± 14	264 ± 7	268 ± 13	*p<0.05*	*NS*
Inflammatory marker					
Haptoglobin *(g/l)*	0.36 ± 0.04	2.10 ± 0.38	1.59 ± 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 ± SEM.
NS: Non significant