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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ABSTRACT

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

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

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

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

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

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

1. INTRODUCTION

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

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

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

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

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

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

The aims of this study therefore were to determine whether maternal supplementation with CIT and ARG 1) increased the availability of CIT or ARG in fetal circulation; 2) enhanced placental amino acid transport, and 3) impacted the fetal availability of essential amino acids in a model of IUGR induced by protein restriction.

2. METHODS

2.1 Animals and experimental design

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

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

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

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

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

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

2.2 Amino acid analysis

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

2.3 RNA isolation and RT-qPCR.

To assess amino acid transporter relative mRNA abundance, total RNA was isolated from four placentas in 6 dams per group. Placental tissues were homogenized and RNA was extracted in 1 mL trizol reagent solution. RNA samples were treated with DNase I (Promega, 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 ± 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, Kruskall-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

3. RESULTS

3.1 Gestation performance and food intake, fetal and placental growth

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

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

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

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

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

3.2 Effects of arginine and citrulline supplementation on fetal citrulline and arginine

Plasma citrulline concentrations was ≈5-fold higher in the LP+CIT mothers and fetuses, compared with other groups ($p < 0.05$). Arginine concentrations rose 2.4 fold in maternal plasma and 2 fold in fetal plasma in response to CIT supplementation. Supplementation with citrulline was as effective as arginine supplementation in raising arginine concentrations in 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

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

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

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

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

DISCUSSION

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

4.1 Effect of citrulline and arginine on fetal growth.

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

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

4.2 Effect of citrulline and arginine on fetal plasma arginine and citrulline

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

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

4.3 Effect of citrulline and arginine on placental amino acid transport

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

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

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

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Valérie Amarger : conceptualization, data analysis, manuscript writing

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

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References

1. Resnik R: Intrauterine growth restriction. Obstet Gynecol 2002;99:490-496.
2. Gardosi J, Mul T, Mongelli M, Fagan D: Analysis of birthweight and gestational age in antepartum stillbirths. Br J Obstet Gynaecol 1998;105:524-530.
3. Pallotto EK, Kilbride HW: Perinatal outcome and later implications of intrauterine growth restriction. Clin Obstet Gynecol 2006;49:257-269.
4. Lackman F, Capewell V, Richardson B, daSilva O, Gagnon R: The risks of spontaneous preterm delivery and perinatal mortality in relation to size at birth according to fetal versus neonatal growth standards. Am J Obstet Gynecol 184;946-953.
5. Barker DJ, Bull AR, Osmond C, Simmonds SJ: Fetal and placental size and risk of hypertension in adult life. BMJ 1990;301:259-262.
6. Barker DJ: Maternal nutrition, fetal nutrition, and disease in later life. Nutrition 1997; 13:807-813.
7. Hales CN, Barker DJ: The thrifty phenotype hypothesis. British medical bulletin 2001; 60:5-20.
8. Wu G, Bazer FW, Cudd TA, Meininger CJ, Spencer TE: Maternal nutrition and fetal development. J Nutr 2004;134:2169-2172.
9. Cetin I, Alvino G, Radaelli T, Pardi G: Fetal nutrition: a review. Acta Paediatr Suppl 2005; 94:7-13.
10. Battaglia FC, Regnault TR: Placental transport and metabolism of amino acids. Placenta 2001;22:145-161.
11. Sanchez R, Fischer P, Cuniberti L, Masnatta LD, Ramirez AJ: Vascular oxidative stress is associated with insulin resistance in hyper-reninemic nonmodulating essential hypertension. J Hypertens 2007;25:2434-2440.
12. Jansson T: Amino acid transporters in the human placenta. Pediatr Res 2001; 49:141-147.
13. Grillo MA, Lanza A, Colombatto S: Transport of amino acids through the placenta and their role. Amino Acids 2008;34:517-523.
14. Cetin I, Marconi AM, Bozzetti P, Sereni LP, Corbetta C, Pardi G, Battaglia FC: Umbilical amino acid concentrations in appropriate and small for gestational age infants: a biochemical difference present in utero. Am J Obstet Gynecol 1988; 158:120-126.
15. Kwon H, Ford SP, Bazer FW, Spencer TE, Nathanielsz PW, Nijland MJ, Hess BW, Wu G: Maternal nutrient restriction reduces concentrations of amino acids and polyamines in ovine maternal and fetal plasma and fetal fluids. Biol Reprod 2004;71:901-908.
16. Malandro MS, Beveridge MJ, Kilberg MS, Novak DA: Effect of low-protein diet-induced intrauterine growth retardation on rat placental amino acid transport. Am J Physiol 1996;271(1 Pt 1):C295-303.
17. Cetin I, Alvino G: Intrauterine growth restriction: implications for placental metabolism and transport. A review. Placenta 2009;30 (Suppl A):S77-82.
18. Avagliano L, Garo C, Marconi AM: Placental amino acids transport in intrauterine growth restriction. J Pregnancy 2012;972562.
19. Jansson T, Scholtbach V, Powell TL: Placental transport of leucine and lysine is reduced in intrauterine growth restriction. Pediatr Res 1998;44:532-7.
20. Shibata E, Hubel CA, Powers RW, von Versen-Hoeynck F, Gammill H, Rajakumar A, Roberts JM: Placental system A amino acid transport is reduced in pregnancies with

392 small for gestational age (SGA) infants but not in preeclampsia with SGA infants.
393 Placenta 2008;29:879-882.
394 21. Mahendran D, Donnai P, Glazier JD, D'Souza SW, Boyd RD, Sibley CP: Amino acid
395 (system A) transporter activity in microvillous membrane vesicles from the placentas
396 of appropriate and small for gestational age babies. Pediatr Res 1993;34:661-665.
397 22. Glazier JD, Cetin I, Perugino G, Ronzoni S, Grey AM, Mahendran D, Marconi AM,
398 Pardi G, Sibley CP: Association between the activity of the system A amino acid
399 transporter in the microvillous plasma membrane of the human placenta and severity
400 of fetal compromise in intrauterine growth restriction. Pediatr Res 1997;42:514-519.
401 23. Jansson N, Pettersson J, Haafiz A, Ericsson A, Palmberg I, Tranberg M, Ganapathy V,
402 Powell TL, Jansson T: Down-regulation of placental transport of amino acids precedes
403 the development of intrauterine growth restriction in rats fed a low protein diet. J
404 Physiol 2006;576(Pt 3):935-946.
405 24. Rosario FJ, Jansson N, Kanai Y, Prasad PD, Powell TL, Jansson T: Maternal protein
406 restriction in the rat inhibits placental insulin, mTOR, and STAT3 signaling and
407 down-regulates placental amino acid transporters. Endocrinology 2011;152:1119-
408 1129.
409 25. Kavitha JV, Rosario FJ, Nijland MJ, McDonald TJ, Wu G, Kanai Y, Powell TL,
410 Nathanielsz PW, Jansson T: Down-regulation of placental mTOR, insulin/IGF-I
411 signaling, and nutrient transporters in response to maternal nutrient restriction in the
412 baboon. FASEB J 2014;28:1294–305.
413 26. Pantham P, Rosario FJ, Nijland M, Cheung A, Nathanielsz PW, Powell TL, Galan HL,
414 Li C, Jansson T: Reduced placental amino acid transport in response to maternal
415 nutrient restriction in the baboon. Am J Physiol 2015;309:R740-R746.
416 27. Pantham P, Rosario FJ, Weintraub ST, Nathanielsz PW, Powell TL, Li C, Jansson T:
417 Down-Regulation of Placental Transport of Amino Acids Precedes the Development
418 of Intrauterine Growth Restriction in Maternal Nutrient Restricted Baboons. Biol
419 Reprod 2016;95:98.
420 28. Belkacemi L, Nelson DM, Desai M, Ross MG: Maternal undernutrition influences
421 placental-fetal development. Biol Reprod 2010;83:325-331.
422 29. Curis E, Nicolis I, Moinard C, Osowska S, Zerrouk N, Benazeth S, Cynober L:
423 Almost all about citrulline in mammals. Amino Acids 2005;29:177-205.
424 30. Lassala A, Bazer FW, Cudd TA, Li P, Li X, Satterfield MC, Spencer TE, Wu G:
425 Intravenous administration of L-citrulline to pregnant ewes is more effective than L-
426 arginine for increasing arginine availability in the fetus. J Nutr 2009;139:660-665.
427 31. Wu G, Bazer FW, Davis TA, Kim SW, Li P, Marc Rhoads J, Carey Satterfield M,
428 Smith SB, Spencer TE, Yin Y: Arginine metabolism and nutrition in growth, health
429 and disease. Amino Acids 2009;37:153-168.
430 32. Krause BJ, Hanson MA, Casanello P: Role of nitric oxide in placental vascular
431 development and function. Placenta 2011; 32:797-805.
432 33. Wu Z, Hou Y, Hu S, Bazer FW, Meininger CJ, McNeal CJ, Wu G: Catabolism and
433 safety of supplemental l-arginine in animals. Amino Acids 2016;48:1541-1552.
434 34. Xiao XM, Li LP: L-Arginine treatment for asymmetric fetal growth restriction. Int J
435 Gynaecol Obstet 2005;88:15-18.
436 35. Lassala A, Bazer FW, Cudd TA, Datta S, Keisler DH, Satterfield MC, Spencer TE,
437 Wu G: Parenteral administration of L-arginine prevents fetal growth restriction in
438 undernourished ewes. J Nutr 2010;140:1242-1248.
439 36. Lassala A, Bazer FW, Cudd TA, Datta S, Keisler DH, Satterfield MC, Spencer TE,
440 Wu G: Parenteral administration of L-arginine enhances fetal survival and growth in
441 sheep carrying multiple fetuses. J Nutr 2011;141:849-855.

37. Vosatka RJ, Hassoun PM, Harvey-Wilkes KB: Dietary L-arginine prevents fetal growth restriction in rats. Am J Obstet Gynecol 1998;178:242-246.
38. Bourdon A, Parnet P, Nowak C, Tran NT, Winer N, Darmaun D: l-Citrulline Supplementation Enhances Fetal Growth and Protein Synthesis in Rats with Intrauterine Growth Restriction. J Nutr 2016;146:532–541.
39. Tran NT, Amarger V, Bourdon A, Misbert E, Grit I, Winer N, Darmaun D: Maternal citrulline supplementation enhances placental function and fetal growth in a rat model of IUGR: involvement of insulin-like growth factor 2 and angiogenic factors. J Matern Fetal Neonatal Med 2017;30:1906-11.
40. Roucher VF, Desnots E, Nael C, Agnoux AM, Alexandre-Gouabau MC, Darmaun D, Boquien CY: Use of UPLC-ESI-MS/MS to quantitate free amino acid concentrations in micro-samples of mammalian milk. Springerplus 2013;1186, 2(622).
41. Cohen E, Baerts W, van Bel F: Brain-Sparing in Intrauterine Growth Restriction: Considerations for the Neonatologist. Neonatology 2015;108:269-76.
42. Vadillo-Ortega F, Perichart-Perera O, Espino S, Avila-Vergara MA, Ibarra I, Ahued R, Godines M, Parry S, Macones G, Strauss JF: Effect of supplementation during pregnancy with L-arginine and antioxidant vitamins in medical food on pre-eclampsia in high risk population: randomised controlled trial. BMJ 2011; 2011(19).
43. Schwartz R, Gruppuso PA, Petzold K, Brambilla D, Hiilesmaa V, Teramo KA: Hyperinsulinemia and macrosomia in the fetus of the diabetic mother. Diabetes Care 1994;17:640-8.
44. Harrington B, Glazier J, D'Souza S, Sibley C: System A amino acid transporter activity in human placental microvillous membrane vesicles in relation to various anthropometric measurements in appropriate and small for gestational age babies. Pediatr Res 1999;45:810–4.
45 Bortolus R, Chatenoud L, Di Cintio E, Rossi P, Benzi G, Surace M, Parazzini F: Placental ratio in pregnancies at different risk for intrauterine growth. Eur J Obstet Gynecol Reprod Biol 1998;80:157–8.
46. Lurie S, Feinstein M, Mamet Y: Human fetal-placental weight ratio in normal singleton near-term pregnancies. Gynecol Obstet Invest 1999;48:155–7.
47. Lackman F, Capewell V, Gagnon R, Richardson B: Fetal umbilical cord oxygen values and birth to placental weight ratio in relation to size at birth. Am J Obstet Gynecol 2001;185:674–82.
48. Macdonald EM, Koval JJ, Natale R, Regnault T, Campbell MK: Population-based placental weight ratio distributions. Int J Pediatr 2014;2014:291846.
49. Hayward CE, Lean S, Sibley CP, Jones RL, Wareing M, Greenwood SL, Dilworth MR: Placental Adaptation: What Can We Learn from Birthweight:Placental Weight Ratio? Front Physiol 2016;7:28.
50. Winer N, Branger B, Azria E, Tsatsaris V, Philippe HJ, Roze JC, Descamps P, Boog G, Cynober L, Darmaun D: L-Arginine treatment for severe vascular fetal intrauterine growth restriction: a randomized double-bind controlled trial. Clin Nutr 2009; 28:243-248.
51. Cetin I, Marconi AM, Corbetta C, Lanfranchi A, Baggiani AM, Battaglia FC, Pardi G: Fetal amino acids in normal pregnancies and in pregnancies complicated by intrauterine growth retardation. Early Hum Dev 1992;29:183-186.
52. Marconi AM, Paolini CL, Stramare L, Cetin I, Fennessey PV, Pardi G, Battaglia FC: Steady state maternal-fetal leucine enrichments in normal and intrauterine growth-restricted pregnancies. Pediatr Res 1999;46:114-119.

53. Paolini CL, Marconi AM, Ronzoni S, Di Noio M, Fennessey PV, Pardi G, Battaglia FC: Placental transport of leucine, phenylalanine, glycine, and proline in intrauterine growth-restricted pregnancies. J Clin Endocrinol Metab 2001;86:5427-5432.
54. Ross JC, Fennessey PV, Wilkening RB, Battaglia FC, Meschia G: Placental transport and fetal utilization of leucine in a model of fetal growth retardation. Am J Physiol 1996, 270:E491-503.
55. Glazier JD, Cetin I, Perugino G, Ronzoni S, Grey AM, Mahendran D, Marconi AM, Pardi G, Sibley CP: Association between the Activity of the System A Amino Acid Transporter in the Microvillous Plasma Membrane of the Human Placenta and Severity of Fetal Compromise in Intrauterine Growth Restriction. Pediatr Res 1997;42:514-519.
56. Mando C, Tabano S, Pileri P, Colapietro P, Marino MA, Avagliano L, Doi P, Bulfamante G, Miozzo M, Cetin I: SNAT2 expression and regulation in human growth-restricted placentas. Pediatr Res 2013;74:104-110.
57. Jansson T, Ylven K, Wennergren M, Powell TL: Glucose transport and system A activity in syncytiotrophoblast microvillous and basal plasma membranes in intrauterine growth restriction. Placenta 2002;23:392-9.
58. Aiko Y, Askew DJ, Aramaki S, Myoga M, Tomonaga C, Hachisuga T, Suga R, Kawamoto T, Tsuji M, Shibata E: Differential levels of amino acid transporters System L and ASCT2, and the mTOR protein in placenta of preeclampsia and IUGR. BMC Pregn and Childb 2014;14:181-181.
59. Weckman AM, McDonald CR, Baxter JB, Fawzi WW, Conroy AL, Kain KC: Perspective: L-arginine and L-citrulline Supplementation in Pregnancy: A Potential Strategy to Improve Birth Outcomes in Low-Resource Settings. Adv Nutr 2019;10:765-77.

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

	Group				
	NP	LP	LP+CIT	LP+ARG	*P*
mean litter size	10±0.9[a]	13.8±0.7[b]	11.5±0.9[b]	13.1±0.9[b]	0.03
fetal weight, g	5.48±0.07[a]	4.01±0.03[b]	4.22±0.05[c]	4.48±0.04[d]	<0.001
placental weight, g	0.617±0.01[a]	0.392±0.04[b]	0.391±0.008[b]	0.413±0.01[b]	<0.001
Placenta efficiency[2]	9.1±0.2[a]	10.3±0.1[b]	10.9±0.2[b]	11.1±0.2[c]	<0.002
Brain/body weight ratio	0.038 ± 0.002	0.047 ± 0.002[c]	0.044 ± 0,003[c]	0.046 ± 0.003[c]	<0.05

528 [1]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 [2]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).

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

537

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 [1]Dfference assessed using Kruskall-Wallis test.

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

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

[1]Differenced analyzed using Kruskall-Wallis test

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).

560

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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568

Fig 1.

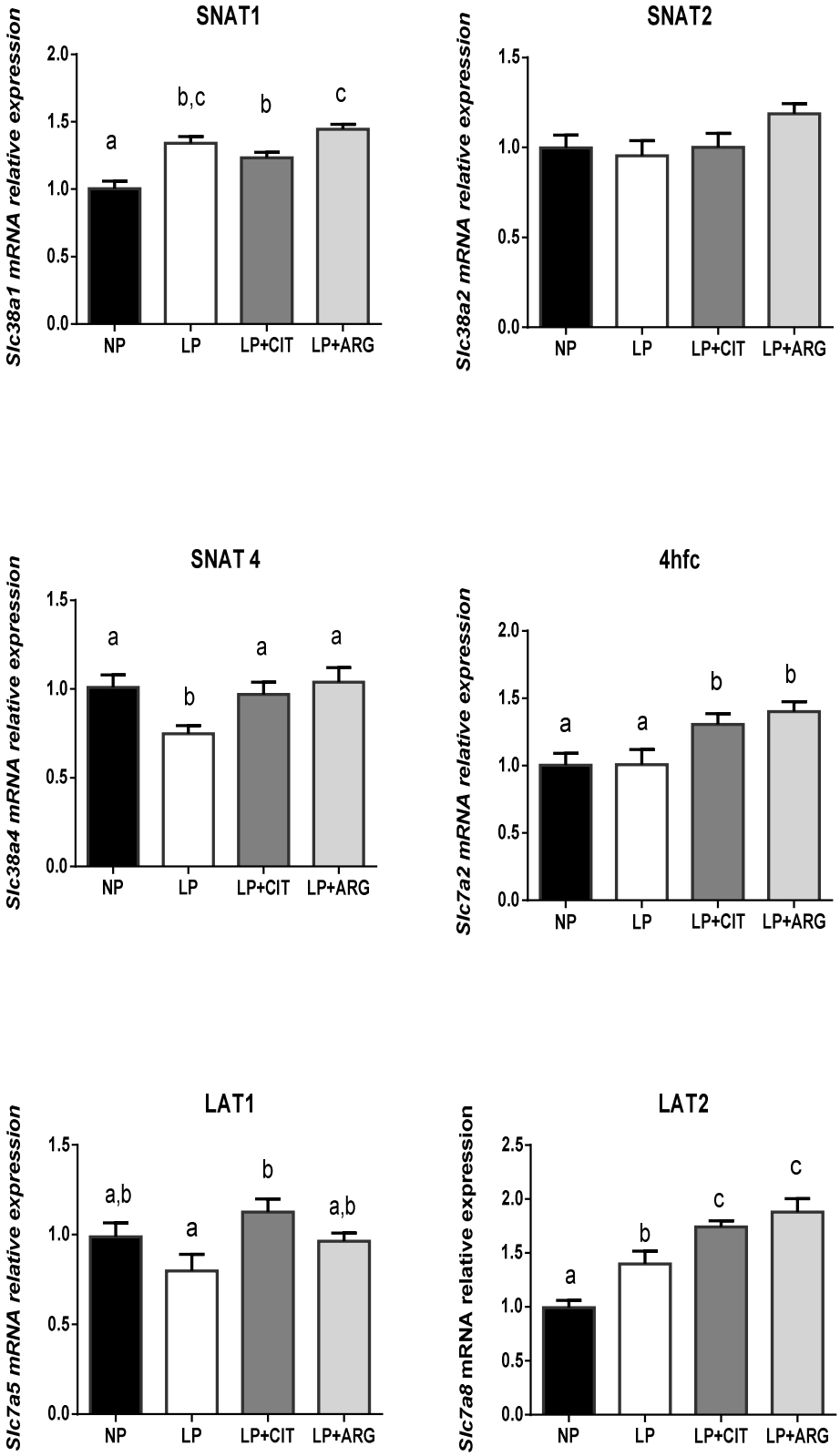
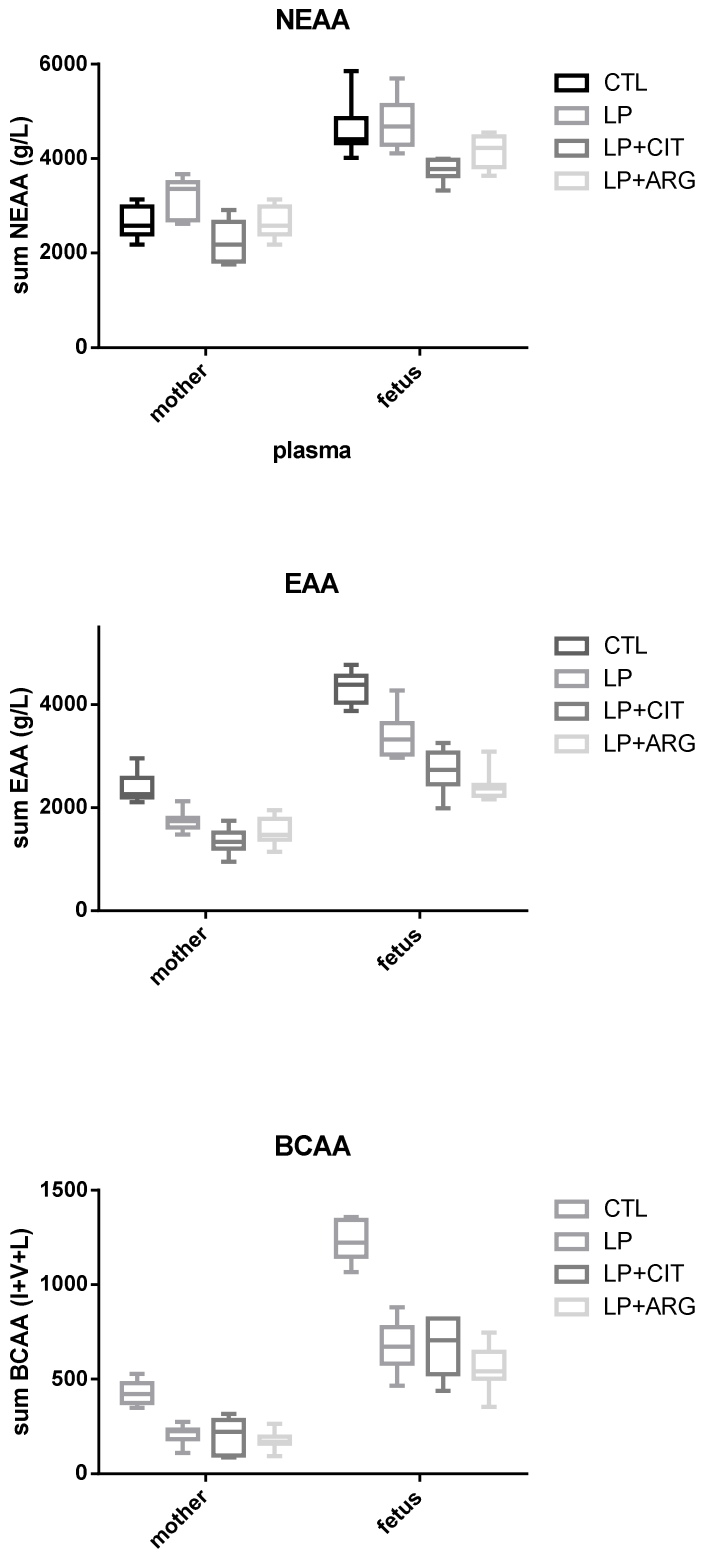

SNAT1
Slc38a1 mRNA relative expression
2.0
1.5
1.0
0.5
0.0
a
b,c
b
c
NP
LP
LP+CIT
LP+ARG
SNAT2
Slc38a2 mRNA relative expression
1.5
1.0
0.5
0.0
NP
LP
LP+CIT
LP+ARG
SNAT 4
Slc38a4 mRNA relative expression
1.5
1.0
0.5
0.0
a
b
a
a
NP
LP
LP+CIT
LP+ARG
4hfc
Slc7a2 mRNA relative expression
2.0
1.5
1.0
0.5
0.0
a
a
b
b
NP
LP
LP+CIT
LP+ARG
LAT1
Slc7a5 mRNA relative expression
1.5
1.0
0.5
0.0
a,b
a
b
a,b
NP
LP
LP+CIT
LP+ARG
LAT2
Slc7a8 mRNA relative expression
2.5
2.0
1.5
1.0
0.5
0.0
a
b
c
c
NP
LP
LP+CIT
LP+ARG

Fig 2.


NEAA
sum NEAA (g/L)
6000
4000
2000
0
mother
fetus
plasma
CTL
LP
LP+CIT
LP+ARG
EAA
sum EAA (g/L)
4000
2000
0
mother
fetus
CTL
LP
LP+CIT
LP+ARG
BCAA
sum BCAA (I+V+L)
1500
1000
500
0
mother
fetus
CTL
LP
LP+CIT
LP+ARG