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Added sugar intake during pregnancy: Fetal behavior, birth outcomes, and placental DNA methylation

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Abstract

Pregnancy is a critical time for the effects of environmental factors on children's development. The effect of added sugar intake on fetal development and pregnancy outcomes remains understudied despite increasing dietary intake in the United States. This study investigated the effect of added sugar on fetal programming by examining the association between maternal added sugar consumption, fetal movement, birth outcomes, and placental DNA methylation. Further, primary human fibroblasts were cultured under normal or high glucose conditions to assess the effect of high glucose exposure on cells' DNA methylation. We found that higher added sugar intake across pregnancy was associated with reduced 3rd-trimester fetal movement ($p < .05$) and shorter gestation ($p < .01$). Our sample size was not powered to detect the alteration of individual placental CpG with genome-wide significance. However, a secondary analysis suggested that added sugar consumption was associated with differential methylation of functionally related gene families across pregnancy. Consistent with this, high glucose exposure in primary cultured human fibroblasts altered the methylation of 17% of all CpGs, providing converging evidence for an effect of sugar on DNA methylation. Our results suggest that diets high in added sugar during pregnancy may have implications for offspring health via prenatal programming effects measurable before birth.

KEYWORDS
added sugar, birth outcomes, DNA methylation, fetal behavior, placenta, pregnancy

1 | INTRODUCTION

The average consumption of added sugar has increased remarkably in recent years and more than half of the U.S. population now exceeds the recommended daily limit (>10% of calories per day; Steele et al., 2016) with pregnant women consuming on average 50% more added sugar than the daily recommended levels (Cioffi et al., 2018). Added sugars are incorporated into food and beverages during their preparation and the greatest source of sugar intake in the U.S. diet comes from sugar-sweetened beverages (Cioffi et al., 2018; Steele et al., 2016). In the general population, excessive added sugar intake has been associated with higher risk of weight gain, obesity, type

two diabetes, and metabolic syndrome (Popkin & Hawkes, 2016; Te Morenga et al., 2013; Vartanian et al., 2007).

Studies in animals and humans informed by the "developmental origins of health and disease" (DOHaD) model show that metabolic insults during pregnancy, such as maternal diabetes, obesity, and overnutrition increase the offspring's risk of developing non-communicable diseases, including type two diabetes (Gluckman et al., 2008). Health outcomes related to increased added sugar consumption during pregnancy, such as hyperglycemia and maternal diabetes, have been associated with pregnancy complications, long term offspring risk for obesity (Dabelea et al., 2000; Okubo et al., 2014), type two diabetes (Dabelea et al., 2000), and metabolic syndrome (Boney et al., 2005). Despite this, studies on the impact of added sugar intake during pregnancy on pregnancy and offsprings outcomes are scarce, but some evidence suggest higher risks gestational diabetes (Donazar-Ezcurra et al., 2018), excessive gestational weight gain (Maslova et al., 2015), preeclampsia (Borgen et al., 2012; Clausen et al., 2001; Cohen et al., 2012), increased offspring weight (Renault et al., 2015), small for gestational age offspring (Lenders et al., 1994), and earlier birth (Grieger et al., 2014; Lenders et al., 1994), all of which are risk factors for long term altered health in the offspring (Gluckman et al., 2008; Hanson & Gluckman, 2014).

Higher risk of neurodevelopmental impairments have been associated with obesogenic and hyperglycemia environments in pregnancy (Adane et al., 2016; Van Lieshout, 2013; Robles et al., 2015), but little is known about the consequences of added sugar in pregnancy specifically. In mice, a study showed an increased risk of offspring hyperactivity behavior secondary to maternal high sucrose diet (Choi et al., 2015). In humans, one study found lower cognitive scores in children of women who had high sucrose intake during pregnancy (Cohen et al., 2018). Fetal behavioral assessment is a promising method for detecting individual differences in development during prenatal exposure, with the advantage of removing the confound of postnatal environmental factors in the investigation of the determinants of child development. For example, greater fetal movement predicts increased psychomotor activity in infancy and reduced behavioral inhibition at 2 years old (DiPietro et al., 2002). While acute induction of maternal hyperglycemia has been associated with a decrease in fetal movement (Edelberg et al., 1987), no previous study has examined the effects of habitually elevated added sugar consumption in relation to this early index of behavioral development. This is of particular significance since added sugar consumption is a modifiable behavior.

Among the biological mechanisms proposed to explain *in utero* programming, epigenetic adaptation to environmental exposure is a candidate (Monk et al., 2019). Fetal development is dependent on placental function, which includes exchanges of nutrients, gases, and waste during pregnancy. Adequate placenta function relies on its proper development which is regulated by epigenetic mechanisms including DNA methylation. Epigenetic processes enable a static genome to adapt to the fluctuating maternal environment. Maternal diabetes and hyperglycemia, which manifest as elevated levels of blood sugar, have been found to influence the placental DNA methylation of several genes involved in metabolism (Bouchard et al., 2010, 2012; Cardenas et al., 2018; Côté et al., 2016; Houde et al., 2013; Lesseur et al., 2014; Xie et al., 2015). Despite this, to our knowledge, no previous research has investigated the association between added sugar dietary consumption and placenta DNA methylation.

In the present study, we first assessed the association between habitual added sugar consumption during pregnancy—defined as the average intake over pregnancy—with fetal movement and birth outcomes. Then we conducted an exploratory analysis to examine whether habitual added sugar consumption is associated with differential placental DNA methylation. We considered added sugar intake at each trimester in relation to differentially methylated CpG sites using gene set enrichment analysis and functional annotation (Subramanian et al., 2005) to identify candidate pathways and biological processes that could be associated with added sugar intake. Finally, to determine experimentally if sugar could directly affect DNA methylation levels, primary cultured fibroblasts were grown in either normal or high glucose environment and their DNA methylation levels were compared.

2 | METHODS

2.1 | Participants

Healthy pregnant women ($n = 170$, ages 20–45) were recruited during the years 2011–2016 through the Department of Obstetrics and Gynecology at Columbia University Medical Center. Exclusion criteria were multiparity, medication use, and tobacco or recreational drug use.

Participants provided written, informed consent prior to participating in the study. All procedures were approved by the Institutional Review Board of the New York State Psychiatric Institute/Columbia University Medical Center and all methods were performed in accordance with relevant guidelines and regulations.

2.2 | Study procedures

The participants completed the Automated Self-Administered 24-hr Dietary Recall (ASA24) at three timepoints of pregnancy (early second, late second, and third trimesters) and underwent fetal neurobehavioral assessment in the third trimester (34–37 weeks). A placenta sample near the fetal surface was collected at birth and analyzed for a randomly selected sub-sample of women ($n = 107$) who had similar demographic characteristics to the complete sample.

2.3 | Dietary recall

Information about caloric intake, total sugar intake, and added sugar intake (defined as white sugar, brown sugar, raw sugar, corn syrup,

corn syrup solids, high fructose corn syrup, malt syrup, maple syrup, pancake syrup, fructose sweetener, liquid fructose, honey, molasses, dextrose, and dextrin, eaten separately or in processed/prepared foods) was collected using the ASA24, an internet-based questionnaire developed by the National Cancer Institute (Subar et al., 2012). This questionnaire collects information on food intake over the preceding 24 hr using detailed probes and portion-size food images. Its user-friendly interface has been validated for participants with at least some secondary education. The ASA24 estimates relative macronutrient and micronutrient levels using the three following databases: the USDA's MyPyramid Equivalents Database (MPED), the USDA's Food and Nutrient Database for Dietary Surveys (FNDDS), and the USDA's Center for Nutrition Policy and Promotion's MPED Addendum.

2.4 | Fetal data collection

Fetal movement and heart rate were acquired using a Toitu MT 325 fetal actocardiograph (Toitu Co., Ltd., Tokyo, Japan) via a single transabdominal Doppler transducer, while participants were in a semi-recumbent position for 20 min (described in previous work Doyle et al., 2015; Gustafsson et al., 2018; Werner et al., 2007)). The fetal data were collected from the Toitu's output port, digitized at 50 Hz using a 16-bit A/D card (National Instruments 16XE50), and analyzed offline. Data were analyzed using custom made MAT-LAB 8.3 scripts (Mathworks Inc., Natick, MA). Datapoints detected below the first interquartile range (IQR) or above the third IQR (fetal movement, $N = 5$) were considered as outliers and deleted prior analysis.

To ensure that fetuses included in the study were developing typically, we measured their mean heart rate and heart rate variability (standard deviation) as described previously (Doyle et al., 2015; Gustafsson et al., 2018; Werner et al., 2007).

2.5 | Demographic, maternal, and birth outcomes information

Birth weight and delivery information were collected from the medical record. Gestational age at birth was determined by ultrasound examinations and last reported menstrual cycle. Pre-pregnancy weight and height were self-reported and this information was verified in participants' medical records when possible. Maternal stress was assessed using the perceived stress scale (PSS) in the third trimester of pregnancy (Cohen et al., 1983).

2.6 | Analysis of CpG methylation in placentas

A 1 cm³ sample of the placenta was collected (using scissors, midway between the umbilical cord and the outer edge of the placenta) and genomic DNA was extracted. DNA integrity was assessed by agarose gel electrophoresis with ethidium bromide staining and

PicoGreen (Life Technologies, Carlsbad, CA). Starting with 500 ng of genomic DNA, bisulfite conversion, and methylation profiling were performed according to the manufacturer's instructions for Illumina Human Methylation 450K BeadChips. All assays were performed at the Roswell Park Cancer Institute Genomics Shared Resource. After background correction and normalization to internal controls, Genome Studio software was used to calculate the fractional methylation (AVG_Beta) at each CpG ($N = 453,144$ CpG sites). All probes querying CpGs that overlapped common single-nucleotide polymorphisms (SNPs) (SNPs with minor allele frequency ≥1% in dbSNP build 138) were removed.

2.7 | Analysis of CpG methylation in primary cultured fibroblast

To determine if sugar exposure itself could directly change DNA methylation levels, primary cultured fibroblasts were obtained from a healthy male donor. The participant provided written, informed consent prior to participating in the study. All procedures were approved by the Institutional Review Board of the New York State Psychiatric Institute/Columbia University Medical Center and all methods were performed in accordance with relevant guidelines and regulations.

Cells were grown at sub-confluency and DNA was extracted from over 28 passages. In one condition, cells were exposed to high glucose (25 mM); in the other, the cells were grown in normal glucose levels (5mM). DNA methylation was measured at 8 timepoints separated by 10 days. 250 ng of DNA was used for measurement of DNA methylation on the Infinium EPIC 850K BeadChips. DNA methylation data were processed in R (Version 3.5.0). Preprocessing quality control included checking for correct sex prediction, probe quality, sample intensities, and excluding SNPs and non-CpG probes. All samples passed our quality control and no samples were excluded. Then, data were pre-processed by functional normalization (2-PCs). RCP and ComBat adjustments were applied for probe-type and plate bias using the R package SVA which excluded 68 out of the 866,836 CpG probes. The remaining 866,768 probes were used for further analysis.

2.8 | Data processing and statistical analysis

Statistical analyses were performed using SAS statistical software 9.3 (SAS Institute Inc., Cary, NC), R (version 3.4.1), and Prism 7.0 (Graphpad). Fetal movement and heart rate variables were screened for extreme values using the inter-quartile range outlier labeling rule, and extreme values were removed (Hoaglin et al., 1986). Descriptive statistics were calculated using mean and standard deviation for continuous variables and count/percent for categorical variables. To assess the effect of habitual added sugar consumption over pregnancy on fetal movement and birth outcomes, the average intake drawn from the three ASA24-hr recalls administered at each trimester of

pregnancy was computed. Then, multiple linear regression models were used to examine associations between average added sugar intake and outcomes of interest while controlling for relevant covariates. Association between average added sugar intake and fetal movement was controlled for birthweight, sex, pregnancy complication, maternal age, maternal calories intake, maternal pre-pregnancy weight, and maternal reported stress in the third trimester. Association between average added sugar and gestational age was controlled for birthweight, sex, pregnancy complication, maternal age, maternal calorie intake, and maternal pre-pregnancy weight.

2.9 | Genome-wide DNA methylation and gene enrichment analysis

2.9.1 | Added sugar and placental DNA methylation

The associations between added sugar intake and genome-wide placental DNA methylation was examined using partial correlation, while adjusting for relevant covariates in the subset of participants having both DNA methylation and food diary recall data ($N = 73$). The choice of covariate was based on prior literature (Appleton et al., 2013; Monk et al., 2016; Robakis et al., 2020).

While no single CpG passed Benjamini-Hochberg corrected genome-wide significance for altered fractional methylation as a function of added sugar intake, we nonetheless brought forward the highest ranked CpGs for exploratory analyses. Gene set enrichment analysis and functional annotation of differentially methylated loci were performed using The Database for Annotation, Visualization, and Integrated Discovery v6.7 (DAVID; Huang et al., 2009), a publicly available platform to explore the functional significance of gene lists. This approach was chosen to understand the effect of dietary added sugar intake on placental biology. After exclusion of the CpGs without a corresponding annotated gene provided by Illumina, each site was ranked by the strength of the association (unadjusted p-value) between added sugar across pregnancy and at each trimester and methylation level. To capture biologically meaningful signatures of regulation that may not be revealed in single CpGs, the top 300 sites were selected, the corresponding neighboring genes were mapped, and the gene list was used for functional annotation analysis in DAVID. From each gene list (one per timepoint), overrepresented biological processes and functions were identified, and their significance was established based on Bonferroni-corrected p values to adjust for multiple testing. Adjusted p values are reported in the figures.

Additionally, to identify differentially methylated regions (DMR), the BumpHunter (v1.26.0) method (Jaffe et al., 2012) was used to identify segments of the genome presenting quantitative alteration in DNA methylation levels. The association between added sugar intake (in each trimester and averaged across pregnancy) and methylated regions was performed with partial correlation to adjust for relevant covariates. Candidate regions were selected with a threshold of 0.15 (corresponding to a minimum of 15% change in DNA methylation). To assess statistical significance and account for multiple testing, we performed 1,000 bootstrapping and report a family-wise error rate (FWER) value, representing the number of regions in permuted (null) data sets that had an area value as extreme as our observed exposure-associated DMR.

2.9.2 | High glucose and DNA methylation in primary cultured fibroblast

DNA methylation levels assessed at 8 timepoints across lifespan were averaged before looking at the mean difference at each CpG between high and normal glucose exposure. Mixed effect models followed by multiple comparison correction with the false discovery rate (FDR) (Benjamini & Hochberg, 1995) method were used to evaluate the site-by-site difference between glucose exposure groups while controlling for the effect of time. Gene set enrichment analysis and functional annotation of differentially methylated loci were performed using DAVID (Huang et al., 2009) for both the top 300 most significant loci and on all loci significant after FDR correction.

Finally, as an exploratory analysis, the overlap between differentially methylated CpG in the placenta and in primary cultured fibroblast was compared, as well as the top 15 gene enrichment categories obtained with functional annotation.

3 | RESULTS

3.1 | Characteristics of the study sample

Demographic, maternal, fetal heart rate and movement, pregnancy complications, and birth outcome data are summarized in Table 1. Average pre-pregnancy weight, infant birth weight, and gestational age at birth were consistent with population norms. Average third-trimester fetal movement, fetal mean heart rate, and standard deviation of fetal heart rate were comparable to previous reports (Doyle et al., 2015).

3.2 | Added sugar, fetal behavior, and birth outcomes

After adjusting for potential covariates (birthweight, sex, pregnancy complications, maternal age, average maternal calorie intake, and maternal pre-pregnancy weight), higher average added sugar intake across pregnancy was associated with reduced fetal movement during the 3rd trimester (β(SE) = −27,572 (113,196), p = .0411; Figure 1a). No significant associations were found between added sugar intake and fetal heart rate both for the whole group or stratified by sex (data not shown).

Regarding birth outcomes, higher dietary intake of added sugar was associated with shorter gestational age at birth (β(SE) = −0.041 (0.014), p = .0048; Figure 1b). No significant associations were found

TABLE 1 Demographic, maternal, and fetus/newborn characteristics

Demographics characteristics	N		%
Maternal ethnicity			
Not Hispanic	54		32
Hispanic/Latina	116		68
Incomes/years			
$0–$50,000	77		45
Above $51,000	93		55
Maternal education			
High school or below	67		40
Greater than high school	102		60
Maternal characteristics	**N**	**Means (SD)**	**Min−max**
Age, years	170	30 (6)	20–45
Pre-pregnancy weight, kg	170	69 (16)	34–141
Height, cm	157	162 (6)	147–180
Dietary intake/day:			
Average calories	170	2,399 (932)	750–6,941
Average added sugar, tsp[a]	170	13 (8)	0.1–37
Added sugar early 2nd tr	99	13 (10)	0.2–48
Added sugar late 2nd tr	141	13 (11)	0.1–65
Added sugar 3rd tr	126	13 (9)	0.3–60
Depressive symptoms 3rd tr	136	21 (7)	4–46
Pregnancy complications	**N**		**%**
Infection	2		1
Preeclampsia/hypertension	5		3
Vascular complications	1		0.5
Diabetes Mellitus	4		2
Others	38		23
Fetus/newborn characteristics	**N**		**%**
Sex			
Male	85		51
Female	81		48
	N	**Means (SD)**	**Min−max**
Birthweight, g	160	3,308 (515)	1,310–4,470
Birth length, cm	134	51 (4)	22–57
Gestational age, weeks	169	39 (2)	16–41
Fetal movement	118	9,516,213 (8,399,061)	1,237–33,997,200
Fetal heart rate, mean	134	139 (10)	94–159
Fetal heart rate, SD	134	8 (3)	0.1–26

[a]tsp: Teaspoon equivalents of added sugars. Added sugars are defined as white sugar, brown sugar, raw sugar, corn syrup, corn syrup solids, high fructose corn syrup, malt syrup, maple syrup, pancake syrup, fructose sweetener, liquid fructose, honey, molasses, dextrose, and dextrin that are eaten separately or as ingredients from processed or prepared foods. Abbreviation: tr, trimester.

between added sugar intake and birth weight both for the whole group or stratified by sex (data not shown).

## 3.3 | Added sugar and placenta genome-wide DNA methylation

Associations between added sugar and placenta genome-wide CpG DNA methylation were examined using partial correlation and controlling for birthweight, sex, pregnancy complications, maternal age, average maternal calorie intake, and maternal pre-pregnancy weight. After correction for multiple comparisons, no individual CpG achieved significance for altered methylation as a function of added sugar intake at each trimester or across pregnancy. The results of the associations between added sugar and genome-wide DNA methylation were sorted by unadjusted p-value and the 300 most significant associations (listed in Table S1) were selected for further analysis. The direction of these associations for the average and early 2nd, late 2nd, and 3rd trimesters, respectively, was: 19% negative and 81% positive (average); 1% negative and 99% positive (1st), 22% negative and 78% positive (2nd), and 14% negative and 86% positive (3rd).

Results of enrichment gene analysis of genome-wide DNA methylation association are shown in Figure 2. This analysis indicated that the amount of added sugar during pregnancy was associated with differential methylation of functionally related gene families, but that this differed across trimesters. In the early 2nd trimester, higher added sugar consumption was associated with differential methylation of genes preferentially involved in establishing cell-cell junctions, ion transport, and transmembrane signaling. This signature is consistent with the establishment of the maternal-fetal barrier (Malassine & Cronier, 2005; Rothbauer et al., 2017) and of differentiated tissues (Cooper, 2000; Shlyonsky et al., 2005). In the late 2nd trimester, genes affected were enriched for morphogenesis, cellular projections, and neurogenesis, consistent with nervous system development (Eccles, 1970; Zhu et al., 2016). Finally, in the 3rd trimester, sugar levels showed an association with genes involved in intracellular signaling and post-translational modifications (phosphoprotein and glycoprotein), consistent with the regulation of established or mature tissue (Carnino et al., 2020; Pirola et al., 2012; Sato et al., 2012).

A secondary analysis aiming to identify DMR using the BumpHunter method yielded one result that passed statistical correction. Added sugar intake in the third trimester was associated with increased DNA methylation of a cluster of 8 CpG sites within the gene ADD2 (FWER < 0.05) (see Table 2). All DMR results obtained at the r > .15 threshold—including those that did not pass statistical FWER correction—are presented in Table S2.

3.4 | High sugar exposure and DNA methylation in human cells

The cross-sectional association found between added sugar consumption and placental DNA methylation could be indirectly
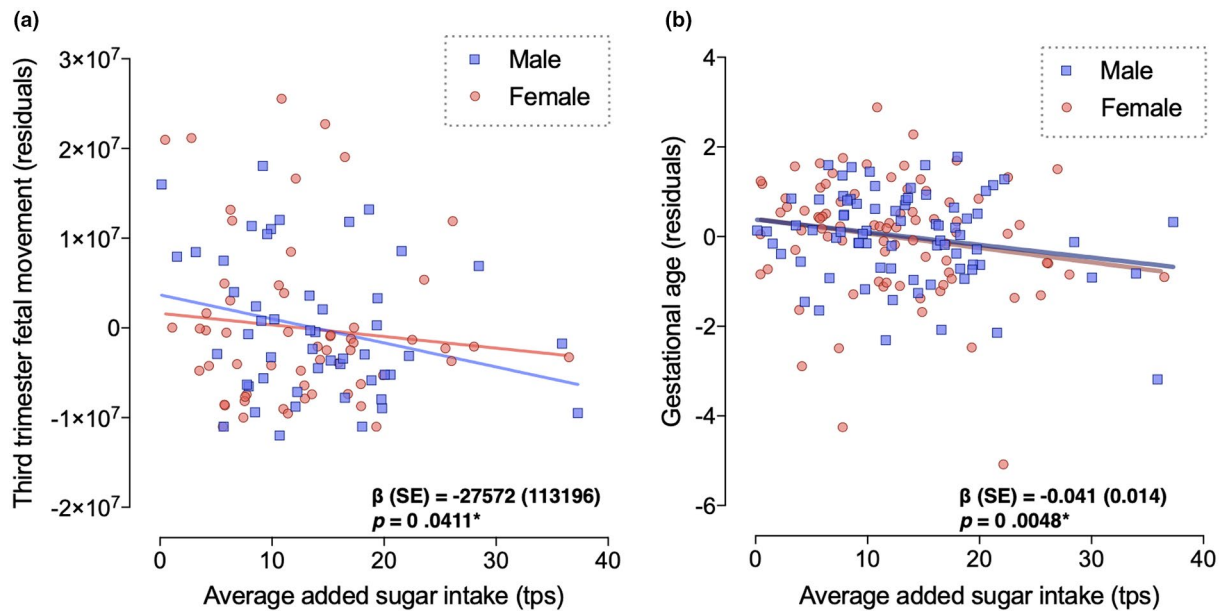
FIGURE 1 Association between average (early 2nd tr, late 2nd tr, 3rd tr) added sugar intake during pregnancy, fetal movement and gestational age. Results from linear regression between added sugar (expressed as teaspoon equivalents (tps)) and (a) third trimester fetal movements, adjusted for birthweight, sex, pregnancy complication, maternal age, maternal calories intake, maternal pre-pregnancy weight and maternal reported stress in the third trimester, $n = 106$; and (b) gestational age adjusted for birthweight, sex, pregnancy complication, maternal age, maternal calorie intake & maternal pre-pregnancy weight, $n = 159$. Scatter plots of residuals. *p-values $< .05$

mediated by behavioral or other physiological factors. For example, women who consume more added sugar may also be malnourished (e.g., micronutrient deficiency) or engage in less physical activity, thus possibly affecting DNA methylation via mechanisms other than sugar. To determine if sugar exposure itself could directly change DNA methylation levels, we tested the effect of high glucose exposure (25 mM) on primary cultured human fibroblasts, compared to cells grown in normal glucose levels (5 mM). High glucose exposure altered the methylation of 17% of all CpGs. Among those, 39% were hypermethylated and 61% were hypomethylated (Figure 3). The change in methylation (delta beta) was on average 14% for hypermethylated CpGs and −20% for hypomethylated CpGs. To illustrate the global alteration of methylation between high and normal glucose conditions, we plotted the beta value distribution of both groups (Figure S1a) and of the difference between high and normal glucose exposures (Figure S1b). Overall, in comparison with the normal glucose exposure group, the high glucose group presented more hypomethylation. To assess global methylation patterns evolution over time, we performed principal component analysis (PCA). Observation of principal component 1 (PC1) (explaining 23.77% of the variance) over the time of cell growth shows that high glucose exposure has a stepwise effect on the methylome and that this effect is stable across time (Figure S1c).

The top 300 differentially methylated genes between high and normal glucose conditions were significantly enriched for alternative splicing, phosphoprotein, cell junction, and development-related proteins (Figure 3). Gene families associated with differentially methylated CpG candidates showed substantial overlap between placenta

and cultured fibroblast: cell junction, cell membrane, cytoplasm, developmental protein, glycoprotein, plasma membrane, and polymorphism (see Table 3). Other less specific findings included categories such as alternative splicing, splice variant, and phosphoprotein, which overlapped but also were found to appear in random site analysis. The overlap between differentially methylated CpGs found in cultured fibroblasts (all FDR significant sites) and in association with added sugar (top 300 most significant) was limited (<1%) (see Table S4). Since the lack of overlap between the two studies may be partially explained by different versions of the DNA methylation array used, we also assessed the overlap in the gene name of differentially methylated CpG (all FDR significant sites vs. top-300) and found from 50% (early 2nd tr) to 100% (3rd tr) overlap in gene names (see Table S5).

4 | DISCUSSION

To our knowledge, this is the first study to suggest an effect of pregnant women's habitually high added sugar consumption on offspring behavior *in utero*. We found that greater average added sugar intake was associated with a decrement in fetal motor activity and with earlier birth. In addition, our exploratory analysis of placental DNA methylation showed added sugar intake was associated with variations in DNA methylation, with different functionally related families of genes being affected at different stages of pregnancy.

We report that elevated habitual added sugar consumption across pregnancy is associated with less fetal movement in the 3rd trimester. In early pregnancy, high maternal carbohydrate intake has been

(a) Average pregnancy

(b) Early 2nd trimester

(c) Late 2nd trimester
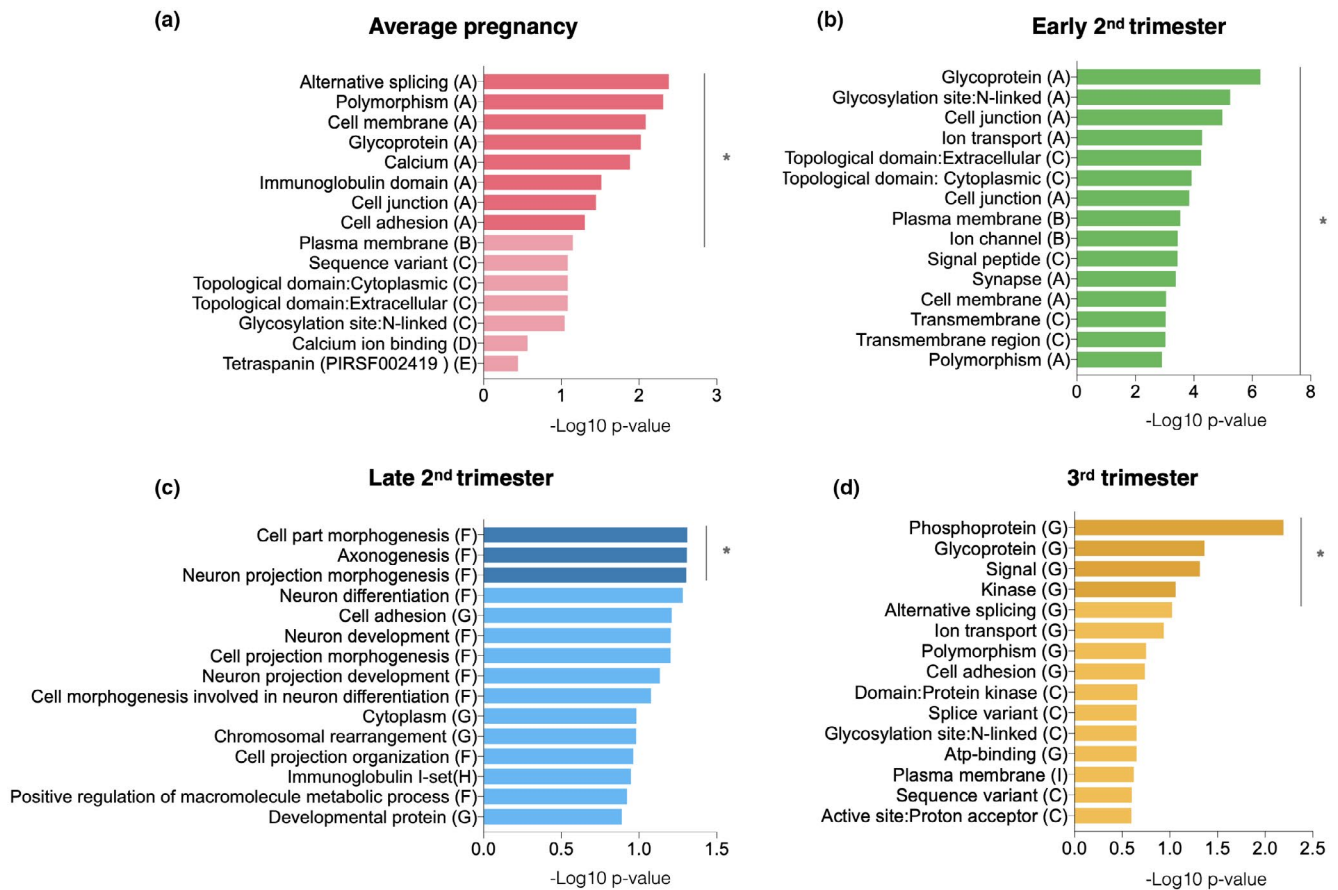
(d) 3rd trimester

FIGURE 2 Added sugar and DNA methylation: 15 most significant functional gene enriched categories at (a) average of all trimester; (b) early second trimester; (c) late second trimester and (d) third trimester. Analysis performed using DAVID Bioinformatics Resources 6.7. *Benjamini corrected *p*-values < .05. Keywords database used: (A) UniProt Knowledgebase Protein Database (UniProtKB); (B) UniProt/ Swiss-Prot Protein Identification Resource ; (C) Gene Ontology project Molecular Function; (D) Gene Ontology project Biological Process ; (E) Gene Ontology project Cellular Component; (F) InterPro Database; (H) SwissProt-PIR keywords (G) UniProtKB Functional Categories

TABLE 2 Significant differentially methylated regions (DMRs) associated with added sugar prenatal exposure

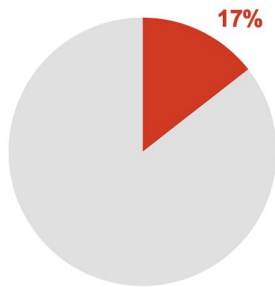| Predictor	r	FWER	Genomic location	Distance	Gene symbol	CpGs
Added sugar intake during the third trimester.	0.24	0.044	Chr2: 70995134–70995527	393	ADD2	cg01290229_2_70995134 cg05659187_2_70995522 cg15170605_2_70995459 cg24347663_2_70995349 cg03384579_2_70995440 cg08857144_2_70995351 cg23427269_2_70995444 cg25631352_2_70995527

Note: Correlation coefficient from partial correlation analysis adjusted for birthweight, sex, pregnancy complications, maternal age, average maternal calorie intake, and maternal pre-pregnancy weight. Abbreviation: FWER, Family-wise error rate.

found to be associated with increases in fetal movement (Goldstein et al., 2003). Later in pregnancy, several studies have found no alteration of the incidence of gross body movements after acute intravenous glucose administration (Bocking et al., 1982; Divon et al., 1985; Harper et al., 1987; Natale et al., 1983; Nijhuis et al., 1986). In line with our findings, a previous report showed decreased fetal movements secondary to sust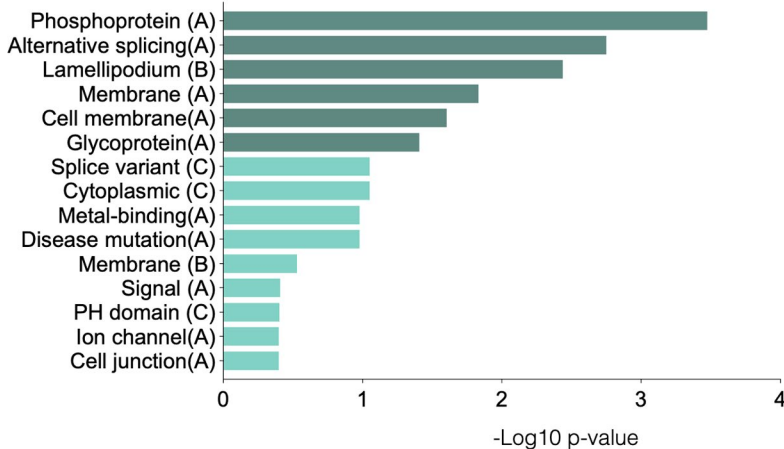ained maternal hyperglycemia (using the hyperglycemic clamp technique; Edelberg et al., 1987). Taken together with findings from the present study, this suggests that maternal added sugar intake may reduce fetal movement via sustained hyperglycemia, yet precise mechanisms for this effect are not known.

Fetal motor activity in the 3rd trimester has been shown to be associated with fetal movement observed in the neonatal period: at 36 weeks, higher occurrence of fetal movement has been associated

(a)

146,024 FDR Significant CpG Sites

17%

(b)

Top 300 FDR Significant CpG Sites

Phosphoprotein (A)
Alternative splicing(A)
Lamellipodium (B)
Membrane (A)
Cell membrane(A)
Glycoprotein(A)
Splice variant (C)
Cytoplasmic (C)
Metal-binding(A)
Disease mutation(A)
Membrane (B)
Signal (A)
PH domain (C)
Ion channel(A)
Cell junction(A)

0   1   2   3   4

-Log10 p-value

FIGURE 3 Genome wide DNA methylation of human fibroblast exposed to high or normal glucose environment across lifespan. (a) Proportion of differentially methylated CpG sites after FDR correction (*p*-values < .05) between normal or high glucose exposure over the lifespan. (b) 15 most significant functional gene enriched categories for the top 300 FDR significant CpG. Among the top 300, 22% were hypomethylated (mean delta beta = 28) and 78% were hypermethylated (mean delta beta = −27). Analysis performed using DAVID Bioinformatics Resources 6.7. *Benjamini corrected *p*-values < .05. Keywords database used: (A) UniProt Knowledgebase Protein Database (UniProtKB); (B) Gene Ontology project Cellular Component; (C) UniProt/Swiss-Prot Protein Identification Resource

TABLE 3 Overlapping gene families associated with differentially methylated CpG candidates as a function of added sugar in human placenta and high glucose in cultured fibroblast

Fibroblast and high glucose[a]	Placenta and added sugar[b]			
Top 300	Early 2nd tr	Late 2nd tr	3rd tr	Average
Cell junction	X			X
Signal			X	
Splice variant			X	
Glycoprotein	X		X	X
Cell membrane	X			X
Alternative splicing	X		X	X
Phosphoprotein			X	

[a]FDR Significant CpG Sites.

[b]Top 300 significant CpG sites not FDR corrected.

with more advanced motor development in the neonatal period (DiPietro et al., 2010). Fetal movement has also been inversely associated with infant distress to limitations at 1 year and behavioral inhibition at 2 years (DiPietro et al., 2002). Among a sample of women with type-one diabetes, abnormal fetal motor activity has been associated with higher diabetes severity and with lower neurodevelopmental score in the offspring (Kainer et al., 1997). Maternal health-related factors associated with added sugar intake such as obesity and gestational diabetes have also been associated with lower developmental scores in infants (Adane et al., 2016; Van

Lieshout, 2013; Robles et al., 2015). To extend our understanding of the long-term implications of prenatal programming effects of maternal added sugar intake, future studies should assess whether higher sugar intake consumption associated with lower fetal motor activity in turn predicts developmental impairments in childhood.

In line with previous reports, our findings show that increased added sugar consumption is associated with earlier birth. This is consistent with a previous report showing that high sugar intake in teenage pregnancy was associated with increased risk of reduced gestational age at birth (Lenders et al., 1994). Similarly, increased sugar

and fat intake prior to conception has been associated with higher risk of preterm birth (Grieger et al., 2014). Our study was not designed to assess preterm birth specifically and therefore did not include enough preterm babies for statistical modeling. However, it is worth emphasizing that developmental risk can reside outside the timeframe of preterm birth (<37 weeks). Indeed, increased risk of morbidity has been observed in babies born between 37 and 38 weeks and 6 days compared with infants born after 39 weeks (Spong, 2013).

Regarding the effect of added sugar intake on DNA methylation, the results of our exploratory analysis suggest that sugar intake at each timepoint is associated with differential regulation of specific gene families. Across development, chromatin remodeling occurs to control gene expression (making the chromatin shapes vary (open/condensed)). Our findings suggest that added sugar intake at each trimester could preferentially affect the DNA methylation of genes that are most active (i.e., with open chromatin) at each stage of placental and fetal development. The two main lines of evidence supporting this hypothesis are: (a) differentially methylated candidate CpGs and their associated genes show very little overlap across timepoints, suggesting that added sugar influences different genes based on gestation time, that sugar may affect different gene targets based on timing, and that analyzing all trimesters as a whole may "wash out" or dilute the specific effects; and (b) the enriched gene families associated with added sugar intake at the early 2nd, late 2nd, and 3rd trimesters appear to follow the biological progression from the establishment of basic tissue/cellular structures (early 2nd trimester), to morphogenesis and neural maturation (2nd trimester), to intracellular signaling and post-translational modifications (3rd trimester) that have been described in a previous report of placental gene expression (Mikheev et al., 2008; Sitras et al., 2012; Winn et al., 2007).

Using DMR analysis, we found that increased added sugar intake in the third trimester was associated with increased methylation of a cluster of 8 CpG sites within the gene ADD2 (FWER < 0.05). The protein encoded by this gene, β-Adducin, is expressed at high levels in the brain and hemopoietic tissue and at low levels in the placenta (Fagerberg et al., 2014). Adducins are proteins found in cytoskeleton junctional complexes, which bind and regulate actin filaments and actin-spectrin complexes. Interestingly, in mice, knock out of the ADD2 gene leads to reduced synaptic assembly and plasticity, motor coordination performance and learning behaviors (Bednarek & Caroni, 2011; Porro et al., 2010), suggesting a role of this gene in fetal neurodevelopment. Our finding is calling for the future experimental study to determine whether prenatal high glucose exposure could affect the fetal brain methylation of the ADD2 gene and in turn brain development.

To our knowledge, only one study has investigated placental DNA methylation across the three trimesters of pregnancy (Novakovic et al., 2011), finding that the most differentially methylated genes across the three trimesters were associated with immune regulation (Novakovic et al., 2011). In the current study, this class of genes was not found to be enriched in the top 300 differentially methylated candidate CpG associated genes most associated with added sugar. The developmental consequences of added sugar related to changes in placental DNA methylation remain to be investigated further.

The experimental findings on cultured cells showed that high glucose alone is sufficient to alter CpG methylation. Although not definitive, these results provide preliminary evidence, suggesting that the association between added sugar and DNA methylation in the human placenta may be directly mediated by a metabolic mechanism, rather than via secondary physiological processes or by dietary or behavioral factors. Differentially methylated genes were significantly enriched for biological pathways including cell membrane, plasma membrane glycoprotein, and development-related proteins, providing converging evidence that excess sugar exposure alone may induce DNA methylation changes, partially overlapping with those associated with dietary added sugar in human placenta at different trimesters. That being said, the human placenta and cultured fibroblasts are different biological systems. The observed overlap between both systems should, therefore, be interpreted with caution. Nevertheless, the effect of excess glucose on DNA methylation provides proof-of-concept evidence that sugar levels could influence DNA methylation in similar ways in different cell types. Theoretically, the epigenetic effect of sugar could possibly contribute to the indirect effects of added sugar on placental and fetal development although more work is needed to establish this as a viable mechanism.

The current study is limited by its sample size, which did not allow sufficient power for the identification of DNA CpG methylation changes in the placenta at the individual CpG level after correction for multiple comparisons. Further, placental DNA CpG methylation was assessed on different cell types, and future studies should assess the effect of sugar on sorted cell populations to allow the detection of changes belonging to cell types. Also, it is important to highlight that the presented experimental findings showing the direct effect of high glucose on DNA CpG methylation were obtained on human fibroblasts, which limit their translation to placenta cells. The human fibroblast experiment was limited by the lack of biological replicates, but the use of repeated methylation analysis at 8 timepoints provided confidence in the effect of glucose exposure on CpG methylation patterns. Our reliance on 24-hr food recall may have reduced the internal validity of our findings; nonetheless, 24-hr recall reduces error compared to the longer interval of recollection for food frequency questionnaires utilized in other studies (Murphy & Poos, 2002). To avoid recall bias and add objective measures to the results, future studies should complement measures of self-report of added sugar intake with glucose peripheral blood assays. Another limitation is the self-report of maternal pre-pregnancy weight.

5 | CONCLUSION

Our data add to literature showing that maternal diets high in added sugar during pregnancy may have implications for offspring health via prenatal programming effects measurable before birth. We have observed associations between added sugar intake and shorter duration of gestation as well as reduced fetal motor activity.

Additionally, while preliminary, our secondary exploratory analysis suggests that the influence of added sugar on placental DNA methylation should be investigated further as a possible pathway for the transduction of high sugar into developmental outcomes.

6 | COMPETING INTERESTS

The author(s) declare no competing interests.

AUTHORS' CONTRIBUTION

All the authors participated in interpreting the results of this study, writing and reviewing the manuscript. TF and SL performed the statistical analysis relating to placental DNA methylation. CT performed the statistical analysis, gene annotation analysis, and drafted the manuscript. SF helped with the data collection. GS ran the in vitro fibroblast glucose experiment and DNA preparation. The corresponding 850k methylation data were preprocessed by AC and along with GS performed the statistical and gene annotation analysis.

DATA AVAILABILITY STATEMENT

The methylation array data used in this manuscript are available under accession number GSE144977 (placenta) and GSE131280 (fibroblast). The other data will be available upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.