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SNAT2 expression and regulation in human growth-restricted placentas

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BACKGROUND: Amino acid placental delivery is reduced in human intrauterine growth–restricted (IUGR) fetuses, and the activity of placental amino transporters has been consistently shown to be decreased in *in vitro* studies. We hypothesized lower placental expression and localization of sodium-coupled neutral amino acid transporter 2 (*SNAT2* (also known as *SLC38A2*)), altered levels of intron-1 methylation, and altered distribution of single-nucleotide polymorphisms in human IUGR vs. normal pregnancies.

METHODS: We studied 88 IUGR and 84 control placentas from singleton pregnancies at elective caesarean section. *SNAT2* expression was investigated by real-time PCR and immunohistochemistry. Intron-1 methylation levels were analyzed by pyrosequencing, and single-nucleotide polymorphism distribution was analyzed by allelic discrimination.

RESULTS: mRNA levels were significantly decreased in IUGR placentas with reduced umbilical blood flows. Syncytiotrophoblast immunostaining was lower in IUGR placentas than in control placentas. Methylation levels were steadily low in both IUGR and control placentas. SNP genotype and allele frequencies did not differ between the two groups.

CONCLUSION: This is the first study investigating *SNAT2* expression and regulation mechanisms in human IUGR placentas. We confirm previous results obtained in rats and cell cultures that support the fundamental role of *SNAT2* in fetal growth and well-being, as well as a possible role of oxygen levels in regulating *SNAT2* expression, indicating the relevance of hypoxia in IUGR.

Reduced oxygen and nutrient placental delivery has been clearly demonstrated in intrauterine growth–restricted (IUGR) pregnancies (1–7). Amino acids are needed during fetal growth for protein synthesis and also for energy production, supplying 20–40% of the fetal–placental unit energy requirements (8). The placenta itself consumes a large amount of amino acids, accounting for its active metabolism (9).

We previously reported reduced amino acid concentrations as well as altered fetal–maternal amino acid gradients in IUGR pregnancies sampled at the time of caesarean section or at cordocentesis (3,4). Previous *in vivo* studies measured by stable isotope tracers found decreased essential amino acid placental transport rates (5,6), and *in vitro* experiments confirmed reduced activity of placental amino acid transport systems (10,11) in IUGR and small-for-gestational-age pregnancies as compared with normal pregnancies.

Amino acid transporters play an important part in regulating fetal plasma amino acid composition (12). The System A family, which contains three highly homologous protein subtypes (sodium-coupled neutral amino acid transporter 1 (SNAT1; also known as SLC38A1), SNAT2 (also known as SLC38A2), and SNAT4 (also known as SLC38A4), promotes the transport of neutral amino acids from the mother to the fetus, which is vital for the development and growth of both the fetus and the placenta and may function in the transport of amino acids at the blood–brain barrier. In particular, *SNAT2* is expressed both in fetus and placenta and has a crucial role in the control of fetal growth (13). A number of studies report reduced System A membrane activity per milligram of microvillous membrane in human IUGR placentas (10,14,15). Of note, in rats, placental *Snat2* downregulation has been demonstrated to precede the development of intrauterine growth restriction (13), suggesting that its reduction is a cause, and not a consequence of the disease. However, *SNAT2* expression and regulation mechanisms have never been investigated in the human placental tissue.

Snat2 regulation is mediated by several factors (13,16–19), particularly hypoxia and amino acid extracellular concentrations. *SNAT2* intron-1 is a noncoding region within the *SNAT2* gene that spans 54 nucleotides and contains sequences that are able to respond to amino acid deficiency and activate *SNAT2* gene expression (20). Moreover, intron-1 contains CpG islands (CpGs), which are repeated CG sequences in which the C nucleotide is the site of DNA methylation.

Here, we measured *SNAT2* gene expression and protein localization in human IUGR placentas compared with control placentas. We then measured methylation levels of the CpG

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islands located within the placental *SNAT2* intron-1 to verify possible differences in methylation profile between IUGR and normal pregnancies. Finally, we investigated the association with intrauterine growth restriction of a single-nucleotide polymorphism (SNP) located within a *SNAT2* intron-1 CpG island, the ancestral nucleotide of which is a cytosine belonging to the CpG island.

RESULTS

Cord Blood Analysis

Blood from IUGR umbilical arteries and veins had significantly lower pO_2 than that of controls ($P < 0.001$) (**Table 1**). Similarly, oxygen saturation and content were significantly lower and lactate was significantly higher in IUGR umbilical veins and arteries than in controls. These differences increased progressively with the severity of intrauterine growth restriction.

SNAT2 Gene Expression in IUGR and Control Placentas

SNAT2 mRNA levels from 49 IUGR and 41 control placentas were analyzed by real-time PCR.

SNAT2 gene expression levels were significantly lower in IUGR placentas, with a 27% reduction compared with controls ($P = 0.03$) (**Figure 1a**). However, after dividing the IUGR placentas into three severity groups, *SNAT2* expression levels were significantly lower than controls only in the two most severe groups (that is, IUGR2 and IUGR3) ($P = 0.007$) (**Figure 1b**).

SNAT2 mRNA levels decreased in relation to the oxygen content in the umbilical vein, and this relation was statistically significant ($P = 0.03$) (**Figure 1c**). A significant positive relationship was observed in the study population between *SNAT2* mRNA levels and maternal age, as well as with gestational age and fetal and placental weights. However, after analyzing differences in the IUGR population with gestational age ranging between 30 and 39 wk, representing all three groups of severity, we reported a significant difference in gestational age between the severity groups, but no significant correlation between *SNAT2* expression and gestational age. There were no significant gender-related differences in *SNAT2* gene expression.

Table 1. Cord blood analysis in IUGR and control pregnancies

		Average	SD	*P*
UV: pO_2 (mm/Hg)	Controls	25.9	6.7	**
	IUGR	17.6	4.5	
UA: pO_2 (mmHg)	Controls	12.5	4.1	**
	IUGR	8.1	3.5	
UV: O_2 content (mmol/l)	Controls	4.0	1.8	**
	IUGR	2.2	1.2	
UA: O_2 content (mmol/l)	Controls	1.2	0.9	*
	IUGR	0.6	0.6	
UV: O_2 saturation (%)	Controls	45.5	19.1	**
	IUGR	24.9	13.6	
UA: O_2 saturation (%)	Controls	13.2	9.8	*
	IUGR	7.4	6.2	
UV: lactate (mmol/l)	Controls	1.5	0.4	**
	IUGR	2.2	1.3	
UA: lactate (mmol/l)	Controls	1.7	0.6	*
	IUGR	2.5	1.6	

IUGR, intrauterine growth–restricted; UA, umbilical artery; UV, umbilical vein.
*$P < 0.05$; **$P < 0.001$.

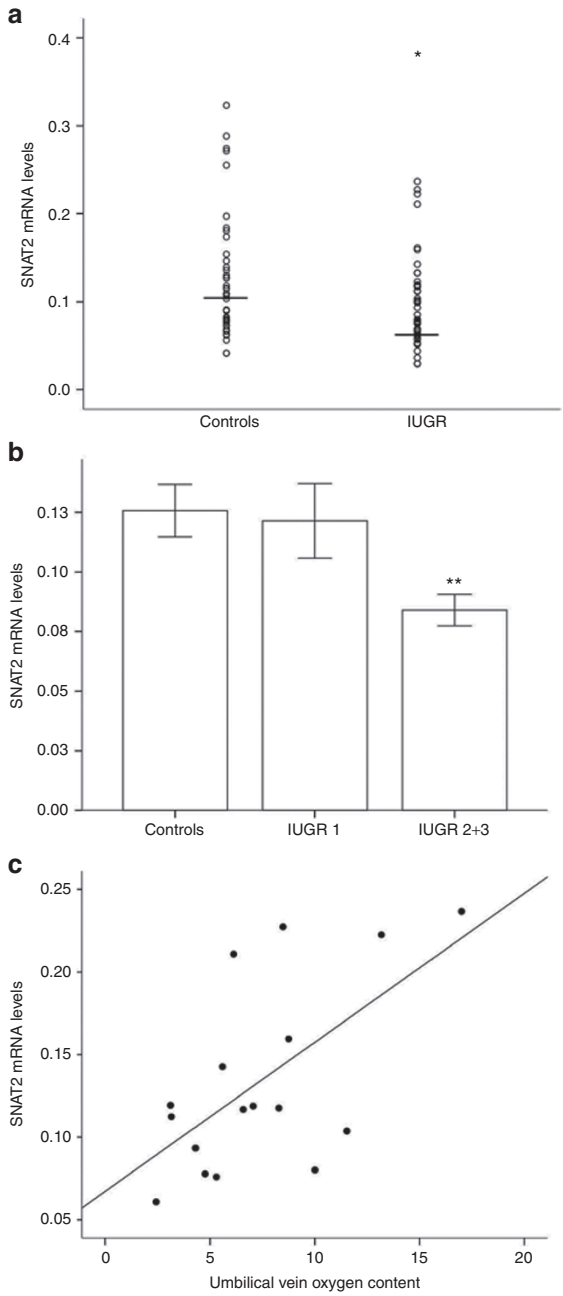

Figure 1. Sodium-coupled neutral amino acid transporter 2 (*SNAT2*) gene expression. (**a**) *SNAT2* gene expression in control and intrauterine growth–restricted (IUGR) placentas. Bars represent median values. mRNA levels in IUGR placentas are significantly lower compared with control placentas. (**b**) *SNAT2* gene expression in control and IUGR placentas divided by severity. Data are presented as mean ± SE. mRNA levels decrease with IUGR severity. (**c**) Significant relation between *SNAT2* mRNA levels and umbilical vein oxygen content in the IUGR group. *$P < 0.05$ and ** $P < 0.01$.

SNAT2 Localization in IUGR and Control Placentas

SNAT2 protein was expressed in placental tissues from both IUGR patients and controls. Cytoplasmic SNAT2 immunoreactivity was observed in the amniotic epithelium, in the wall of the chorionic and stem vessels, and in the villous and extravillous trophoblast. Immunostaining was present in both the cyto- and syncytiotrophoblast.

No differences in SNAT2 spatial distribution were observed between cases and controls in all samples examined. In our population, IUGR placentas frequently presented abnormalities of villous branching and Tenney–Parker changes. In these conditions, considerable variation was detected in staining intensity between normal and IUGR placentas (Figure 2a,b). In control placentas, immunostaining was predominantly in the syncytiotrophoblast, with stronger staining on the microvillous membrane. In IUGR cases, the syncytiotrophoblast showed a weak SNAT2 immunoreactivity, with no increase in intensity of staining on the microvillous membrane, whereas the cytotrophoblast showed intense immunoreactivity. Semiquantitative analysis by blind scoring of randomly selected slides showed that the IUGR type 1 (IUGR1) group exhibited similar distribution (3 ± 0) and syncytiotrophoblast intensity (3 ± 0) to controls (2.6 ± 0.5 and 2.8 ± 0.4, respectively), whereas the IUGR2 and IUGR3 groups had significantly lower values for both distribution (1.3 ± 0.6) and intensity (1.6 ± 0.6) ($P < 0.05$).

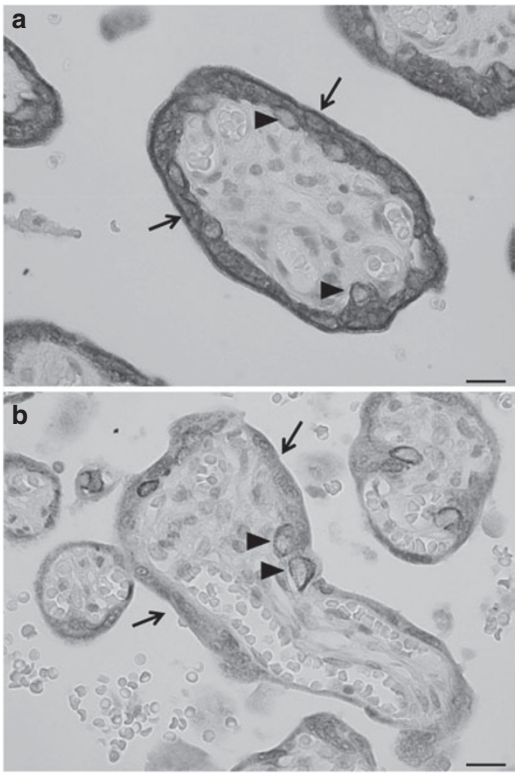

Figure 2. Immunohistochemical expression of sodium-coupled neutral amino acid transporter 2 (SNAT2) in intrauterine growth–restricted (IUGR) and normal placentas. (**a**) In controls, immunostaining is in trophoblast cells, both in the cytotrophoblast (arrowhead) and in the syncytiotrophoblast, with intense staining on the microvillous membrane (arrow; semiquantitative score 3+). Scale bar: 20 µm. (**b**) Example of placenta from the IUGR type 3 (IUGR3) group with weak SNAT2 immunoreactivity in the syncytiotrophoblast (arrow; semiquantitative score 1+). Cytotrophoblast (arrowhead) showed strong staining also in cases of severe intrauterine growth restriction. Scale bar: 20 µm.

SNAT2 Intron-1 Methylation Levels in IUGR and Control Placentas

Methylation analyses were conducted on DNA extracted from 37 IUGR placentas and 28 control placentas, as well as from 10 peripheral blood samples taken from healthy adults, which were used as external controls.

Average methylation levels were 3.02% in healthy adult peripheral blood (range: 2.50–3.69%) (Figure 3a), with steadily low levels for all of the observed CpGs. Control placentas presented the same profile as external controls, with average methylation levels of 3.16% (range: 1.92–7.15%) (Figure 3b). Similarly, IUGR placentas presented steadily low methylation, with average levels of 3.08% (range: 2.00–6.53%) (Figure 3c) and no differences between the three IUGR severity groups (data not shown). Therefore, altogether, the CpG sites in the

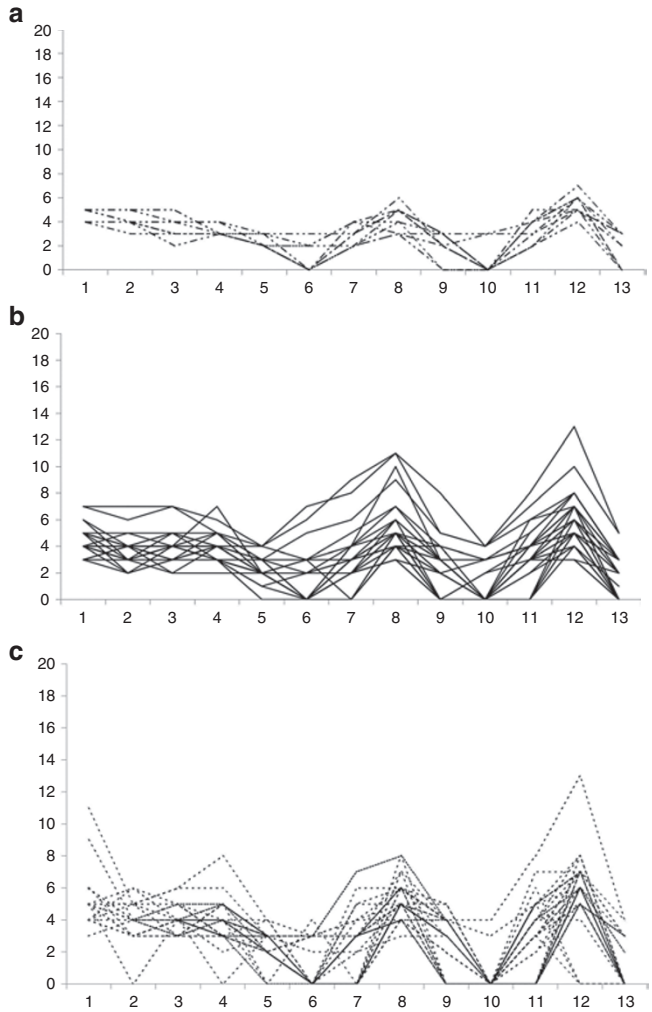

Figure 3. Methylation levels (%) of the 13 analyzed CpG islands within the sodium-coupled neutral amino acid transporter 2 (*SNAT2*) intron-1 region. Each sample is represented by a different line, which draws the methylation fluctuation in the analyzed loci (percentage values in each CpG site). (**a**) Peripheral blood from10 healthy adults (external controls); (**b**) 28 control placentas; and (**c**) 35 intrauterine growth–restricted (IUGR) placentas.

SNAT2 intron-1 region were all hypomethylated, with no significant difference between the analyzed groups.

SNP Allelic Discrimination

The intron-1 rs10880961 SNP, in which the ancestral allele (C) can be replaced by a G, was genotyped in 68 IUGR and 68 control placentas. The data were analyzed under a codominant model (considering separately the genotypes CC, CG and GG), a dominant model (considering together all cases in which the genotype contained the C allele: CC+CG vs. GG), and a recessive model (considering together all cases in which the genotype did not contain the C allele: CC vs. CG+GG), and evaluating the allele frequency. **Table 2** shows the genotypes and the allele frequencies in the two studied groups.

No association between the polymorphism and the IUGR pathology was found in any of the three mentioned models. Allele frequencies were not significantly different between the IUGR placentas and controls.

DISCUSSION

Several *in vivo* and *in vitro* studies report alterations of placental amino acid transport in human IUGR fetuses (3,5,6). The functional role of SNAT2, a neutral amino acid transporter, has been extensively described in placental tissues of different species and in human placental cells *in vitro* (13,16–19).

In this study, we have characterized the expression of *SNAT2* in human growth-restricted placentas for the first time, following on work done in other species and cell culture. In our large sample of IUGR placentas, *SNAT2* gene expression was significantly lower compared with control placentas. This suggests that previous data on reduced fetal amino acid concentrations (4) and reduced placental amino acid transport activity (10) in intrauterine growth restriction are related to a downregulation of *SNAT2* transcription. We also investigated *SNAT2* gene expression depending on the severity of intrauterine growth restriction. We divided the IUGR placentas according to a previously described classification (2) based on the fetal heart rate and the umbilical artery pulsatility index. According to this classification, in our population, the IUGR placentas were progressively hypoxic and lactacidemic with increasing severity.

SNAT2 mRNA levels were significantly lower only in the most severe cases, in which there was a reduction in the umbilical blood flow, suggesting that reduced *SNAT2* gene expression may be related to reduced placental oxygenation. These data confirm our previous results, which showed lower System A activity in IUGR2 and IUGR3 placental microvillar membrane vesicles (10). Nevertheless, a previous study (13) demonstrated that in pregnant rats fed a low protein diet, *Snat2* downregulation precedes the onset of intrauterine growth restriction. A possible limit of the current study, which was performed on human pregnancies, is that our data are a picture of the placenta at delivery, and therefore cannot clarify whether *SNAT2* expression is reduced in severe intrauterine growth restriction before the reduction in fetal growth. However, the positive correlations between *SNAT2* mRNA levels and fetal and placental weights seem to confirm the relevant function of this protein in supplying amino acids from the mother to the fetus.

Table 2. Genotype and allele frequency of the SNP located in a CpG island within the *SNAT2* intron-1 region

	Genotype frequency			Allelic frequency	
	CC	CG	GG	C	G
IUGR	25%	37%	38%	0.43	0.57
Controls	18%	36%	46%	0.38	0.04

IUGR, intrauterine growth–restricted; SNAT2, sodium-coupled neutral amino acid transporter 2; SNP, single-nucleotide polymorphism.

In our population, gestational age, which was significantly lower in IUGR pregnancies, was also positively related with *SNAT2* gene expression. This is a limit of all studies investigating human IUGR vs. control term placentas, as IUGR pregnancies are electively delivered earlier in the interest of the fetus. However, we analyzed the relationship between gestational age and *SNAT2* mRNA levels in the IUGR population with gestational age ranging between 30 and 39 wk. In this range, all three IUGR severity groups were represented. Although the IUGR2 and IUGR3 groups had a significantly lower gestational age than the IUGR1 group, there was no significant correlation between SNAT2 expression and gestational age.

Decreased uptake of amino acids by the placenta as a result of reduced *SNAT2* expression may also have relevant effects, because the human placenta has a very high protein turnover rate (8). Moreover, SNAT2 mediates the transport of neutral α-amino acids, such as glutamine. Indeed, we previously reported that umbilical venous glutamine concentration is significantly lower in small-for-gestational-age patients compared with controls (21). Glutamine is a protective factor against mitochondrial lipid peroxidation and has an important role in antioxidant activity (22). It also has important metabolic functions in the transfer of carbon atoms, as a fuel for rapidly dividing cells, and as a precursor for many biologically active molecules. Moreover, glutamine stimulates signaling pathways, such as the hexosamine pathway, which influence cellular signaling, protein turnover, and gene expression (23). Therefore, lower placental glutamine concentrations possibly deriving from *SNAT2* decrease may lead to mitochondrial oxidative stress and cell distress, resulting in alterations in cell metabolism and the biosynthesis of molecules (such as progesterone) that are fundamental for pregnancy maintenance (24). Indeed, these are typical characteristics leading to the IUGR condition, and mitochondrial alterations have been reported in human IUGR placentas (25).

Moreover, further negative consequences for the placenta could be related to decreased amino acid concentrations as a result of lower *SNAT2* expression, in turn leading to a lack of fundamental elements that normally are consumed in high quantities by the placental active metabolism (9).

Immunohistochemical data confirmed that in human placentas, *SNAT2* is expressed in the villous trophoblast, in both the syncytio- and cytotrophoblast (26). However, in IUGR placentas, SNAT2 staining intensity seemed to be lower in the syncytiotrophoblast. As expected, IUGR placentas presented

abnormal villous branching and an increased number of syncytial knots (the so-called Tenney–Parker changes). An increased number of syncytial knots is frequently observed in IUGR placentas (27), and it may reflect conditions of cellular distress (28). Hypoxia frequently affects IUGR babies, and it could increase the formation of syncytial knots (28) and decrease the expression of *SNAT2* (19), as was found in our study population.

Another important regulatory element for *SNAT2* expression is represented by the intron-1 region located in the *SNAT2* gene. This region has been reported to carry amino acid response elements that respond to amino acid deficiency and activate *SNAT2* gene expression (20). We found that the *SNAT2* intron-1 is rich in CpG islands; these nucleotide sequences are sensitive to DNA methylation, which is one of the conditions that regulate epigenetic gene expression. Therefore, we investigated the methylation and SNP status of *SNAT2* to determine whether they differ between control and IUGR pregnancies. Although these variables did not differ, these findings are informative on the lack of methylation changes of *SNAT2* in the placenta, although methylation can occur (and can have a significant impact on gene expression) outside CpG islands. It is well recognized that CpG island hypermethylation in gene control regions results in stable transcriptional repression (29), whereas hypomethylation is a guarantee for gene expression, giving to the gene the possibility of being expressed (30). Therefore, our data reporting the hypomethylation of control samples confirm the great importance of placental *SNAT2*. However, they indicate that the reduction of *SNAT2* expression in IUGR placentas is not due to epigenetic modifications in *SNAT2* intron-1.

We also performed an allelic discrimination for a SNP located within the intron-1 region, the ancestral allele of which is a cytosine that can occur as a guanine in a particular CpG island. In this case, a sequence that ancestrally is CG would occur instead as GG, which is not a target for methylation. Therefore, we wondered whether the absence of a CG sequence, which occurs in the polymorphic forms, might represent a risk factor for the disease. However, the genotype and the allele frequency were not different between the IUGR and control group, even when considering the data under different models. Therefore, we concluded that in the study population, intrauterine growth restriction is not associated with the SNP located at this region.

The *SNAT2* downregulation that we reported in this study may thus be due to the deregulation of other regulatory systems involved in the modulation of *SNAT2* expression, such as the previously mentioned oxygen levels. Indeed, the oxygen levels in both the umbilical artery and vein were significantly decreased in the IUGR samples, particularly in the more severe groups, with a significant relationship between O_2 content and *SNAT2* expression. This is in agreement with previous studies showing *SNAT2* downregulation in trophoblast cultures under hypoxic conditions (19).

A further pathway leading to *SNAT2* downregulation might be represented by mammalian target of rapamycin complex 1, which has been reported to be inhibited in human IUGR placentas (31,32). Potentially, decreased *SNAT2* expression could also be the result of a shorter *SNAT2* mRNA half-life and not of defects in the translation rate; however, our study did not address this hypothesis.

Finally, because the other *SNAT* gene isoforms (*SNAT1* and *SNAT4*) may contribute to System A amino acid transporter activity in the human placental trophoblast (26), the analysis of their expression in this study population might clarify the role of System A amino acid transporter deregulation in the etiopathogenesis of intrauterine growth restriction.

In conclusion, this is the first study investigating *SNAT2* expression and regulation mechanisms in human IUGR placental tissues. Our results confirm previous studies on rats and human placental cells (13,19), supporting the fundamental role of *SNAT2* in fetal growth and well-being, and the possible role of oxygen levels in regulating *SNAT2* expression, indicating the relevance of hypoxia in intrauterine growth restriction.

METHODS

The study was approved by the local Institutional Review Board (Luigi Sacco Hospital), and all pregnant patients gave their informed consent.

Study Population

We studied a total of 172 singleton pregnancies (88 IUGR pregnancies and 84 controls) at elective cesarean section. Gestational age was calculated from the last menstrual period and confirmed by routine ultrasonography at 11–12 wk of gestation (33). Exclusion criteria for both groups were fetal or maternal infections, maternal drugs or alcohol abuse, fetal malformations, chromosomal abnormalities, maternal chronic hypertension, maternal cardiovascular or autoimmune diseases, and diabetes.

We identified IUGR fetuses *in utero* through repeated longitudinal measurements that demonstrated a reduction in fetal growth velocity, and we further classified them according to umbilical arterial Doppler velocimetry and fetal heart rate (FHR) tracings (2,34), as previously described (35). Growth restriction was confirmed at birth if the neonatal weight was below the 10th percentile according to Italian standards for birth weight and gestational age (36).

IUGR fetuses were classified into three groups of increasing clinical severity on the basis of Doppler velocimetry of the umbilical artery and FHR, as previously proposed (2). Briefly, the IUGR1 group had a normal pulsatility index and normal FHR; the IUGR2 group had an abnormal pulsatility index and normal FHR; and the IUGR3 group had an abnormal pulsatility index and abnormal FHR.

Normal pregnancies were those of healthy mothers with normal BMI, no pregnancy complication, and normal fetal growth confirmed by birthweight between the 10th and the 90th percentile for Italian references (37).

Maternal and fetal characteristics for controls and intrauterine growth restriction are presented in **Table 3**. Maternal age and BMI were not significantly different between the two groups. As expected, gestational age and fetal and placental weights were significantly lower in the IUGR group.

Procedures

Placental sample collection. Placental samples were collected for RNA and protein extraction; fragments were washed using PBS (Dulbecco's Phosphate Buffered Solution, Euroclone, Milan, Italy), then cut into small pieces, which were then checked by optical microscope in order to eliminate any residual maternal decidua fragments. Selected chorionic villi were then frozen in liquid nitrogen for protein extraction, or put into RNA-stabilizing solution (RNA later, Ambion, Austin, TX) for RNA extraction; in this latter case, samples were stored at 4 °C for 24 h, and then stored at −20 °C.

For immunohistochemistry experiments, placental fresh full thickness samples were fixed in 10% neutral buffered formalin (Bio-optica Milano S.p.a., Milan, Italy) for 12–24 h before paraffin embedding.

Table 3. Characteristics of the study population

		Average	SD	*P*
Maternal age (years)	Controls	33.9	4.4	NS
	IUGR	33.3	4.7	
Maternal BMI	Controls	21.5	2.5	NS
	IUGR	23.0	3.8	
Gestational age at delivery (d)	Controls	270.2	10.2	**
	IUGR	231.8	25.5	
Fetal weight (g)	Controls	3250.7	434.4	**
	IUGR	1414.5	584.4	
Placental weight (g)	Controls	518.3	115.7	**
	IUGR	235.4	98.4	

IUGR, intrauterine growth–restricted; NS, not significant.

**$P < 0.001$.

Biochemical analyses. Umbilical venous and arterial blood was sampled from a doubly clamped segment of the cord immediately after fetal extraction. All samples were collected in heparinized syringes and kept on ice until the end of analysis. Blood gases and lactate concentration were measured on a GEM Premier 3000 (Instrumentation Laboratory, Brussels, Belgium). Oxygen content was calculated according to the following formula (2): oxygen content (mmol/l) = hemoglobin (g/l) * oxygen saturation * 0.005982.

SNAT2 gene expression: quantitative real-time PCR analysis. *SNAT2* mRNA level placentas were analyzed using real-time PCR. Total RNA was isolated from tissues using Trizol reagent (Invitrogen, Life Technologies, Cergy, France) following the manufacturer's instructions and treated with DNA-free kit (Ambion) to remove potentially contaminating DNA. RNA concentration was determined using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). For *SNAT2* expression studies, 500 ng of total RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), and the obtained cDNA served as a template for quantitative real-time PCR, on the basis of TaqMan methodology, using the 7500 Fast Real-Time PCR System (Applied Biosystems).

The amount of *SNAT2* RNA was calculated using the $2^{-\Delta Ct}$ method relative to the hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) and succinate dehydrogenase complex-subunit A (*SDHA*) housekeeping genes, which were selected from a pool of tested housekeeping genes because they showed a similar amplification efficiency in a scale of RNA concentrations (38). All the assays were provided by Applied Biosystems. All samples were reverse transcribed in duplicate, and cDNA was run in triplicate to allow assessment of sample homogeneity and technical variability. Log_{10}-transformed results were used to obtain normally distributed values, as previously described (39).

Real-time data were analyzed using the Sequence Detector software (Applied Biosystems).

SNAT2 ***protein localization: immunohistochemistry.*** Immunohistochemical studies were carried out on 4-µm-thick, formalin-fixed, paraffin-embedded placental tissue sections using a Novolynk Polymer Detection System (Novocastra Laboratories, Newcastle, UK). Primary antibodies (affinity-purified rabbit IgG) were custom-made (Eurogentec, Seraing, Belgium) using the following amino acid sequences of SNAT2 as antigen: SNLGKKKYETEFHPG (residues 55–69).

Sections were deparaffinized in bioclear for 20 min, then washed twice in ethanol. Slides were placed in a water bath containing 0.01 mol/l sodium citrate at pH 6.0 for 30 min at 95 °C. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in distilled water for 10 min. Staining was performed with 3,3′ diaminobenzidine as a chromogen. For SNAT2 staining, the primary antibody was applied at a concentration of 1:1,000 in 0.5% BSA and sodium azide and incubated for 30 min at room temperature. Antibody specificity was confirmed by preincubation of the SNAT2 antibody with its antigenic peptide, and slides on which the primary antibody was absent were included as negative controls.

Protein intensity and distribution were assessed in five randomly selected areas at ×40 magnification using a semiquantitative 0–3 plus-scale: 0 = no staining; 1 = weak intensity of staining, focal distribution (positivity in <25% of syncytiotrophoblast); 2 = moderate staining, distribution in 25–50% of syncytiotrophoblast; and 3 = strong staining, diffuse distribution (>50% of syncytiotrophoblast). Areas with abundant necrosis or infarction were excluded from semiquantitative evaluation.

Intron-1 CpG methylation levels: pyrosequencing. *SNAT2* intron-1 is a region within the *SNAT2* gene that is relevant for its expression regulation because it is an amino acid concentration "sensor" (20). Therefore, we analyzed the *SNAT2* intron-1 region sequence using a CpG island searcher (40) and found several CG sites (CpG islands) at which the cytosine is potentially a target for DNA methylation.

Quantitative methylation level analyses were performed using a pyrosequencing approach on a 365-base-pair region within *SNAT2* intron-1 (13 CpG islands in total).

DNA methylation analysis. Genomic DNA was isolated by Trizol reagent (Invitrogen – Life Technologies, Cergy, France) following standard procedures. Sodium bisulfite conversion of DNA (700 ng) was performed using the EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA). Bisulfite-converted DNA was eluted with 30 µl of Elution Buffer (Thermo Fisher Scientific, Rockford, IL) and the concentration was determined by the NanoDrop ND1000 spectrophotometer (NanoDrop Technologies).

A PCR reaction was carried out using 20 ng of bisulfite-treated DNA, 10 pmol forward primer 5′-GAGGGATAATAGAGAGGGT-3′, and 10 pmol reverse biotinylated primer 5′-CTTCTTCATACT AAACACTA-3′, allowing the amplification of a 365-base-pair product located inside *SNAT2* intron 1 and containing 13 CpG sites. PCR conditions were as follows: 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 20 s for 45 cycles.

PCR products were used for the quantitative DNA methylation analyses by the Pyro Mark ID instrument (Biotage AB, Uppsala, Sweden) in the PSQ HS 96 System (Biotage AB), using the PyroGold SQA reagent kit (Biotage AB) according to the manufacturer's instructions.

Raw data were analyzed by Q-CpG software v1.0.9 (Biotage AB), which calculates the ratio of converted Cs (Ts) to unconverted Cs at each CpG, giving the percentage of methylation.

For each sample, the methylation value reported represents the mean methylation value of the 13 CpG sites and was obtained by at least two independent PCR and pyrosequencing experiments.

Intron-1 SNP: allelic discrimination by real-time PCR. The intron-1 rs10880961 SNP was genotyped using the Real Time 7500 Fast System Instrument (Applied Biosystems). The reaction mix (total volume 15 µl) contained 100 ng DNA, 1X SNP Genotyping Assay Mix, and 1X Taqman Genotyping Master Mix (Applied Biosystems). Amplification conditions were 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

Statistical analysis. Data concerning the clinical characteristics of the population, presented as mean ± SD, were compared using an unpaired Student's *t*-test.

SNAT2 gene expression data in normal and IUGR pregnancies were compared using a nonparametric Mann–Whitney *U*-test and a Kruskal–Wallis test.

SNAT2 immunohistochemical scores were compared using a nonparametric Mann–Whitney *U*-test. The statistical analysis of methylation levels was performed using an unpaired Student's *t*-test.

The χ^2 test for independent samples was used to determine whether the intron-1 SNP was associated with intrauterine growth restriction. The data were analyzed under a codominant, a dominant, and a recessive model, evaluating the allele frequency. Allele frequencies were calculated as follows:

Frequency = ((number of homozygote samples × 2) + (number of heterozygote samples))/(total number of samples × 2)

Differences between cases and controls were considered statistically significant when $P < 0.05$. The correlation between values was performed using the Spearman correlation. Correlations were considered significant when $P < 0.05$. All tests were performed using the statistical package SPSS (IBM SPSS Statistics, version 17.00 for Windows, Chicago, IL).

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