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## Maternal supplementation with citrulline or arginine during gestation impacts fetal amino acid availability in a model of intrauterine growth restriction (IUGR)

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1 **Maternal supplementation with citrulline or arginine during gestation impacts fetal**  
2 **amino acid availability in a model of intrauterine growth restriction**

3

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15 **ABSTRACT**

16 *Background.* Supplementing maternal diet with citrulline or arginine during gestation was  
17 shown to enhance fetal growth in a model of IUGR induced by maternal dietary protein  
18 restriction in the rat.

19 *Objective.* The aims of this study were to determine in the same model whether maternal  
20 supplementation with citrulline or arginine would increase 1) citrulline and arginine  
21 concentration in fetal circulation; 2) the expression of placental amino acid transporters, and  
22 3) the fetal availability of essential amino acids.

23 *Methods.* Pregnant rats (n=8) were fed either an isocaloric control (20% protein, NP) or a low  
24 protein (LP, 4% protein) diet, either alone or supplemented with 2g/kg/d of L-citrulline  
25 (LP+CIT) or isonitrogenous Arginine (LP+ARG) in drinking water throughout gestation.  
26 Fetuses were extracted by C-section on the 21<sup>st</sup> day of gestation. The gene expression of  
27 system A (*Slc38a1*, *Slc38a2*, and *Slc38a4*) and L (*Slc7a2*, *Slc7a5*, *Slc7a8*) amino acid  
28 transporters was measured in placenta and amino acid concentrations determined in maternal  
29 and fetal plasma .

30 *Results.* Maternal LP diet decreased fetal (4.01±0.03 vs. 5.45±0.07 g, p<0.0001) and  
31 placental weight (0.617±0.01 vs. 0.392±0.04g, p<0.001), by 26 and 36% respectively,  
32 compared with NP diet . Supplementation with either CIT or ARG increased fetal birth  
33 weight by ≈ 5 or 11%, respectively (4.21±0.05 and 4.48±0.05 g vs. 4.01±0.03 g, p<0.05). CIT  
34 supplementation produced a 5- and 2-fold increase in fetal plasma citrulline and arginine,  
35 whereas ARG supplementation only increased fetal arginine concentration. . LP diet led to  
36 lower placental SNAT 4 mRNA, and higher LAT2 and SNAT1 expression, compared with  
37 NP. SNAT4, 4hFC, LAT2 mRNA were up-regulated in LP+CIT and LP+ARG group  
38 compared with the un-supplemented LP group. Higher level of LAT1 mRNA was also  
39 observed in the LP+CIT group than in the LP group (p<0.01). SNAT2 expression was

40 unchanged in response to CIT or ARG supplementation. Fetal amino acid concentrations were  
41 decreased by LP diet, , and were not restored by CIT or ARG supplementation.

42

43 *Conclusions* The current findings confirm supplementation with citrulline or arginine  
44 enhances fetal growth in a rat model of IUGR. They further suggest that: 1) citrulline and  
45 arginine administered orally to the pregnant mother may reach fetal circulation; 2) citrulline  
46 effectively raises fetal arginine availability; and 3) although it failed to increase the  
47 concentrations of essential amino acids in fetal plasma, citrulline or arginine supplementation  
48 upregulates the gene expression of several placental amino acid transporters,.

49

50

## 51 1. INTRODUCTION

52 Intrauterine growth restriction (IUGR), defined as a birth weight < 10<sup>th</sup> percentile for  
53 gestational age, is a common obstetrical complication [1] which exposes infants not only to  
54 an increased risk of stillbirth [2], neonatal mortality, and morbidity [3], requiring neonatal  
55 intensive care [4], but to a higher risk of developing metabolic or cardiovascular diseases in  
56 adulthood as well [5-7].

57 Whether IUGR is due to maternal undernutrition– or impaired utero-placental perfusion–the  
58 main causes of IUGR in developing and industrialized countries, respectively– insufficient  
59 nutrient availability is the main factor leading to fetal undernutrition [8,9].

60 Fetal amino acid supply is thought to be the driving force for fetal growth, as it determines  
61 protein accretion.

62 In normal pregnancy, the concentration of most amino acids in fetal blood largely exceeds  
63 those in maternal blood, as amino acids cross the placenta by active transport through several  
64 amino acid transporters systems [10]: system A, [11] which transports small, amino acids  
65 such as alanine or glycine[12], and system L which exchanges essential amino acids such as  
66 leucine against non-essential amino acids. [13]

67 In both humans [14] and animals [15,16], IUGR is associated with a decrease in the fetal  
68 plasma concentration of essential amino acids, (e.g., branched-chain amino acids). and  
69 alterations in placental nutrient transport and metabolism [17]. In human IUGR, a down  
70 regulation of placental system A and system L has been described both *in vitro* and *in vivo*  
71 [18-22]. Down regulation of placental amino acid transporters was observed in models of  
72 IUGR in rats [23, 24] and baboons [25, 26]. Such alterations occur before the onset of fetal  
73 growth failure, which suggests that impaired placental amino acid transport may be a cause  
74 rather than a consequence of growth restriction [23, 27].

75

76 Citrulline is a non-essential amino acid that is not incorporated in protein and is produced  
77 endogenously in the small intestine; it escapes hepatic uptake, and is taken up by kidney  
78 where it is quantitatively converted to Arginine [29, 30]. Arginine, a conditionally essential  
79 amino acid for fetuses and growing mammals [31], is the sole endogenous source of nitric  
80 oxide (NO) which is involved in the regulation of placental growth and blood flow [32].  
81 Arginine supplementation was therefore tested as a safe approach [33] to treat IUGR in  
82 humans [34] and in animal IUGR models induced by underfeeding [35], multiple  
83 pregnancy [36], or inhibitors of NO synthesis [37]. In earlier studies, we showed that  
84 maternal supplementation with citrulline or arginine during gestation was able to stimulate  
85 fetal growth and muscle protein synthesis in a rat model of IUGR induced by dietary protein  
86 restriction [38], and the expression of genes involved in placental growth such as insulin-  
87 like growth factor 2 (Igf2), and angiogenesis, such as Vegf and Flt-1 [39]. Yet several  
88 questions remained open. Do citrulline and arginine reach fetal bloodstream, as shown in an  
89 ovine model [30]? Does the effect of citrulline improve placental amino acid transport? Does  
90 it increase the bioavailability of essential amino acids to the fetus??  
91 The aims of this study therefore were to determine whether maternal supplementation with  
92 CIT and ARG 1) increased the availability of CIT or ARG in fetal circulation; 2) enhanced  
93 placental amino acid transport, and 3) impacted the fetal availability of essential amino  
94 acids in a model of IUGR induced by protein restriction.

95

96

## 97 2. METHODS

### 98 *2.1 Animals and experimental design*

99 The study was carried out in accordance with current institutional guidelines in France and the  
100 EU Directive 2010/63/EU for animal experiments, and after approval from the animal ethics  
101 committee of Pays de La Loire [N°CEEA.2010.8].

102 Eight-week old, primiparous timed-pregnant Sprague-Dawley rats [n=7 to 8 per group],  
103 were purchased from Janvier [Le Genest Saint Isle, France], delivered to the animal facilities  
104 at gestation day 2 [GD2], and housed in individual cages in a room with constant air  
105 humidity, temperature ( $22\pm 2^{\circ}\text{C}$ ), and a 12 h light/dark cycle with *ad libitum* access to chow  
106 [Arie Block, Woerden, The Netherlands] and water.

107 Upon arrival, pregnant rats were randomly assigned to one of four regimens (1) a control,  
108 semi-purified diet with an adequate protein content [NP, 20% casein]; (2) an isocaloric diet  
109 with a low protein content [LP; 4% casein] to induce IUGR; (3) a low protein diet, along  
110 with a 2 g/kg/d L-citrulline [0.48 g nitrogen/kg/d; obtained from Inresa, Bartenheim, France]  
111 supplementation in drinking water (LP+CIT diet) and (4) a LP diet, along with an  
112 isonitrogenous amount of L-arginine in drinking water (LP+ARG).

113 Composition of diets is shown in Supplemental **Table 1**. Amino acid solutions were  
114 prepared twice a week, stored at 4°C and added to drinking water. Arginine solution pH was  
115 adjusted to 7.0 with NaOH.

116 Maternal body weight, food and water consumption were recorded 3 times per week. On  
117 GD21, pregnant rats underwent C-section under general anesthesia as described [39] The  
118 fetuses and placentas were rapidly extracted and dried, and litter size, placental weight and  
119 pup birth weight were recorded. Blood samples were drawn simultaneously from mother by  
120 cardiac puncture and from fetuses by decapitation, collected into heparinized tubes and pooled  
121 per litter. Placentas were washed in 0.9% NaCl, cut in four parts and quickly frozen in liquid

122 nitrogen. Blood samples were centrifuged at 4000g for 10 min at 4°C. Placental and plasma  
123 samples were stored at -80 °C until used for analysis. At the end of the procedure, females  
124 rats were euthanized by an overdose of isoflurane.

## 125 ***2.2 Amino acid analysis***

126 Amino acid analysis was performed by ultra-high performance liquid chromatography-tandem  
127 mass spectrometry (UPLC-MSMS) as described for milk [40] and adapted for small volumes  
128 of plasma. Briefly, 20 µL of maternal or fetal plasma were mixed with 80µL of ultrapure  
129 water obtained from a Milli-Q® purifier [Millipore, Eschborn, Germany] and 50µL of labeled  
130 internal standard pool. Isotope-labeled amino acid internal standards were obtained from  
131 Cambridge Isotope Laboratories Inc. (Andover, USA), Tracer Technologies Inc. (Waterloo,  
132 Canada), or Eurisotop (Saint-Aubin, France). Samples were deproteinized with 10%  
133 sulfosalicylic acid, and centrifuged at 10,000 g for 15 min at 4°C. Free amino acids contained  
134 in the supernatant phase were collected and 10 µL were derivatized by adding 70 µL of  
135 AccQ•Tag™ Ultra Borate Buffer and 10 µL of supernatant and 20 µL of AccQ•Tag™ Ultra  
136 reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) [Waters Corporation, Milford,  
137 MA, USA] were incubated for 10 min at 55°C with. 1µL of sample was injected in duplicate  
138 into an Acquity H-Class® UPLC system (Waters Corporation, Milford, USA) equipped with  
139 a quaternary solvent manager, an autosampler maintained at 4°C, a Waters AccQ•Tag™  
140 Ultra® column (2.1 mm × 10 mm, 1.7 µm particles) with a pre-filter heated at 55°C, and  
141 coupled with a tandem quadrupole detector.

## 142 ***2.3 RNA isolation and RT-qPCR.***

143 To assess amino acid transporter relative mRNA abundance, total RNA was isolated from  
144 four placentas in 6 dams per group. Placental tissues were homogenized and RNA was  
145 extracted in 1 mL trizol reagent solution. RNA samples were treated with DNase I (Promega,  
146 Madison, WI, USA). Following extraction, total RNA was quantified and purity were



147 determined by UV spectrophotometry at 260 and 280 nm with NanoVue Spectrophotometer.  
148 The integrity of total RNA was confirmed by agarose gel electrophoresis. Quantitative real-  
149 time PCR analyses were performed with a 96-well plate carried out using SYBR Green  
150 detection on a CFX Connect™ Real Time PCR Detection System [Biorad, Hercules, CA,  
151 USA] First-strand cDNA was synthesized from 1 µg of total RNA with random hexamer  
152 primers and *M-MLV* reverse transcriptase [Invitrogen, Life Technologies, Carlsbad, USA].  
153 Primers were designed using Perlprimer software (Sourceforge, <https://sourceforge.net/>).  
154 Sequences are shown in Supplemental Table 2.

155 In preliminary experiments, analysis using Bestkeeper, geNorm and NormFinder algorithm  
156 showed *Ywhaz* and GAPDH to be the most constant housekeeping genes under our  
157 experimental conditions, and to be expressed in placenta at levels of the same magnitude as  
158 our genes of interest. Data were normalized against the mean of *Ywhaz* and GAPDH  
159 expression in NP pups. Fold changes were calculated using the formula  $2^{-\Delta Ct}$ . The mean of  
160 normalized expression from 4 placentas of 6 dams per group is reported.

#### 161 ***2.4 Biochemical parameters.***

162 Glucose and lipids were analyzed in an automate analyzer.

#### 163 ***2.5 Statistical analysis.***

164 Values are means  $\pm$  SEM. After normal distribution and equal variances were confirmed,  
165 differences between the four experimental groups were analyzed by one-way ANOVA using  
166 Prism 6.0® [GraphPad Software, San Diego, CA]. Fisher protected least significant difference  
167 (PLSD) method was applied for *post hoc* intergroup comparisons. In case of non-gaussian  
168 distribution, a non parametric, Kruskal-Wallis test was performed to determine significance  
169 and followed by Mann - Whitney U test. Alpha level for statistical significance was  $p \leq 0.05$ .

170

### 171 3. RESULTS

#### 172 *3.1 Gestation performance and food intake, fetal and placental growth*

173 (Supplemental Table 3). On GD21, maternal body weight gain was lower in the LP group  
174 than in the NP group ( $163 \pm 10$  vs  $61 \pm 7$  g,  $P < 0.05$ ). Neither CIT nor ARG supplementation  
175 enhanced maternal gestational weight gain.

176 Average daily food intake was higher in the NP pregnant rats ( $\text{g} \times \text{d}^{-1}$ ) than in the LP+CIT  
177 ( $22.9 \pm 0.8$  vs.  $18.7 \pm 0.8$   $\text{g} \times \text{d}^{-1}$ ;  $P < 0.05$ ) but did not significantly differ between the 3  
178 groups fed a low protein diet (Supplemental Table 3).

179 Fetal and placental weights are detailed in Table 1. Feeding dams a low protein diet was  
180 associated with a 26% and 35% reduction in fetal weight ( $4.01 \pm 0.03$  vs.  $5.45 \pm 0.07$  g,  
181  $p < 0.0001$ ), and placental weight, respectively, compared with the NP group. Though  
182 supplementation with amino acids did not restore fetal weight to those observed in NP  
183 group, CIT and ARG supplementation, increased fetal birth weight by  $\approx 5$  and 11%,  
184 respectively, compared to LP group ( $4.22 \pm 0.05$  and  $4.48 \pm 0.05$  g vs  $4.01 \pm 0.03$  g,  $p < 0.05$ ).  
185 Neither CIT nor ARG supplementation enhanced placental weight.

186 The fetal/placental weight ratio was significantly ( $P < 0.01$ ) increased in the 3 groups fed low  
187 protein diet, whether or not they were supplemented with amino acids.

188 Mean number of fetus was lower in dams fed the 20% protein diet than in dams fed the 4%  
189 protein diet. There was no effect of amino acid supplementation on litter size.

#### 190 *3.2 Effects of arginine and citrulline supplementation on fetal citrulline and arginine*

191 Plasma citrulline concentrations was  $\approx 5$ -fold higher in the LP+CIT mothers and fetuses,  
192 compared with other groups ( $p < 0.05$ ). Arginine concentrations rose 2.4 fold in maternal  
193 plasma and 2 fold in fetal plasma in response to CIT supplementation. Supplementation with  
194 citrulline was as effective as arginine supplementation in raising arginine concentrations in  
195 both maternal ( $p=0.45$ ) and fetal plasma ( $p=0.22$ , LP+CIT vs. LP+ARG).

196 ***3.3 Effect of citrulline and arginine on mRNA expression of amino acid transporters in***  
197 ***placenta (Fig 1)***

198 The expression of SNAT 2, 4hFC, LAT1 mRNA relative to GAPDH and Ywhaz mRNA for  
199 each sample was unaltered in LP group compared with the NP group.

200 Maternal low protein diet led to a decrease in the SNAT 4 placental mRNA. In contrast,  
201 LAT2 and SNAT1 mRNA were higher in LP than in NP placentas.

202 SNAT4, 4hFC, LAT2 mRNA was significantly up-regulated in LP+CIT and LP+ARG group  
203 compared with the unsupplemented LP group and. Higher level of LAT1 mRNA was also  
204 observed in the LP+CIT group than in the LP group ( $p < 0.01$ ).

205 SNAT2 expression was unchanged in response to CIT or ARG supplementation.

206 ***3.4 Effect of citrulline and arginine on maternal and fetal plasma amino acids***  
207 ***concentrations (Table 2 and 3, Fig 2).***

208 Concentrations of alanine and lysine in maternal plasma were not affected by maternal protein  
209 restriction. Concentrations of taurine, serine, glycine, phenylalanine, glutamate and glutamine  
210 were higher in LP than in NP mothers. All other maternal plasma amino acid concentrations  
211 including arginine, citrulline and branched amino acids (BCAA, leucine, isoleucine, valine)  
212 were decreased in the LP mothers compared with the NP group.

213 In fetal plasma, the concentration of most amino acids, including the majority of essential  
214 amino acids—except for lysine, phenylalanine, and tryptophan—was significantly lower in the  
215 LP than the NP group. Serine, glycine, glutamine, and phenylalanine were higher in LP  
216 fetuses. Tryptophan, arginine, citrulline, glutamate and alanine, were similar in the plasma  
217 from LP and NP fetuses.

218 In maternal plasma, ARG or CIT supplementation was associated with lower concentrations  
219 of glutamine, glycine and glutamic acid compared with the unsupplemented LP group.

220 Neither CIT nor ARG supplementation altered the concentration of most other amino acids

221 including BCAA or EAA, except for tryptophan which was higher, and lysine that was lower  
222 in LP+CIT mothers compared with the LP group (Table 2).

223 In fetal plasma, concentrations of glutamine, glycine and taurine were decreased in the  
224 LP+CIT group compared with the LP group [as observed in maternal plasma].(Table 3)

### 225 ***3.5 Biochemical parameters in maternal and fetal plasma (Supplemental Table 4)***

226 Maternal plasma glucose was similar in the 4 groups. Regardless of diet, glucose  
227 concentrations were lower in fetal than in maternal plasma. LP diet was associated with a  
228 lower plasma glucose, compared with the NP group, and was not affected by either CIT or  
229 ARG supplementation.

230

231

## 232 **DISCUSSION**

233 The findings of the current study confirm supplementation with either citrulline or arginine  
234 enhances fetal growth in an animal model of intrauterine growth restriction. They further  
235 demonstrate that both, citrulline and arginine administered orally to the pregnant mother  
236 effectively raise fetal arginine availability. Finally, we provide evidence for an upregulation  
237 of placental amino acid transporters mRNA by citrulline and arginine.

### 238 *4.1 Effect of citrulline and arginine on fetal growth.*

239 As expected, maternal undernutrition impaired fetal growth, with a relative preservation of  
240 brain growth (Table 1), as observed in human IUGR [41]. The LP diet was associated with a  
241 lower concentration of glucose and of most essential amino acids in fetal plasma; as reported  
242 in human IUGR [14]. Consistent with our earlier studies [38,39], maternal supplementation  
243 with arginine or citrulline enhanced fetal growth. In contrast to our initial study, arginine was  
244 more effective than citrulline in the current study. Differences in study design may account  
245 for the discrepancy since: 1) supplementation was initiated from the 2<sup>nd</sup>, rather than the 8<sup>th</sup>  
246 day of gestation in our earlier study; and 2) food intake was 15% higher in LP+ARG group  
247 than in LP+CIT group, although the difference did not reach statistical significance.  
248 Alternatively, arginine may exert its effect in a specific time window in the earlier part of  
249 gestation; accordingly, in a recent clinical trial arginine prevented pre-eclampsia only when  
250 administered in the first trimester of pregnancy [42]. Finally, fetal plasma glucose,  
251 (Supplemental Table 4) tended to be higher in the arginine- than the citrulline-supplemented  
252 group, and a higher fetal plasma glucose may increase insulin secretion by fetal pancreas.  
253 Elevation of fetal insulin has long been known to drive fetal growth, as evidenced by the  
254 occurrence of macrosomia in infants from diabetic mothers [43]. We therefore speculate that  
255 arginine supplementation may enhance growth through enhanced fetal insulin secretion. As  
256 we did not measure fetal plasma insulin, such hypothesis remains to be tested. .

257 The fetal to placenta weight ratio, was higher in groups fed an LP diet, and further increased  
258 by arginine and citrulline supplementation. Interpreting fetal/placental weight ratio, however,  
259 is complex. Fetal/placental weight ratio correlates with amino acid transport system A  
260 activity measured on placental vesicles from infants with adequate weight for gestational age  
261 [44]. This suggests such ratio may reflect placental transport efficiency. The increased  
262 birth/placental weight ratio commonly observed in human IUGR and in animal models of  
263 IUGR is thought to reflect an adaptative response of placental function to IUGR [45-48]. Yet  
264 impaired placental amino acid transport has been documented in animal and human IUGR  
265 [17-23, 25-27]. In the current report, both fetal /placental weight ratio and the gene  
266 expression of SNAT4, 4hFC, LAT2 mRNA were enhanced by CIT or ARG, but SNAT2 was  
267 not, and actual amino acid fluxes from mother to fetus were not measured. It therefore  
268 remains unclear whether the higher fetal/placental weight ratio reflects enhanced placental  
269 function.

270

#### 271 *4.2 Effect of citrulline and arginine on fetal plasma arginine and citrulline*

272 Though our earlier studies documented an anabolic effect of citrulline on fetal growth, the  
273 specific site(s) of action of citrulline remained elusive. Citrulline may impact maternal  
274 metabolism, placenta, or fetal metabolism itself. The current report demonstrates that  
275 citrulline administered orally to the pregnant mother 1) raises the concentration of citrulline in  
276 fetal circulation, and 2) is as effective as arginine itself to increase arginine bioavailability in  
277 the fetus. This is consistent with data comparing intravenous citrulline vs. arginine infusion in  
278 pregnant ewes [30]. Citrulline may thus exert its effect—either *per se* or through its  
279 conversion to arginine—directly on fetal tissues. One potential advantage of citrulline over  
280 arginine is the fact that only arginine supplementation was associated with a rise in maternal  
281 plasma urea concentration (Table 7). Such rise is consistent with the fact that arginine may be

282 substantially extracted in maternal liver and degraded to urea by hepatic arginase. Extensive  
283 arginine catabolism was one of the hypotheses raised to explain the lack of efficacy of oral  
284 arginine supplementation in clinical trials [49]. In contrast, citrulline is known to escape  
285 hepatic uptake, and may be a better candidate for clinical trials in human IUGR.

286

#### 287 *4.3 Effect of citrulline and arginine on placental amino acid transport*

288 In the current study, we observed a positive effect of citrulline and arginine on the expression  
289 of placental amino acid transporter LAT1 and LAT2 in charge of large neutral amino acids  
290 such as BCAA, as well as SNAT4. As a change in mRNA expression does not necessarily  
291 translate into a parallel change in protein expression or transport activity, these observations  
292 would obviously warrant confirmation using immunocytochemistry and Western blotting.

293 Previous work showed that cord blood amino acid concentrations are significantly reduced in  
294 human IUGR, compared to normal pregnancy [50]. Literature suggests impaired amino acid  
295 transport is a common feature of fetal growth restriction, regardless of its cause: down-  
296 regulation of placental A and L amino acid transporters has been reported both in human  
297 IUGR due to altered placental blood flow, as well as in animals models of IUGR induced by  
298 dietary protein restriction. *In vivo* and *in vitro* studies have shown lower transport of labelled  
299 amino acids or a reduced activity or expression of placental amino acid transporters in  
300 syncytiotrophoblast membrane from IUGR pregnancies [20, 22, 23, 51-57]. The fact that such  
301 alterations occurred before the onset of growth deceleration suggests that alterations in amino  
302 acid transport play a causative role in the growth failure associated with maternal  
303 underfeeding. Accordingly, treatment of rats fed a normal diet with an inhibitor of the A  
304 transport system was sufficient to produce growth restriction in rats [58]. In that context, the  
305 enhanced expression of placental amino acid transporters, supplementation may contribute to

306 the anabolic effect of citrulline and arginine on fetal growth through improved amino acid  
307 transport to the fetus.

#### 308 *4.4 Effect of citrulline and arginine on fetal plasma amino acids, and fetal energy substrates.*

309 In the current study, the enhanced expression of placental amino acid transporters observed  
310 with CIT or ARG supplementation did not translate into higher concentrations of essential  
311 amino acids in fetal plasma. It should be borne in mind, however, that the concentration of an  
312 essential amino acid in fetal plasma, only reflects the balance between the appearance of such  
313 amino acid from placental transfer, or from fetal protein breakdown on one hand, and its  
314 utilization for fetal protein synthesis and oxidation, on the other hand. We therefore speculate  
315 that fetal amino acid concentrations remained unaltered because increased utilization of  
316 essential amino acids for protein synthesis may offset the increased placental transfer of  
317 essential amino acids. In an earlier study [38], we indeed showed that citrulline had a  
318 powerful effect on protein synthesis rate in fetal skeletal muscle. The enhanced growth fetal  
319 observed in the current study supports the hypothesis. Such effect is unlikely to be due to  
320 nonspecific nitrogen supply, since a mixture of non-essential amino acids failed to stimulate  
321 protein synthesis in our earlier study [38].

322

323 In summary, the current study demonstrates that, in an animal model of IUGR, arginine and  
324 citrulline supplementation efficiently raise fetal plasma citrulline and arginine concentrations  
325 in fetal plasma, and enhance the expression of placental amino acid transporters. Such effect  
326 likely contributes to the effect of citrulline and arginine on fetal growth and anabolism, and  
327 suggests that, as recently proposed [59], trials of citrulline or arginine supplementation may  
328 be warranted in human IUGR.

329



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### **Statement of authors' contributions to manuscript.**

330 Aurélie Bourdon: conceptualization, performance of experiments, data analysis, manuscript  
331 writing

332 Jacob Hannigsberg: conceptualization, performance of experiments, data analysis, manuscript  
333 writing

334 Emilie Misbert : conceptualization, performance of experiments, data analysis, manuscript  
335 writing

336 Thang Nhat Tran conceptualization, performance of experiments, data analysis, manuscript  
337 writing

338 Valérie Amarger : conceptualization, data analysis, manuscript writing

339 Véronique Ferchaud-Roucher : data analysis, manuscript writing

340 Norbert Winer : funding acquisition, conceptualization, performance of experiments, data  
341 analysis, manuscript writing

342 Dominique Darmaun : funding acquisition, conceptualization, performance of experiments,  
343 data analysis, manuscript writing

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525 **TABLE 1.** Litter size, fetal and placental weights on day 21 of pregnancy of dams fed either  
 526 a control (NP), or a protein-restricted (LP) diet as such or supplemented with L-ARG  
 527 (LP+ARG) or L-CIT (LP+CIT) in drinking water throughout gestation

	<i>Group</i>				<i>P</i>
	NP	LP	LP+CIT	LP+ARG	
mean litter size	10±0.9 <sup>a</sup>	13.8±0.7 <sup>b</sup>	11.5±0.9 <sup>b</sup>	13.1±0.9 <sup>b</sup>	0.03
fetal weight, g	5.48±0.07 <sup>a</sup>	4.01±0.03 <sup>b</sup>	4.22±0.05 <sup>c</sup>	4.48±0.04 <sup>d</sup>	<0.001
placental					
weight, g	0.617±0.01 <sup>a</sup>	0.392±0.04 <sup>b</sup>	0.391±0.008 <sup>b</sup>	0.413±0.01 <sup>b</sup>	<0.001
Placenta					
efficiency <sup>2</sup>	9.1±0.2 <sup>a</sup>	10.3±0.1 <sup>b</sup>	10.9±0.2 <sup>b</sup>	11.1±0.2 <sup>c</sup>	<0.002
Brain/body					
weight ratio	0.038 ± 0.002	0.047 ± 0.002 <sup>c</sup>	0.044 ± 0.003 <sup>c</sup>	0.046 ± 0.003 <sup>c</sup>	<0.05

528 <sup>1</sup>Data are expressed as means ± SEM, n=7-8 dams/group. Means in a row with letter  
 529 superscripts without a common letter differ, *P*<0.05.

530 <sup>2</sup>Placenta efficiency was estimated by the fetal/placental weight ratio

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534 **TABLE 2.** Amino acid concentrations in maternal plasma at GD21 in pregnant dams fed a  
 535 control diet (NP), a low protein diet alone(LP), or a low protein diet plus citrulline (LP+CIT)  
 536 or plus Arginine (LP+ARG).

Amino acid	Group				<i>P</i>
	NP	LP	LP+CIT	LP+ARG	
	<i>μmol/L</i>				
Histidine	58±3 <sup>a</sup>	26±2 <sup>b</sup>	29±4 <sup>b</sup>	25±2 <sup>b</sup>	<0.001
Taurine	91±18 <sup>b</sup>	259±29 <sup>a</sup>	102±23 <sup>a</sup>	150±26 <sup>a</sup>	<0.001
Serine	278±16 <sup>b</sup>	511±29 <sup>a</sup>	457±49 <sup>a</sup>	428±23 <sup>a</sup>	<0.001
Glutamine	728±37 <sup>b,c</sup>	1091±66 <sup>a</sup>	645±47 <sup>c</sup>	833±64 <sup>b</sup>	<0.001
Arginine	125±8 <sup>a</sup>	96±4 <sup>b</sup>	232±43 <sup>c</sup>	240±44 <sup>c</sup>	<0.001 <sup>1</sup>
Glycine	38±3 <sup>c</sup>	224±33 <sup>a</sup>	145±8 <sup>b</sup>	145±14 <sup>b</sup>	<0.001
Glutamic acid	25±3 <sup>b</sup>	52±4 <sup>a</sup>	30±5 <sup>b</sup>	36±3 <sup>b</sup>	<0.001
Citrulline	57±3 <sup>a</sup>	28±3 <sup>b</sup>	282±16 <sup>c</sup>	35±4 <sup>b</sup>	<0.001 <sup>1</sup>
Threonine	467±20 <sup>a</sup>	178±12 <sup>b</sup>	172±15 <sup>b</sup>	157±15 <sup>b</sup>	<0.001
Alanine	972±121	913±144	680±102	803±113	0.41
Proline	924±68 <sup>a</sup>	385±25 <sup>b</sup>	322±21 <sup>b</sup>	354±25 <sup>b</sup>	<0.001 <sup>1</sup>
Lysine	1142±77 <sup>a</sup>	1148±59 <sup>a</sup>	772±60 <sup>b</sup>	1003±85 <sup>a</sup>	<0.01
Cysteine	39±3 <sup>a</sup>	27±3 <sup>b</sup>	23±2 <sup>b</sup>	21±2 <sup>b</sup>	<0.001
Methionine	102±5 <sup>a</sup>	64±2 <sup>b</sup>	62±1 <sup>b</sup>	64±2 <sup>b</sup>	<0.001 <sup>1</sup>
Valine	230±11 <sup>a</sup>	103±8 <sup>b</sup>	111±16 <sup>b</sup>	93±7 <sup>b</sup>	<0.001
Isoleucine	64±4 <sup>a</sup>	35±6 <sup>b</sup>	36±9 <sup>b</sup>	28±4 <sup>b</sup>	0.001
Leucine	134±8 <sup>a</sup>	67±9 <sup>b</sup>	66±15 <sup>b</sup>	54±6 <sup>b</sup>	<0.001
Phenylalanine	66±2 <sup>b</sup>	73±2 <sup>a</sup>	65±2 <sup>a</sup>	63±1 <sup>a</sup>	0.01
Tryptophane	79±6 <sup>a</sup>	29±2 <sup>b</sup>	39±4 <sup>c</sup>	28±2 <sup>b</sup>	<0.001 <sup>1</sup>

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538 Data are means ± SEM of 6-8 dams, means in a row with superscripts without a common  
 539 letter differ, *P*<0.05 ; the letter ‘a’ denotes the highest value within the row.

540 <sup>1</sup>Difference assessed using Kruskal-Wallis test.

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545 **TABLE 3.** Plasma amino acid concentrations at GD21 in fetuses born to dams fed 20%  
 546 protein diet (NP) , low protein diet alone (LP), or supplemented with citrulline (LP+CIT) or  
 547 Arginine (LP+ARG) throughout gestation.

	<i>Group</i>				<i>P</i>
	NP	LP	LP+CIT	LP+ARG	
	<i>μmol/L</i>				
Histidine	153±8 <sup>a</sup>	30±2 <sup>b</sup>	36±7 <sup>b</sup>	29±3 <sup>b</sup>	<0.001 <sup>1</sup>
Taurine	527±15 <sup>a</sup>	529±35 <sup>a</sup>	382±13 <sup>b</sup>	435±19 <sup>b</sup>	<0.001 <sup>1</sup>
Serine	463±12 <sup>b</sup>	701±55 <sup>a</sup>	619±28 <sup>a</sup>	619±19 <sup>a</sup>	<0.001 <sup>1</sup>
Glutamine	1383±56 <sup>b</sup>	1917±124 <sup>a</sup>	1274±46 <sup>b</sup>	1419±57 <sup>b</sup>	<0.001 <sup>1</sup>
Arginine	180±12 <sup>a,b</sup>	152±16 <sup>a</sup>	308±34 <sup>c</sup>	239±28 <sup>b</sup>	0.007 <sup>1</sup>
Glycine	321±13 <sup>c</sup>	583±29 <sup>a</sup>	488±18 <sup>b</sup>	509±33 <sup>a,b</sup>	<0.001
Glutamic acid	173±11	164±8	143±8	161±11	0.30
Citrulline	43±2 <sup>b</sup>	42±4 <sup>b</sup>	250±119 <sup>a</sup>	45±3 <sup>b</sup>	<0.001 <sup>1</sup>
Threonine	644±33 <sup>a</sup>	260±23 <sup>b</sup>	242±29 <sup>b</sup>	192±15 <sup>b</sup>	<0.001
Alanine	880±86	888±92	652±78	931±85	0.26
Proline	1396±121 <sup>a</sup>	642±50 <sup>b</sup>	580±50 <sup>b</sup>	524±37 <sup>b</sup>	<0.001 <sup>1</sup>
Lysine	1657±74	1891±112	1183±69	1150±85	<0.001
Cysteine	46±3 <sup>a</sup>	22±2 <sup>b</sup>	20±1 <sup>b</sup>	19±1 <sup>b</sup>	<0.001
Tyrosine	200±17	178±22	183±24	186±11	0.89
Methionine	243±18 <sup>a</sup>	138±6 <sup>b</sup>	138±7 <sup>b</sup>	137±6 <sup>b</sup>	<0.001 <sup>1</sup>
Valine	627±20 <sup>a</sup>	304±19 <sup>b</sup>	319±26 <sup>b</sup>	259±18 <sup>b</sup>	<0.001
Isoleucine	178±5 <sup>a</sup>	116±12 <sup>b</sup>	120±14 <sup>b</sup>	95±11 <sup>b</sup>	<0.001
Leucine	424±14 <sup>a</sup>	250±20 <sup>b</sup>	241±19 <sup>b</sup>	204±13 <sup>b</sup>	<0.001
Phenylalanine	243±9 <sup>b</sup>	281±14 <sup>a</sup>	268±1 <sup>a,b</sup>	230±9 <sup>b,c</sup>	0.01
Tryptophane	108±3 <sup>a</sup>	107±8 <sup>a,b</sup>	131±10 <sup>b</sup>	100±7 <sup>a</sup>	0.04

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549 <sup>1</sup>Differenced analyzed using Kruskal-Wallis test

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## 554 FIGURE LEGENDS

555 **Fig 1.** Quantification by RT-PCR of the gene expression of amino acid transporters SNAT1,  
556 SNAT2, SNAT4, LAT1, LAT2 and 4hFC mRNA in placentas collected at GD21 of rats fed  
557 20% protein diet (NP), 4% low protein diet (LP), low protein diet plus citrulline (LP+CIT) or  
558 low protein diet plus arginine (LP+ARG). GAPDH and Ywhaz served as the internal control.  
559 Results are expressed as a percentage of the C group (n = 6 pools of 4 placentas /group).

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561 **Fig 2.** Concentrations of total non-essential amino acids (NEAA; panel A), total essential  
562 amino acids (EAA), and branched-chain amino acids determined by LC-MSMS in maternal  
563 and fetal plasma in the various groups. Arginine and Citrulline concentrations were not used  
564 in the calculation.

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Fig 1.

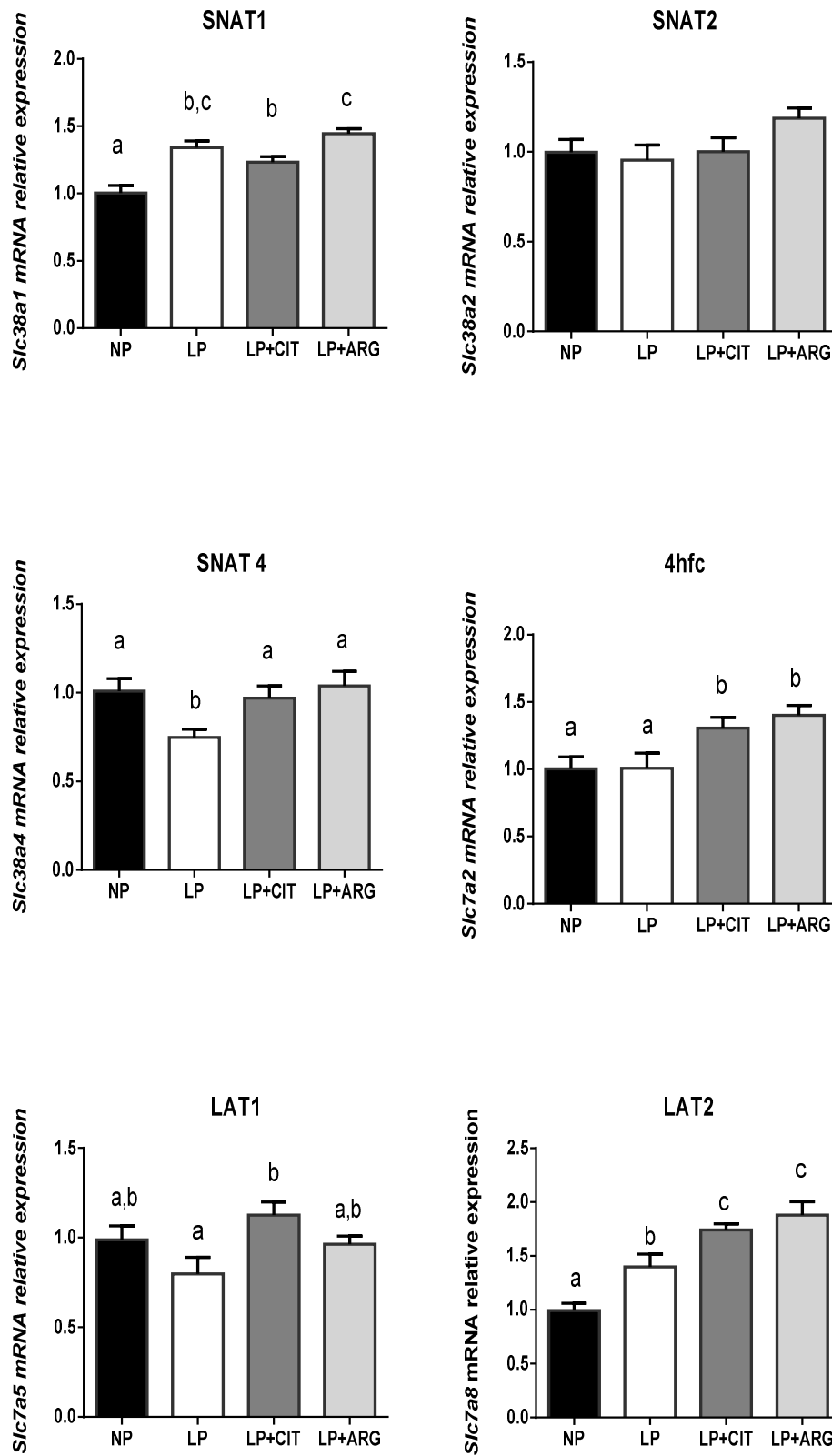


Fig 2.

