

Placental expression of serotonin transporter (*SERT*) gene: associations with maternal overweight/obesity and neonatal anthropometry

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Competing Interests

The authors declare that they have no competing interests.

Abstract

Objectives: We investigated the relationship between maternal pre-pregnancy body mass index (BMI) and expression of key serotonin-regulating genes (namely metabolic enzymes, transmembrane transporters, and receptors) in term placentas, including a possible moderating effect of glucose tolerance status (normal glucose tolerance (NGT) vs. gestational diabetes mellitus (GDM)). Associations between placental expression of serotonin-regulating genes and neonatal anthropometry were also explored.

Methods: The study included 105 women with overweight/obesity (OWO) and 111 women with normal-weight (NW), all giving birth at term by planned cesarean section. Placental tissue was collected from the fetal side using a standardized protocol. Expression of serotonin-regulating genes was quantified by RT-qPCR and/or ELISA.

Results: Pre-pregnancy OWO, GDM or their interaction were not associated with mRNA levels of tryptophan hydroxylase 1 (*TPH1*), monoamine-oxidase A (*MAOA*), organic cation transporter 3 (*OCT3*), and serotonin receptor 2A (*HTR2A*) in the term placenta. However, mRNA levels of plasma membrane monoamine transporter (*PMAT*) were significantly upregulated in association with pre-pregnancy OWO, regardless of GDM status ($p=0.014$). Furthermore, in women with NGT, but not in women with GDM, pre-pregnancy OWO was associated with decreased placental serotonin transporter (*SERT*) mRNA levels ($p=0.001$), while placental SERT protein levels were increased in women with pre-pregnancy OWO and further elevated in women with concurrent GDM ($p=0.005$). In addition, higher placental *SERT* mRNA levels negatively predicted birth weight and newborn length and, in women with NGT, partially mediated the association between pre-pregnancy BMI and birth weight.

Conclusion: The results show associations between maternal pre-pregnancy OWO and altered expression of high- and low-affinity serotonin transport genes (*SERT* and *PMAT*, respectively). Among the genes analyzed, *SERT* may play a role in linking maternal OWO to fetal growth. The results underscore the importance of further functional studies into the placental serotonin system in the context of maternal OWO.

Introduction

Body mass index (BMI), a common measure of overweight (BMI 25.0–29.9 kg/m²) and obesity (BMI ≥ 29.9 kg/m²), has increased significantly in women of childbearing age worldwide [1]. In Europe, 42.5% of adult women had overweight/obesity (OWO) in 2017-2018, with national rates ranging from 35.2% to 53.0% [2]. In Croatia, nearly a third (29.2%) of women who gave birth in 2017 had OWO at the start of pregnancy [3]. Elevated pre-pregnancy BMI is a known risk factor for gestational diabetes mellitus (GDM), preeclampsia, fetal macrosomia, preterm birth, cesarean section and perinatal mortality [4,5]. In addition, it increases the lifelong risk of obesity, diabetes, cardiovascular disease, and mental health disorders in the offspring [6,7]. As obesity rates continue to rise [8], it is critical to identify the biological pathways through which maternal OWO influences pregnancy outcomes to pave the way for interventions that mitigate the negative effects on mother and child.

The placenta plays a central role in these pathways by mediating nutrient and waste exchange between the maternal and fetal circulations and providing essential regulatory factors for maternal physiological adaptations and proper fetal development [9]. Maternal OWO affects the placenta, including its size, structure, vascular function, inflammatory status, endocrine activity, redox balance, glucose and lipid handling, and nutrient transport [10–14], all of which may be critical for obesity-related pregnancy complications and long-term health risks in the offspring. However, the effects of maternal OWO on the signaling systems that regulate placental development and function are poorly understood.

One signaling system of particular relevance is the placental serotonin (5-hydroxytryptamine, 5-HT) system. Serotonin, a small signaling monoamine, controls trophoblast proliferation and apoptosis [15], regulates umbilico-placental blood flow [16], and influences placental hormone production [17]. Studies in mice suggest that placental serotonin system also regulates nutrient transport [18], supplies the embryo/fetus with serotonin needed for proper (neuro)development [19,20], and mediates the effects of maternal inflammation on fetal brain development [21]. Disruption of serotonin homeostasis in the placenta could therefore be an important mechanism linking maternal OWO to altered placental function and unfavorable pregnancy outcomes.

Serotonin homeostasis is maintained by a network of genes/proteins responsible for its synthesis, degradation, transmembrane transport and signal transduction – collectively referred to as serotonin-regulating genes. While different studies have reported the expression of various serotonin-regulating genes in the human placenta (reviewed in [22]), the evidence for serotonin-synthesizing enzymes remains inconsistent, with some studies demonstrating their presence (e.g. [23]) and others their absence (e.g. [24]). On the other hand, serotonin transporter (SERT), a high-affinity serotonin carrier that plays a critical role in regulating local serotonin bioavailability, is abundant in the placenta. It is localized to cytotrophoblasts and the apical (brush border) membrane of syncytiotrophoblasts, where it mediates serotonin uptake from maternal blood [24,25]. In mice, *Sert* deletion causes placental structural abnormalities [15] and impairs histone serotonylation, leading to placental gene expression changes and altered offspring brain development [26], while in humans, maternal use of SERT-inhibiting antidepressants is associated with an increased risk of placental vascular problems [27]. These findings suggest that *SERT* is essential for normal placental function and fetal development, and that its dysregulation may contribute to adverse pregnancy outcomes.

Alterations in placental expression or activity of some of the serotonin-regulating genes have been reported in pregnancy complications such as preeclampsia, GDM, fetal growth restriction, and preterm birth (reviewed in [22]). Understanding these effects is of particular interest given that many drugs targeting serotonin system components are available and already used in various clinical contexts [28]. However, no study to date has examined the relationship between maternal pre-pregnancy OWO and expression of serotonin-regulating genes in the placenta.

We hypothesized that maternal pre-pregnancy OWO might affect expression of serotonin-regulating genes in the placenta, particularly those involved in serotonin transport. Therefore, we aimed to investigate the relationship between maternal pre-pregnancy OWO and expression of serotonin-regulating genes in term human placentas, also examining a possible moderating effect of GDM, one of the most common complications of maternal OWO, typically diagnosed in the late second trimester [29]. Given the evidence from a mouse model for the role of serotonin in regulating placental nutrient transfer [18], our second aim was to investigate whether placental expression of serotonin-regulating genes is associated with neonatal anthropometric outcomes.

Subjects and methods

Study participants

The study participants were part of the PlaNS (Placental and Neonatal Serotonin) birth cohort established in Zagreb, Croatia (project code: IP-2018-01-6547) [30,31]. The inclusion criteria for the cohort were planned cesarean section and the absence of any type of diabetes before pregnancy. Exclusion criteria for the present study were multiple pregnancies, preterm birth, intrauterine growth restriction, macrosomia (birth weight above 4500 g [32]), and congenital anomalies. The study included 111 women with normal weight (NW; BMI 18.0–24.9 kg/m²) and 105 women with OWO (BMI ≥ 25.0 kg/m²). The height and weight data used to calculate pre-pregnancy BMI were obtained from medical records and additionally confirmed in the questionnaires completed by the participants. Both the NW and OWO groups were adjusted to a similar proportion of women with normal glucose tolerance (NGT) and GDM and male and female newborns. GDM was diagnosed according to the International Association of Diabetes and Pregnancy Study Groups (IADPSG) recommendations [33] applied in Croatian clinical setting [34]. All women with GDM followed a diabetic diet. Three (2.7%) women in the NW group and eight (7.6%) women in the OWO group received oral antidiabetics, and one in each group (<1.0%) received insulin. None of the women received antidepressants or other serotonin-targeting medications during pregnancy, and all reported taking standard prenatal supplements. Women were categorized as smokers if they had smoked or quit during pregnancy and as non-smokers if they had never smoked or had quit at least 6 months before pregnancy; unclear cases were excluded. Gestational age was determined based on the last menstruation and adjusted according to established guidelines [35]. Ponderal index was calculated from the newborns' weight and length, both measured immediately after birth in a standardized manner.

Placental sample collection

Placental tissue samples were collected from the fetal side within 5 minutes after birth using a standardized procedure [30,36]. For mRNA analyses, the samples were preserved in RNAlater Solution (Qiagen, Hilden, Germany) and stored at -80°C. For protein analysis, the native placental tissue was snap-frozen and stored at -80°C.

mRNA expression analyses

mRNA levels were quantified using reverse transcription – quantitative real-time PCR (RT–qPCR) in accordance with MIQE guidelines [37]. Total RNA was extracted from 11–13 mg of RNAlater-preserved placental tissue using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). RNA concentration and purity were determined spectrophotometrically (NanoPhotometer® N60/N50, Implen, Munich, Germany). RNA integrity was verified by electrophoresis, as described [36]. RNA samples were stored at -80°C. cDNA was synthesized from 1500 ng of RNA in a total volume of 20 µl, using High Capacity RNA to cDNA Synthesis Kit (Applied Biosystems, Waltham, MA, USA). Control reactions without reverse transcriptase were included to check for genomic DNA contamination. Sequences of primers targeting tryptophan hydroxylase 1 (*TPH1*), tryptophan hydroxylase 2 (*TPH2*), monoamine oxidase A (*MAOA*), monoamine oxidase B (*MAOB*), serotonin transporter (*SERT*), organic cation transporter 3 (*OCT3*), plasma membrane monoamine transporter (*PMAT*), serotonin receptor type 2A (*HTR2A*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), actin beta (*ACTB*) and ubiquitin C (*UBC*) cDNAs are listed in **Table S1**. Primers were purchased from Metabion (Planegg, Germany). qPCR assays were performed with Fast SYBR Green Master Mix, on StepOne Real-Time PCR System (both from Applied Biosystems, Waltham, MA, USA). Each plate included a reference sample and reactions were performed in duplicate to quadruplicate. The specificity of amplicons was monitored by agarose gel electrophoresis and melting curve analysis. Of the three reference genes tested (*ACTB*, *UBC*, *YWHAZ*), *YWHAZ* was the most stable according to RefFinder [38] and was therefore used for normalization. qPCR efficiencies of reference and target genes were comparable (slope < 0.1 for all targets) and relative expression was calculated using the comparative C_q ($\Delta\Delta C_q$) method [39].

SERT protein quantification

Placental tissue (15 mg per sample) was homogenized in membrane protein extraction buffer (Cloud-clone Corp., Katy, TX, SAD) with protease inhibitors (Halt™ Protease Inhibitor Cocktail, 100X), at a ratio of 1 (tissue mass in g) : 20 (buffer volume in mL), using a mechanical homogenizer (Tehtnica, Slovenia), followed by sonication (B. Braun Biotech International, Melsungen, Germany). The homogenates were centrifuged (10 minutes, 10,000 g, 4 °C) and SERT protein concentrations in the resulting supernatants were quantified using the enzyme-linked immunosorbent assay (ELISA) kit (Cloud-clone Corp., Katy, TX,

USA) according to the manufacturer's instructions. SERT protein content was expressed per total protein content determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Foster City, CA, SAD).

Analyses of blood metabolic parameters

Blood metabolic parameters at the end of pregnancy (on the day of cesarean section or 1-3 days before) were assessed in subgroups of women with NW (N=76) and OWO (N=78). Blood samples were collected in the morning after an overnight fast (VACUETTE® TUBE 5 ml CAT Serum Separator Clot Activator, Greiner Bio-One, Kremsmünster, Austria). Glucose, triglyceride, total cholesterol and high-density lipoprotein (HDL) cholesterol concentrations were measured using Alinity C analyzer (Abbott, Chicago, IL, USA). Low-density lipoprotein (LDL) cholesterol concentrations were calculated based on total and HDL cholesterol levels. C-peptide concentrations were measured using Cobas c 501 analyzer (Roche Diagnostics, Indianapolis, IN, SAD).

Statistical analyses

Statistical analyses were conducted using GraphPad Prism v.8 (San Diego, CA, USA) or IBM SPSS Statistics (Chicago, IL, USA). Data normality was assessed by D'Agostino-Pearson test. Potential outliers were identified using Grubb's and ROUT method. Fisher's exact test (FET) was used for frequency comparisons. Group differences were assessed with Student's t-test, Mann-Whitney test, one-way ANOVA, or Kruskal-Wallis test. Bivariate correlations were analyzed using Pearson's or Spearman's correlation. Multiple linear regression analyses were performed to account for covariates and explore more complex relationships. Residuals were tested for normality and homoscedasticity. *TPH1*, *SERT*, *OCT3*, *PMAT* and *HTR2A* mRNA levels were log₁₀-transformed to meet parametric assumptions. Covariates were selected based on theoretical relevance and bivariate associations with the outcome. Moderation and moderated mediation analyses were performed using the Hayes PROCESS macro v4.2 in SPSS [40]. Model 1 was used to investigate the association between maternal pre-pregnancy OWO (predictor) and placental mRNA levels (outcome), with GDM diagnosis as a moderator. Model 8 was used to investigate whether placental *SERT* mRNA levels (mediator) mediate the association between maternal pre-pregnancy OWO (predictor) and newborns' birth weight (outcome), with GDM diagnosis moderating the path from predictor to mediator and the direct (non-mediated) path from predictor to outcome. A bootstrapping

method estimated the index of moderated mediation and conditional indirect effects, with significance determined when the 95% confidence intervals (CIs) excluded zero. Power calculations using G*Power [41] indicated a minimum sample size of 88 to detect an effect size of 0.15 with α of 0.05, a power of 90% and 8 predictors. All statistical tests were two-sided, with a p -value ≤ 0.05 considered statistically significant.

Results

Characteristics of the study participants

According to the study design, both the NW and OWO groups had similar proportions of women with normal glucose tolerance (NGT) and GDM, as well as male and female newborns (**Table 1**). Maternal age, smoking status, gestational age, and neonatal birth length did not differ between the NW and OWO groups. However, the proportion of multiparous women and neonatal birth weight and ponderal index were higher in the OWO group, while gestational weight gain was lower in the OWO group compared to the NW group. In a subgroup of women with available blood metabolic parameters (N=154), C-peptide levels were higher in the OWO group, while total cholesterol and HDL cholesterol levels were lower in the OWO group compared to the NW group (**Table 1**).

Expression of mRNAs of serotonin-regulating genes in human term placentas

Preliminary expression analysis in placenta samples (**Figure S1**) showed low levels of *TPH1* mRNA (encoding the peripheral serotonin-synthesizing enzyme) and no detection of *TPH2* mRNA (encoding the central serotonin-synthesizing enzyme). Of the serotonin-catabolizing enzymes, *MAOA* mRNA (encoding isoform that preferentially oxidizes serotonin) was almost 200 times more abundant than *MAOB* mRNA (encoding isoform that preferentially oxidizes other monoamines). *SERT* and *OCT3* mRNAs (encoding transmembrane carriers with high- and low-affinity for serotonin, respectively) were also abundant, while *PMAT* mRNA (encoding another transmembrane carrier with low-affinity for serotonin) and *HTR2A* mRNA (encoding serotonin receptor type 2A) were present at relatively low levels. Based on these findings, further analyses focused on *TPH1*, *MAOA*, *SERT*, *OCT3*, *PMAT* and *HTR2A*. Interestingly, mRNA levels of the key synthesizing and catabolizing enzymes of serotonin (*TPH1* and *MAOA*, respectively) were inversely correlated and both correlated (positively and negatively, respectively) with

OCT3 and *PMAT* mRNA levels, while *OCT3* and *PMAT* mRNA levels were also positively correlated (**Table S2**).

Table 1. Demographic and clinical characteristics of mothers and newborns stratified by maternal pre-pregnancy BMI status.

	NORMAL WEIGHT (N=111)	OVERWEIGHT/OBESITY (N=105)	p-value
Maternal characteristics			
GDM diagnosis, N (%)	51 (46.0)	56 (53.3)	0.341 ^a
Age at childbirth, years	33.7 [31.1 – 37.9]	33.3 [29.9 – 37.4]	0.545 ^b
Pre-pregnancy BMI, kg/m ²	22.0 [20.9 – 23.1]	30.0 [27.1 – 34.2]	<0.0001 ^c
Gestational weight gain, kg	14 [11 – 17]	11 [8 – 16]	0.002 ^c
Primiparous women, N (%)	51 (46.0)	32 (30.5)	0.025 ^a
Smoking in pregnancy, N (%) ^d	26 (24.3)	28 (27.7)	0.636 ^a
Blood metabolic parameters ^e			
Glucose (mmol/L)	4.00 [3.78 – 4.40]	4.06 [3.79 – 4.50]	0.634 ^c
C-peptide (nmol/L)	0.85 [0.72 – 1.03]	1.13 [0.94 – 1.41]	< 0.0001 ^c
Triglycerides (mmol/L)	2.92 [2.48 – 3.77]	3.26 [2.48 – 4.04]	0.236 ^c
Total cholesterol (mmol/L)	7.34 [6.59 – 7.99]	6.76 [5.88 – 7.80]	0.014 ^b
HDL cholesterol (mmol/L)	1.89 [1.52 – 2.19]	1.66 [1.44 – 1.98]	0.003 ^c
LDL cholesterol (mmol/L)	4.27 [3.51 – 4.90]	3.69 [2.95 – 4.89]	0.124 ^b
Newborn characteristics			
Male sex assigned at birth, N (%)	52 (46.9)	56 (53.3)	0.414 ^a
Gestational age, weeks	39.4 [38.7 – 39.9]	39.1 [38.7 – 39.7]	0.130 ^c
Birth weight, g	3410 [3160 – 3730]	3570 [3265 – 3900]	0.031 ^b
Birth length, cm	50 [48 – 51]	50 [49 – 51]	0.342 ^c
Ponderal index, g/cm ³	2.77 [2.66-2.94]	2.86 [2.69-3.02]	0.048 ^c

Continuous variables are presented as median [interquartile range], and categorical variables as number of subjects (N) and percentage (%). *p*-values were calculated using ^aFisher's exact test, ^bStudent's t-test, ^c Mann-Whitney test (statistically significant values are in bold). ^dData were missing for 4 women with normal weight and 4 women with overweight/obesity. ^eData for subgroups of women with normal weight (N=76) and overweight/obesity (N=78) are shown. BMI, body mass index; GDM, gestational diabetes mellitus.

Bivariate analyses showed association of primiparity with lower *TPH1* (*p*=0.004) and *OCT3* (*p*=0.017) and higher *MAOA* (*p*=0.001) mRNA levels. Smoking during pregnancy was associated with lower *TPH1* mRNA

levels ($p=0.016$). Gestational age was negatively correlated with *SERT* mRNA levels ($p=0.016$), while pre-pregnancy BMI was positively correlated with *PMAT* mRNA levels ($p=0.001$). No significant associations were found for maternal age, GDM diagnosis, gestational weight gain, and fetal sex (all $p>0.05$).

In a subset of women with available blood metabolic parameters at the end of pregnancy, we observed statistically significant, albeit very weak, correlations between certain blood lipids and mRNA levels of serotonin-regulating genes in the placenta: triglycerides were positively correlated with *SERT* mRNA levels and HDL cholesterol with *OCT3* mRNA levels, while LDL cholesterol and total cholesterol were negatively correlated with *PMAT* and *HTR2A* mRNA levels (**Table 2**).

Table 2. Correlation between maternal blood metabolic parameters and placental mRNA levels of serotonin-regulating genes (N=154).

	Glucose	C-peptide	Triglycerides	Total cholesterol	HDL-cholesterol	LDL-cholesterol
TPH1						
r	-0.04	-0.11	-0.08	-0.10	0.07	-0.09
95% CI	-0.17, 0.16	-0.20, 0.13	-0.24, 0.08	-0.25, 0.07	-0.10, 0.23	-0.25, 0.08
p-value	0.940	0.638	0.330	0.240	0.397	0.275
MAOA						
r	-0.05	-0.06	-0.06	0.01	0.01	0.06
95% CI	-0.20, 0.12	-0.21, 0.11	-0.22, 0.11	-0.15, 0.16	-0.15, 0.17	-0.11, 0.22
p-value	0.621	0.506	0.479	0.945	0.924	0.497
SERT						
r	-0.03	-0.01	0.19	0.09	-0.08	0.12
95% CI	-0.24, 0.09	-0.19, 0.13	0.02, 0.34	-0.07, 0.25	-0.24, 0.09	-0.04, 0.28
p-value	0.343	0.708	0.020	0.266	0.348	0.132
OCT3						
r	0.03	-0.02	-0.10	0.09	0.18	0.09
95% CI	-0.24, 0.09	-0.14, 0.19	-0.26, 0.07	-0.08, 0.25	0.02, 0.34	-0.08, 0.25
p-value	0.372	0.726	0.234	0.267	0.025	0.302
PMAT						
r	-0.03	0.08	-0.12	-0.16	-0.13	-0.21
95% CI	-0.10, 0.23	-0.19, 0.14	-0.28, 0.05	-0.32, 0.00	-0.29, 0.03	-0.36, -0.04
p-value	0.412	0.738	0.150	0.049	0.103	0.011
HTR2A						
r	-0.05	-0.04	-0.09	-0.21	0.04	-0.22
95% CI	-0.09, 0.23	-0.21, 0.12	-0.25, 0.07	-0.36, -0.05	-0.12, 0.20	-0.37, -0.06
p-value	0.373	0.569	0.268	0.008	0.631	0.006

Shown are Spearman or Pearson correlation coefficients (r) with 95% confidence intervals (CI). Statistically significant results are in bold.

Relationship between maternal pre-pregnancy BMI and expression of serotonin-regulating genes in term placentas

To investigate the association between maternal pre-pregnancy BMI and expression of serotonin-regulating genes in term placentas, we performed multiple linear regression analyses with pre-pregnancy body weight status (pBWS; NW vs. OWO) as predictor, mRNA levels of serotonin-regulating genes as outcome, and glucose tolerance status (GTS; NGT vs. GDM) as moderator. Maternal pBWS was a significant predictor of placental *SERT* mRNA levels, with OWO associated with lower levels (**Table 3**). The interaction between pBWS and GTS was also significant and conditional effect analysis showed that OWO was associated with lower *SERT* mRNA levels in women with NGT ($B=-0.06$; 95% CI: -0.10, -0.03; $p=0.001$), but not in women with GDM ($B=0.01$; 95% CI: -0.03, 0.05; $p=0.645$). pBWS also predicted *PMAT* mRNA levels, with OWO associated with higher levels, independent of GTS, while pBWS, GTS or their interaction did not predict placental mRNA levels of other genes studied (**Table 3**).

Consistent with the above results, pre-pregnancy BMI was negatively correlated with *SERT* mRNA levels in women with NGT ($r_s=-0.20$; 95% CI: -0.38, -0.01; $p=0.035$), but not in women with GDM ($r_s=-0.01$; 95% CI: -0.21, 0.18; $p=0.883$), while it was positively correlated with *PMAT* mRNA levels in both women with NGT ($r_s=0.24$; 95% CI: 0.04, 0.42; $p=0.014$) and women with GDM ($r_s=0.21$; 95% CI: 0.01, 0.39; $p=0.035$). No significant correlations were found between pre-pregnancy BMI and mRNA levels of *TPH1*, *MAOA*, *OCT3* or *HTR2A* (**Table S3**). Excluding potential outliers from the analyses did not change the results.

To further investigate the relationship between maternal pre-pregnancy BMI and *SERT* expression in term placenta, we quantified placental SERT protein levels in a subset of participants (**Figure 1**). *SERT* mRNA results in this subset aligned with those in the entire cohort (**Figure S2**), showing decreased levels in women with OWO and NGT (**Figure 1A**). However, SERT protein levels were higher in women with OWO compared to women with NW (**Figure 1B**) and increased linearly across the groups of women with NW and NGT, OWO and NGT, and OWO and GDM ($p=0.005$, test for linear trend following ANOVA). Despite this contrasting pattern between mRNA and protein results, a significant positive correlation between mRNA and protein levels was found in all metabolic groups (**Figure 1C**), with similar slopes of regression lines (0.016 for women with NW and NGT; 0.015 for women with OWO and NGT; 0.013 for women with OWO and GDM), indicating that mRNA to protein processes were not significantly affected by maternal metabolic states.

Table 3. Association between maternal pre-pregnancy body weight status (pBWS; normal weight vs. overweight/obesity) and mRNA levels of serotonin-regulating genes in term placentas, with glucose tolerance status (GTS; normal glucose tolerance vs. gestational diabetes mellitus) as a moderator.

	B	95% CI	SE	t	p-value
TPH1 mRNA					
pBWS	0.03	-0.02, 0.09	0.03	1.15	0.251
GTS	0.03	-0.03, 0.08	0.03	0.94	0.349
Interaction pBWS × GTS	-0.05	-0.12, 0.03	0.04	-1.12	0.265
Parity	0.06	0.02, 0.10	0.02	2.72	0.007
Smoking status	-0.06	-0.10, -0.01	0.02	-2.44	0.015
$R^2=0.076; F(5, 202)=3.32, p=0.007$					
MAOA mRNA					
pBWS	-0.04	-0.17, 0.09	0.07	-0.59	0.558
GTS	-0.10	-0.23, 0.03	0.07	-1.46	0.145
Interaction pBWS × GTS	0.15	-0.04, 0.33	0.10	1.52	0.129
Parity	-0.17	-0.27, -0.07	0.05	-3.43	0.001
$R^2=0.066; F(4, 211)=3.73, p=0.006$					
SERT mRNA					
pBWS	-0.06	-0.10, -0.03	0.02	-3.36	0.001
GTS	-0.02	-0.06, 0.02	0.02	-1.00	0.319
Interaction pBWS × GTS	0.07	0.02, 0.13	0.03	2.69	0.008
Gestational age	-0.04	-0.05, -0.02	0.01	-4.93	<0.0001
$R^2=0.156; F(4, 211)=9.74, p<0.0001$					
OCT3 mRNA					
pBWS	0.06	-0.03, 0.14	0.04	1.36	0.177
GTS	0.04	-0.04, 0.13	0.04	1.03	0.302
Interaction pBWS × GTS	-0.12	-0.23, 0.00	0.06	-1.92	0.057
Parity	0.08	0.02, 0.14	0.03	2.46	0.015
$R^2=0.005; F(4, 206)=2.58, p=0.038$					
PMAT mRNA					
pBWS	0.15	0.03, 0.27	0.06	2.49	0.014
GTS	0.05	-0.06, 0.17	0.06	0.91	0.363
Interaction pBWS × GTS	-0.07	-0.24, 0.09	0.09	-0.87	0.387
$R^2=0.038; F(3, 206)=2.73, p=0.045$					
HTR2A mRNA					
pBWS	-0.03	-0.11, 0.06	0.04	-0.65	0.516
GTS	-0.03	-0.11, 0.06	0.04	-0.62	0.533
Interaction pBWS × GTS	0.04	-0.08, 0.16	0.06	0.65	0.515
$R^2=0.003; F(3, 212)=0.19, p=0.904$					

Analyses were adjusted for covariates associated with each mRNA level in bivariate analyses. Significant *p*-values are in bold. Similar results were obtained in unadjusted analyses (**Table S4**) and in analyses adjusted for parity, gestational age and smoking in all models (**Table S5**). B, unstandardized beta coefficient; CI, confidence interval; SE, standard error of B.

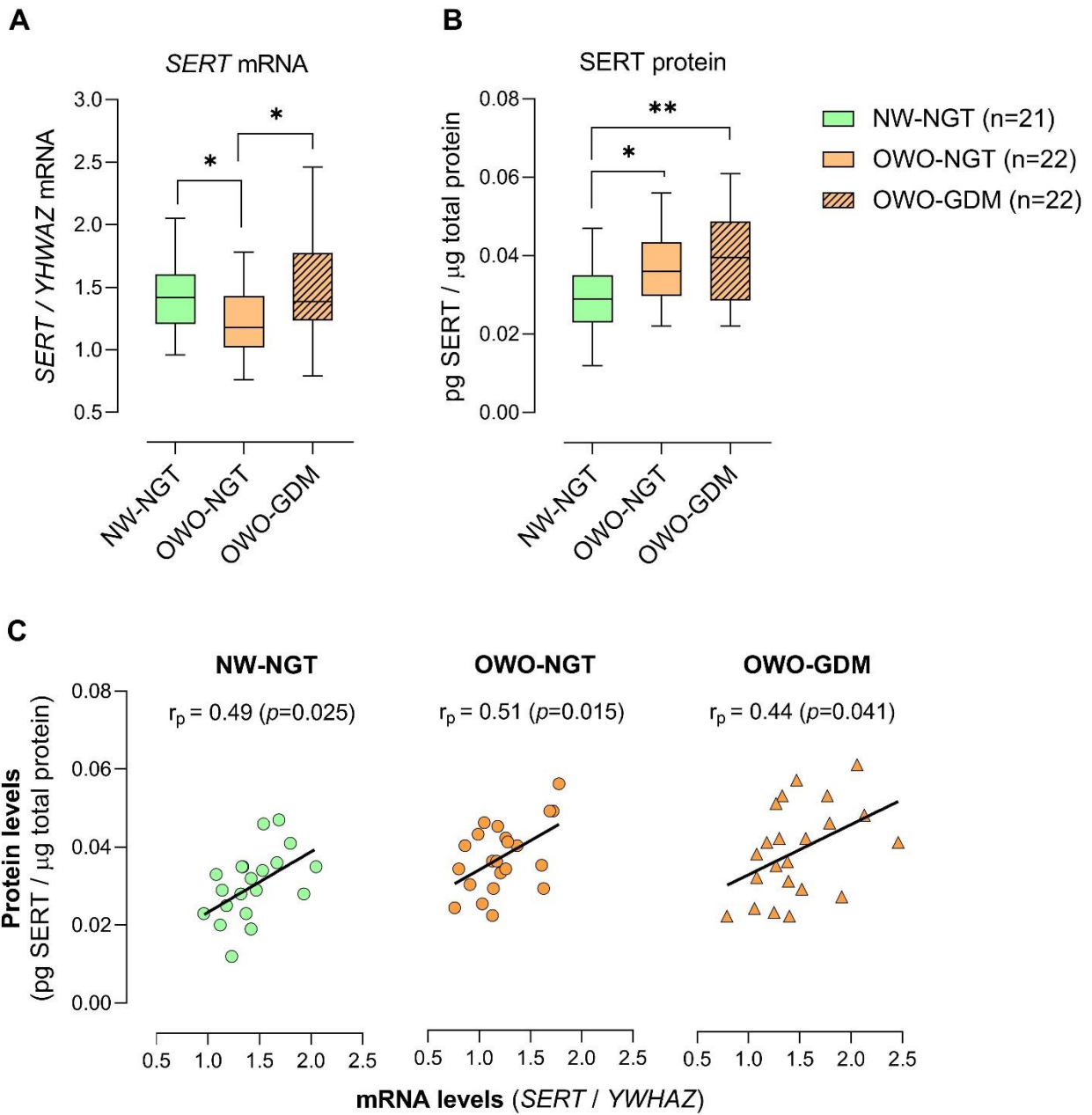


Figure 1. Expression of the serotonin transporter gene (*SERT*) in term placentas as a function of maternal metabolic state. **(A)** *SERT* mRNA levels, **(B)** *SERT* protein levels, and **(C)** their correlation in term placentas of women with normal-weight and normal glucose tolerance (NW-NGT, n=21), overweight/obesity and normal glucose tolerance (OWO-NGT, n=22), and overweight/obesity and gestational diabetes mellitus (OWO-GDM, n=22). **(A, B)** The boxes represent the interquartile range, with Tukey whiskers. Statistical analyses were performed with one-way ANOVA followed by Fisher's LSD test (* $p < 0.05$, ** $p < 0.01$). **(C)** Scatter plots and linear regression lines are shown. r_p , Pearson's correlation coefficient.

Placental mRNA levels of serotonin-regulating genes and neonatal anthropometry

Bivariate analyses showed a weak but significant inverse correlation between placental *SERT* mRNA levels and neonatal birth weight ($r_s=-0.20$; 95% CI: -0.33, -0.06; $p=0.003$) and birth length ($r_s=-0.16$; 95% CI: -0.29, -0.02; $p=0.019$). No correlation was observed between *SERT* mRNA levels and ponderal index, while *TPH1*, *MAOA*, *OCT3*, *PMAT* and *HTR2A* mRNA levels did not correlate with any of the neonatal anthropometric parameters (**Table S6**). In a multiple linear regression analyses adjusting for several relevant covariates, lower *SERT* mRNA levels remained a significant predictor of higher birth weight and length (**Table 4**).

Table 4. Results of the multiple linear regression analyses examining placental *SERT* mRNA levels as a predictor of neonatal birth weight and length (N=216).

Predictor	Birth weight				Birth length			
	B	95% CI	SE	<i>p</i> -value	B	95% CI	SE	<i>p</i> -value
<i>SERT</i> mRNA	-199	-349, -48	76.6	0.010	-0.68	-1.34, -0.02	0.33	0.044
Newborn sex	118	15, 222	52.5	0.025	0.87	0.42, 1.32	0.23	0.0002
Gestational age	120	57, 184	32.3	0.0002	0.28	0.00, 0.56	0.14	0.050
Pre-pregnancy BMI	10.5	2.4, 18.7	4.14	0.012	0.01	-0.02, 0.05	0.02	0.435
Smoking in pregnancy	-106	-225, 12	59.9	0.077	-0.52	-1.0, -0.00	0.26	0.049
	R ² =0.196 F (5, 202)=9.87, $p<0.0001$				R ² =0.140 F (5, 202)=6.60, $p<0.0001$			

Covariates were selected as described in the Methods section. Results were similar when maternal pre-pregnancy body weight status (normal weight vs. overweight/obesity) was included instead of pre-pregnancy body mass index (BMI). Significant *p*-values are in bold. B, unstandardized beta coefficient; CI, confidence interval; SE, standard error of B.

Since maternal pre-pregnancy BMI category was associated with both neonatal birth weight (**Table 1**) and, in women with NGT, placental *SERT* mRNA levels (**Table 3**), while placental *SERT* mRNA levels were also associated with birth weight (**Table 4**), we tested whether placental *SERT* mRNA levels (mediator) mediate the association between pre-pregnancy BMI category (predictor) and birth weight (outcome), with GDM moderating both the association between predictor and mediator (path a) and the direct association between predictor and outcome (path c') (**Figure 2A**). The index of moderated mediation supported a conditional indirect effect of pre-pregnancy BMI category on birth weight via placental *SERT* mRNA levels (B=-38, bootstrapped 95% CI: -94 to -1). Specifically, *SERT* mRNA levels mediated the association between pre-pregnancy OWO and higher birth weight in women with NGT, but not in

women with GDM (**Figure 2B, Table S7**). The direct effect of pre-pregnancy BMI category on birth weight was also significant only in women with NGT (**Figure 2C, Table S7**).

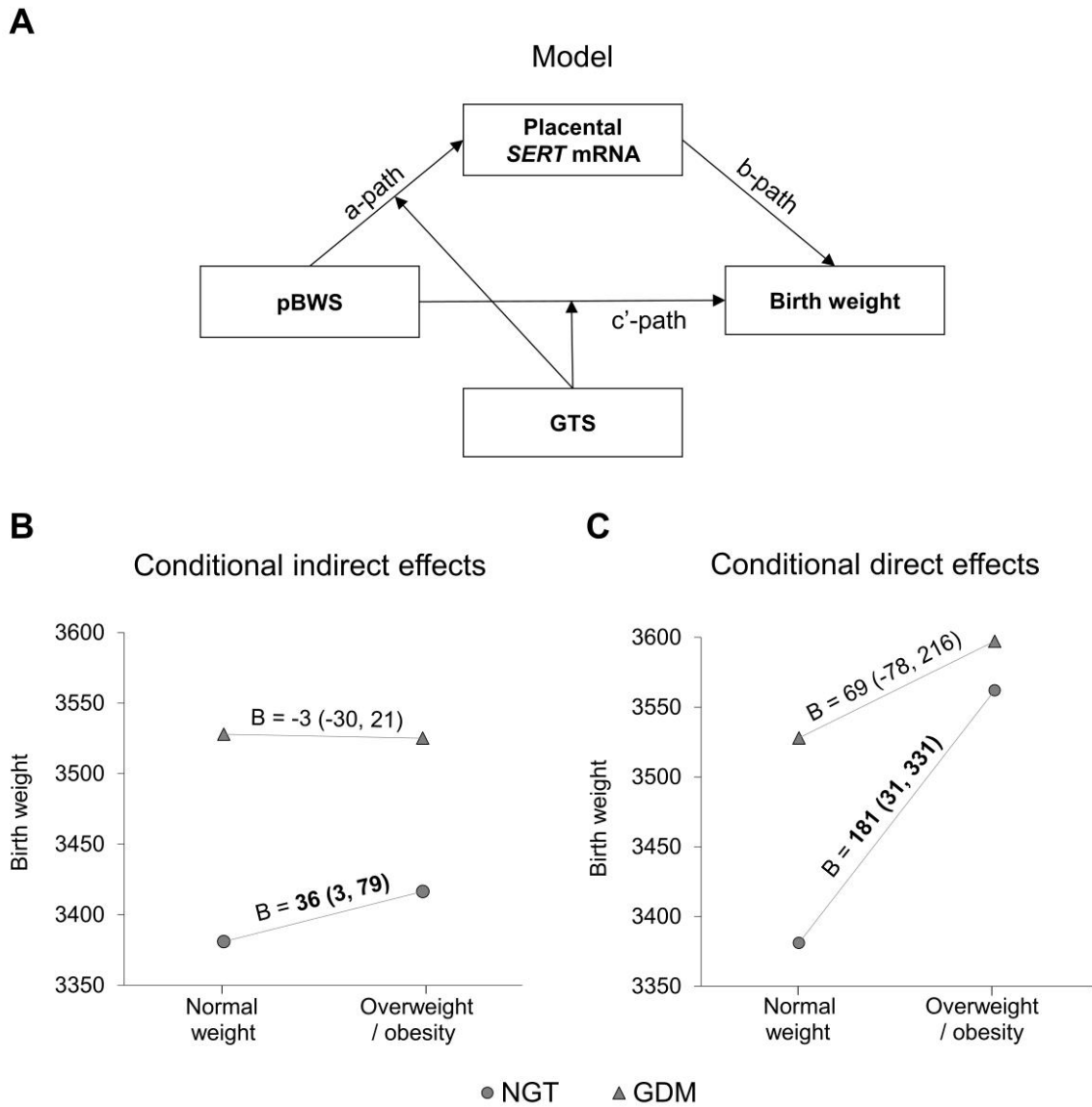


Figure 2. Moderated mediation analysis. **(A)** Conceptual model of placental *SERT* mRNA levels as a mediator between maternal pre-pregnancy body weight status (pBWS; normal-weight vs. overweight/obesity) and birth weight, with glucose tolerance status (GTS; normal glucose tolerance vs. gestational diabetes mellitus) as a moderator. The *c'*-path represents the direct effect of pBWS on birth weight. The model was adjusted for smoking, gestational age, and neonatal sex. **(B, C)** Conditional indirect and direct effects for women with normal glucose tolerance (NGT) and gestational diabetes mellitus (GDM). Shown are unstandardized regression coefficients (B) with 95% confidence intervals; statistically significant results are in bold. See Table S7 for details.

Discussion

This study is the first to systematically investigate, on a well-defined group of mothers and newborns, the relationship of pre-pregnancy BMI and GDM with expression of all relevant serotonin-regulating genes in term human placenta, including metabolic enzymes, transmembrane transporters and receptors for serotonin. We analyzed placentas from healthy full-term newborns born by planned cesarean section, avoiding potential biases due to preterm birth or birth mode.

We found very low expression of *TPH1* mRNA and no detectable levels of *TPH2* mRNA, whereas *MAOA*, *SERT* and *OCT3* mRNAs were abundantly expressed in the placenta. These results indicate a limited capacity for serotonin synthesis in the term placenta and, in agreement with previous reports [24,42,43], emphasize the key role of serotonin catabolism and transmembrane transport in maintaining serotonin homeostasis at the maternal-fetal interface at term.

Our results show disrupted *SERT* expression in placentas exposed to maternal OWO. In women with NGT, pre-pregnancy OWO was associated with downregulation of placental *SERT* mRNA levels. Previous transcriptomic analysis [44] reported widespread downregulation of differentially expressed genes in placentas of women with obesity compared to women with NW, but *SERT* was not among them. This is likely due to the small sample size (n=5 per group) [44], which may have limited the statistical power to detect the modest difference observed in our study between women with NW (n=60) and women with OWO (n=49) who did not have GDM.

The association between pre-pregnancy OWO and decreased placental *SERT* mRNA levels was not found in women with GDM, suggesting that GDM may attenuate the impact of pre-pregnancy BMI on *SERT* transcription or mRNA stability. While no previous study has examined the interaction between OWO and GDM, reports on placental *SERT* expression in relation to GDM are inconsistent [36,45,46]. Our finding that GDM was associated with increased *SERT* mRNA levels only in women with OWO highlights the complex interplay between these conditions and emphasize the need to consider both OWO and GDM when studying placental *SERT* regulation.

Although pre-pregnancy OWO was associated with decreased placental *SERT* mRNA levels in women without GDM, placental *SERT* protein levels were elevated in women with pre-pregnancy OWO and further increased in women with concomitant GDM. Positive correlations between mRNA and protein levels with similar regression slopes in all metabolic groups studied indicate that post-translational rather than pre-translational processes are likely to account for the elevated protein levels despite reduced mRNA levels. Indeed, it has been shown that defective insulin signaling can impair *SERT* trafficking in trophoblasts, leading to accumulation of *SERT* in the endoplasmic reticulum and decreased serotonin uptake [47]. Since we measured only total *SERT* protein levels, further studies are needed to assess how maternal metabolic states affect *SERT* localization and activity.

We also found that placental *SERT* mRNA levels negatively predicted birth weight and, in women with NGT, partially mediated the association between pre-pregnancy BMI and birth weight. Experimental evidence in mice supports a mechanistic role for *Sert* in fetal growth: deletion of *Sert* disrupts placental expression of multiple genes involved in maternal-fetal nutrient transfer [18]. In humans, maternal use of *SERT*-inhibiting antidepressants has been associated with suboptimal fetal growth [48], although meta-analyses are inconclusive [49], highlighting the complexity of this relationship. The finding that placental *SERT* mediates the association between pre-pregnancy BMI and birth weight only in women with NGT is consistent with a stronger influence of maternal obesity than GDM on birth weight [50].

In addition to *SERT*, *PMAT* expression in the placenta was also associated with maternal pre-pregnancy BMI, with higher BMI linked to higher *PMAT* mRNA levels, independent of GDM status. *PMAT* is a low-affinity, high-capacity serotonin carrier expressed in feto-placental endothelial cells that are in direct contact with fetal blood [43], potentially influencing fetal serotonin levels. While the present results indicate that *PMAT* expression is sensitive to maternal BMI status, further studies are needed to determine changes in *PMAT* protein and activity levels.

While placental mRNA levels of other genes were not predicted by pre-pregnancy OWO, GDM or their interaction, we found weak correlations between maternal lipid levels and mRNA levels of *SERT*, *PMAT*, *OCT3* and *HTR2A*. Elevated maternal triglycerides, total cholesterol, and LDL cholesterol were associated with increased placental weight and decreased placental efficiency [51,52]. In our study, higher maternal

triglyceride levels were associated with increased placental *SERT* mRNA levels, while higher total and LDL cholesterol levels were associated with lower *HTR2A* mRNA levels. These changes could attenuate *HTR2A*-mediated signaling by increasing serotonin clearance and decreasing receptor availability. Since *HTR2A* activation stimulates trophoblast proliferation [53,54], such changes may represent an adaptive mechanism to limit excessive placental growth under conditions of maternal lipid imbalance.

Mechanisms through which maternal metabolic conditions such as OWO and GDM could affect placental serotonin regulation may involve maternal metabolic, inflammatory and/or hormonal abnormalities. Our previous in vitro study has shown that alterations in glucose, oxygen and insulin modulate *SERT* expression in a first-trimester trophoblast cell line [55], suggesting that maternal metabolic abnormalities may influence placental serotonin regulation in early pregnancy. The present findings also highlight the potential role of maternal lipids in modulating placental expression of serotonin-regulating genes. Both OWO and GDM are often associated with a pro-inflammatory maternal milieu, which has been shown to alter placental serotonin regulation in mice [21]. In addition, sex hormones involved in both maternal metabolic adaptations and serotonin regulation may be potential mediators. Overall, these observations are consistent with a model in which metabolic, inflammatory, and hormonal changes associated with OWO and GDM alter the expression and/or activity of serotonin-regulating genes in the placenta, thereby influencing serotonin-mediated control of placental function, nutrient transfer, and ultimately fetal growth and development. This provides a framework for future mechanistic studies to test causal relationships between maternal metabolic states, placental serotonin homeostasis, and offspring outcomes.

Our study has several limitations that should be considered when interpreting the results and guiding future research. First, we examined only the expression levels of serotonin-regulating genes without measuring their functional activity. Since the activity of plasma membrane transporters such as *SERT* and *PMAT* is regulated by posttranslational mechanisms, our results should be interpreted as indicators of possible dysregulation rather than definitive evidence of altered function. Future studies assessing transporter activity are needed to fully understand the effects of maternal metabolic states on placental serotonin regulation. Second, our analyses were performed on whole placental tissue, which may obscure changes in serotonin regulation occurring in distinct placental cell populations [55,56]. Finally,

our analyses focused exclusively on term placentas. It is possible that the results would be different at earlier gestational stages, when serotonin plays a more pivotal role in placental and fetal development. Studies involving earlier gestational stages, such as first-trimester placental models, are needed to better understand the temporal dynamics of serotonin regulation and its impact on pregnancy outcomes.

Conclusion

Increased pre-pregnancy BMI is associated with altered expression of high- and low-affinity serotonin transporters in the term placenta. Specifically, it is associated with lower *SERT* mRNA levels, higher *PMAT* mRNA levels, and increased total SERT protein levels, with some of these associations being moderated by maternal GDM. The identification of placental *SERT* as a mediator between pre-pregnancy BMI and birth weight is an important contribution to understanding the molecular pathways linking maternal metabolism to fetal growth. The obtained results form the basis for further research on the placental serotonin system in maternal OWO. Future studies should investigate the functional activity of SERT and PMAT and the consequences of alterations in serotonin system components in the human placenta, including their effects on placental functions, fetal serotonin homeostasis, pregnancy complications, and long-term offspring health. This could lead to the identification of potential therapeutic targets to optimize placental function and mitigate adverse pregnancy outcomes associated with maternal OWO.

List of abbreviations

ACTB	actin beta
BMI	body mass index
ELISA	enzyme-linked immunosorbent assay
FET	Fisher's exact test
GDM	gestational diabetes mellitus
HDL	high-density lipoprotein
HTR2A	serotonin receptor type 2A
IADPSG	International Association of Diabetes and Pregnancy Study Groups
LDL	low-density lipoprotein
MAOA	monoamine oxidase A

MAOB	monoamine oxidase B
NGT	normal glucose tolerance
NW	normal weight
OCT3	organic cation transporter 3
OWO	overweight/obesity
pBWS	pre-pregnancy body weight status
PlaNS	Placental and Neonatal Serotonin
PMAT	plasma membrane monoamine transporter
RT-qPCR	reverse transcription – quantitative real-time PCR
SERT	serotonin transporter
TPH1	tryptophan hydroxylase 1
TPH2	tryptophan hydroxylase 2
UBC	ubiquitin C
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

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Author Contributions

Conceptualization: JŠ; Data curation: MH, MP, JŠ; Formal analysis: MP, MŽ, JŠ; Funding acquisition: JŠ; Investigation: MP, MH, MK; Methodology: MP, MŽ, LČŠ, JŠ; Project administration: JŠ; Writing original draft: MP, JŠ; Writing review & editing: LČŠ, JŠ. All authors approved final version.

Competing Interests

The authors declare that they have no competing interests.

Data Availability Statement

The PlaNS datasets analyzed in the current study are not publicly available because consent for public release of data was not obtained from participants. However, data to generate figures and tables are available from the corresponding author on reasonable request and with appropriate permission from the PlaNS study team and investigators.

Supplementary Information

Supplementary material (single .pdf file) includes: gene-specific primers for RT-qPCR, images of serotonin-regulating gene expression, and tables with intercorrelation of mRNA levels, correlations between pre-pregnancy BMI and mRNA levels, linear regression results (unadjusted and adjusted models), correlations between gene expression and neonatal anthropometry, and results of moderated mediation analysis.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the University Clinical Hospital Centre Zagreb (class: 8.1-18/162-2, number: 02/21 AG, approved on 18.07.2018) and the Bioethics Committee of the Ruđer Bošković Institute, Zagreb (BEP-8761/2-2018, approved on 26.11.2018). Written informed consent to participate in the study was obtained from all participants. All procedures complied with the ethical standards outlined in the Declaration of Helsinki.

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Subject Ontology

Obesity, pregnancy, placenta, 5-hydroxytryptamine