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PEDIATRIC ORIGINAL ARTICLE

Associations between antibiotic exposure during pregnancy, birth weight and aberrant methylation at imprinted genes among offspring

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OBJECTIVES: Low birth weight (LBW) has been associated with common adult-onset chronic diseases, including obesity, cardiovascular disease, type II diabetes and some cancers. The etiology of LBW is multi-factorial. However, recent evidence suggests exposure to antibiotics may also increase the risk of LBW. The mechanisms underlying this association are unknown, although epigenetic mechanisms are hypothesized. In this study, we evaluated the association between maternal antibiotic use and LBW and examined the potential role of altered DNA methylation that controls growth regulatory imprinted genes in these associations.
METHODS: Between 2009–2011, 397 pregnant women were enrolled and followed until delivery. Prenatal antibiotic use was ascertained through maternal self-report. Imprinted genes methylation levels were measured at differentially methylated regions (DMRs) using bisulfite pyrosequencing. Generalized linear models were used to examine associations among antibiotic use, birth weight and DMR methylation fractions.
RESULTS: After adjusting for infant gender, race/ethnicity, maternal body mass index, delivery route, gestational weight gain, gestational age at delivery, folic acid intake, physical activity, maternal smoking and parity, antibiotic use during pregnancy was associated with 138 g lower birth weight compared with non-antibiotic use (β-coefficient $= -132.99$, s.e. $= 50.70$, $P = 0.008$). These associations were strongest in newborns of women who reported antibiotic use other than penicillins (β-coefficient $= -135.57$, s.e. $= 57.38$, $P = 0.02$). Methylation at five DMRs, *IGF2* ($P = 0.05$), *H19* ($P = 0.15$), *PLAGL1* ($P = 0.01$), *MEG3* ($P = 0.006$) and *PEG3* ($P = 0.08$), was associated with maternal antibiotic use; among these, only methylation at the *PLAGL1* DMR was also associated with birth weight.
CONCLUSION: We report an inverse association between *in utero* exposure to antibiotics and lower infant birth weight and provide the first empirical evidence supporting imprinted gene plasticity in these associations.

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INTRODUCTION

Early *in utero* exposures can lead to altered organogenesis and fetal development. Antibiotics, including penicillins, nitrofurans, and cephalosporins, are commonly prescribed for a wide range of ailments during the periconceptional and prenatal periods.[1–3] Over the past 30 years, first-trimester use of prescription drugs increased >60%. In 2010, 94% of pregnant women took at least one medication during pregnancy, and 82% did so in the first trimester; among these medications, antibiotics were within the top 20 most frequently used, amoxicillin being top of the list, followed by azithromycin (No. 10), nitrofurantoin (No. 12), sulfamethoxazole-trimethroprim (No. 14), penicillin (No. 16) and cephalexin (No. 19).[4] Maternal antibiotic use has been associated with changes in infant birth weight,[5–9] but in the few studies that reported associations, the direction of these changes was inconsistent.[5,6] Czeizel *et al.*[5] reported lower birth weights among infants born to women who took antibiotics, whereas Jepsen *et al.*[6] reported higher birth weights among infants born to antibiotic users in pregnancy. Both high birth weight (HBW), including large for gestational age, and low birth weight (LBW), including small for gestational age (SGA), are consistent risk factors for childhood and adult obesity,[10] type 2 diabetes,[11,12] cardiovascular disease[13,14] and some cancers, such as breast[15] and prostate[16] cancers and esophageal adenocarcinomas.[17] For reasons that are as yet unclear, since 1999 the US incidence of large for gestational age births has decreased, whereas that of SGA births has increased.[18]

Because antibiotics are the most commonly prescribed compound during pregnancy[4] and the incidence of SGA is on the rise,[18] understanding the relationship between birth weight and antibiotic use during pregnancy and mechanisms underlying these associations remains important. Improved understanding of this relationship may lead to focused interventions in pregnant

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women who may have other risk factors or exposures for LBW outcomes. Epigenetic mechanisms have been hypothesized to underlie these associations, but potential targets for the study are unknown. Many imprinted genes are critically involved in regulating early growth and development and are regulated by allele-specific methylation at differentially methylated regions (DMRs). The methylation patterns at imprinted DMRs are established in the gamete and early embryo and maintained through germ layer specification and tissue differentiation, resulting in highly similar levels of methylation that is present in all the tissues.[19] Any shift in the process of reprogramming or lack of fidelity in maintaining these marks through the reprograming process can lead to shifts that are evident in somatic tissues. In contrast to most biallelically expressed genes where there are two active alleles, imprinted genes, with only one active allele, are at increased vulnerability to deregulated expression, and this can be mediated through epigenetic changes at the regulatory DMRs.[20–22] As imprinted genes occur in clusters, their regulation may be under network-level control. This class of genes is also over-selected for growth effectors. For example, the *pleomorphic adenoma gene-like 1* (*PLAGL1*) is a paternally expressed gene located at chromosome 6q24-q25, in the same chromosomal region as the imprinted transient neonatal diabetes mellitus locus, that encodes a zinc finger tumor-suppressor protein.[23] In mice studies, *Plagl1* was associated with intrauterine growth restriction, altered bone formation and neonatal lethality. *Plagl1* was also proposed to serve as a major regulatory hub that coordinates the expression of many other genes, including a large number that are imprinted.[24] We chose the nine imprinted marks in this study because these DMRs regulate genes already known to be important to appropriate growth and development,[25,26] neurological function[27] and to social and maternal nurturing behaviors (from studies in mice)[25] (for review, see Murphy *et al.*[28]). In addition, our previous studies have shown that these regions exhibit differential malleability in methylation profiles in response to antidepressant use during pregnancy,[29] maternal depression,[30] smoking,[31] folic acid intake[26] and paternal obesity.[32] To our knowledge, no studies have provided mechanistic insights into the association between antibiotic intake during pregnancy and birth weight, although epigenetic mechanisms have been hypothesized. Here, we report associations between antibiotic intake during pregnancy, birth weight, and newborn methylation status at nine imprinted gene DMRs, including *PLAGL1*, *Insulin-like Growth Factor 2* (*IGF2*) and *noncoding H19, Neuronatin* (*NNAT*), *Delta-like homolog 1* and *noncoding Maternally Expressed Gene 3* (*DLK1/MEG3*), *Paternally Expressed Gene 3* (*PEG3*), the regulatory region regulating both *Epsilon Sarcoglycan* and *Paternally Expressed Gene 10* (*SGCE/PEG10*) and *Paternally Expressed Gene 1/ Mesoderm-Specific Transcript* (*MEST*).[19]

SUBJECTS AND METHODS

Subjects

Pregnant women were enrolled as part of NEST (Newborn Epigenetic STudy), a prospective study of women and their offspring in the Southeastern United States. Methods for enrollment of study participants have been previously described.[30] Briefly, between 2009 and 2011, pregnant women were recruited from five prenatal clinics and obstetric facilities serving Duke University and Durham Regional Hospitals. To be eligible, women had to be $\geqslant$18 years of age, pregnant and intending to use one of the participating obstetric facilities for delivery. Exclusion criteria were plans to relinquish custody of the child and plans to move away from the area in the subsequent 3 years. Women with established HIV-I infection were also excluded due to uncertainty about how this might alter DNA methylation profiles.

We consented and enrolled 1700 (66.7% response rate) of the 2548 women who were approached. Women enrolled in the study were similar to the 848 women who declined with respect to age ($P=0.66$) but more likely to be Asian and Native American women than African American or White ($P<0.001$). Among the 1700 women who consented, we excluded infant deaths before, during or soon after birth ($n=115$), 281 who were either illiterate, underage, refused further participation or could not be found, so that 1304 (76.7%) remained in the study. An additional 50 had missing information on antibiotic use, such that an effective sample size of 1254 remained for these analyses. These analyses are limited to the 397 women and their infants for whom data on infant DNA methylation were available. The study protocol was approved by the Duke University Institutional Review Board.

Data collection

Participants completed a self-administered questionnaire at the time of enrollment that included sociodemographic characteristics, reproductive factors, lifestyle factors such as cigarette smoking, anthropometric measurements and antibiotic use in the 6 months before enrollment; a time period that includes periconception. Medical records were abstracted at delivery to obtain information on birth outcomes, including birth weight and length. Infant cord blood specimens were collected at birth.

Assessment of birth outcome. Birth weight in grams and gestational age at delivery were abstracted from medical records following delivery.

Assessment of antibiotic exposure. To ascertain antibiotic use, participants were first asked a 'Yes/No' question: 'In the past six months did you take antibiotics?'; if the answer was 'Yes', pregnant women were asked to provide the name of the antibiotic used. To ensure inclusion of the periconceptional period, a vulnerable period for the fetus, the questionnaire was administered at enrollment so that antibiotic exposure included the 3 months period before conception. The mean gestational age at enrollment was 12 weeks (range 4–32 weeks). Study participants were unaware of the study hypothesis.

Measurement of co-variables. Current maternal body mass index (BMI) may reduce the effect of antibiotic use or may alter birth weight.[14] Maternal BMI was calculated from self-reported weight and height at the time of enrollment and converted to kilograms and meters and expressed in kilograms per square-meter ($kg\,m^{-2}$). Because birth weight also varies by race/ethnicity, women were asked to self-report their race/ethnicity. Maternal level of education attained, also a risk factor for birth weight, was self-reported.

DNA methylation analysis

Infant cord blood specimens were collected in EDTA-containing tubes and centrifuged using standard protocols to allow for collection of plasma and buffy coat for DNA extraction (Qiagen; Valencia, CA, USA); samples were stored at $-80^{\circ}C$ until required. DNA was extracted using Puregene reagents according to the manufacturer's protocol (Qiagen), and quantity and quality assessed using a Nanodrop 1000 Spectrophotometer (Thermo Scientific; Wilmington, DE, USA). Infant genomic DNA (800 ng) was modified by treatment with sodium bisulfite using the Zymo EZ DNA Methylation kit (Zymo Research; Irvine, CA, USA). Bisulfite treatment of denatured DNA converts all unmethylated cytosines to uracils but leaves methylated cytosines unchanged, allowing quantitative definition of cytosine methylation status. Pyrosequencing was performed using one of two Pyromark Q96 MD pyrosequencers (Qiagen). Nine imprinted DMRs for infants were analyzed: the *IGF2* DMR, the *H19* DMR, the *NNAT* DMR, the *DLK1/MEG3* DMR, the *DLK1/MEG3-IG* DMR, the *PLAGL1* DMR, the *SGCE/PEG10* DMR, the *PEG3* DMR, and the *MEST* DMR. Pyrosequencing assay design, genomic coordinates, assay conditions and assay validation have been previously described in detail.[19,33] Briefly, assays were designed to query established imprinted gene DMRs using the Pyromark Assay Design Software (Qiagen). PCR conditions were optimized to produce a single, robust amplification product by adjusting annealing temperature and magnesium chloride concentrations. Defined mixtures of fully methylated and unmethylated control DNAs were used to show a linear increase in detection of methylation values as the level of input DNA methylation increased (Pearson's $r>0.99$ for all DMRs). Once optimal conditions were defined, each DMR was analyzed using the same amount of input DNA from each specimen (40 ng, assuming complete recovery following bisulfite modification), keeping the thermocycler and pyrosequencer constant. Percentage of methylation for each CpG cytosine was determined using Pyro Q-CpG software (Qiagen). We interrogated between 3 and 10 CpG sites per DMR: 3 for *IGF2*, 4 for *H19*, 4

for *MEST*, 10 for *PEG3*, 6 for *PLAGL1*, 6 for *SGCE/PEG10*, 4 for *MEG3-IG*, 3 for *NNAT*, and 8 for *MEG3*. There was a high correlation between the values of CpGs within a DMR site (Cronbach's alphas for these regions were 0.95–0.99).

Statistical analyses

Infant birth weight showed no evidence of departure from normality and was used as both a continuous and a categorical variable. Categories were LBW (<2500 g), normal birth weight (2500–$\leqslant$4000 g), and HBW (>4000 g). We also calculated birth weight for gestational age *z*-scores as previously described.[34] We examined the distribution of factors that have been previously associated with birth weight and DNA methylation at birth, including maternal age (categorized as <25, 25–29, 30–34 and $\geqslant 35$ years); BMI dichotomized at <30, 30–35 and >35 kg m^{-2}; race/ethnicity (White, African American, Hispanic, others); maternal education (less than high school, high school graduate and some college-/college graduate-level education); maternal smoking (yes, no and quit during pregnancy); early prenatal folic acid intake (yes or no); gestational weight gain obtained from medical records; and gestational age at delivery, in relation to birth weight. Antibiotic use was dichotomized into non-users and users and further categorized by penicillins use and any other antibiotic use, including metronidazoles, nitrofurans, macrolides, tetracyclines, cephalosporines and quinolones. Factors previously associated with birth weight were compared with respect to antibiotic use during pregnancy using chi-square tests. Normality for each DMR was examined using the Kolmogorov–Smirnov test. With the exception of *PLAGL1* ($P<0.01$), *PEG3* ($P<0.01$), *DLK1/MEG3-IG* ($P<0.01$) and *MEST* ($P=0.03$), all other DMRs showed no evidence of departure from normality ($P>0.05$).[20] We used *F*-tests for parametric and Kruskal–Wallis and Wilcoxon–Mann–Whitney tests to examine DNA methylation levels by antibiotic use. Linear regression models were used to examine the associations between antibiotic use and birth weight outcomes. DMR methylation values were included in mixed models to allow unrestrained entry of individual CpGs into refined models, one at a time, to examine the extent to which antibiotic use influences methylation levels. All statistical analyses were conducted in SAS v9.3 (SAS Institute, Cary, NC, USA).

RESULTS

Table 1 summarizes the demographic characteristics of study participants. Twenty one percent ($n=82$) of the 397 women reported antibiotic use during pregnancy. Of these 82, 26% reported use of penicillins and 74% either did not report antibiotic names (48.6%) or reported use of nitrofurans (9%), metronidazoles (7%), quinolones (4%), tetracylclines (3%), macrolides (2%) and cephalosporines (0.4%). Most women (90%) were <35 years old, and maternal age did not vary by antibiotic use during pregnancy ($P=0.50$). About a third of reported antibiotic users ($n=27$) were obese (BMI >30 kg m^{-2}), similar to 32% who did not report antibiotic use ($P=0.92$). More African-American women reported no use of antibiotics (41%), compared with White (19%) and Hispanic (31%) women ($P=0.28$). Overall, women's education level ($<$high school, high school graduate and some college/college graduate) was not associated with antibiotic use ($P=0.97$). More antibiotic users (17.07%) reported folic acid intake compared with non-users (7%) ($P=0.007$), while the proportions of antibiotic users who reported maternal smoking ($P=0.83$), being exposed to environmental smoke ($P=0.73$), and engaging in physical activity >30 min a day ($P=0.93$) were similar compared with non-users. Twenty eight percent of primiparous women reported use of antibiotics compared with 29% who did not report antibiotic use. Mean gestational age at enrollment was 12 weeks (s.d. $=4.70$), mean gestational weight gain was 12.96 kg (s.d. $=8.35$), and infants were all singletons. Infants not exposed to antibiotics and exposed infants were different with respect to delivery route ($P=0.02$), but their gender distribution was similar ($P=0.22$). A slightly higher proportion of infants whose mothers reported antibiotic use during pregnancy were born LBW (5%) versus infants not exposed to antibiotics (3%) ($P=0.47$). Conversely, slightly more unexposed than exposed infants (8% versus 5%) were born with HBW ($P=0.15$; Table 1).

Associations between antibiotic use and birth weight

Mean birth weight in this cohort was 3346 g (s.d. $=479$, range 1500–5500 g). Table 2 shows coefficient estimates (β's) and their s.e.s. for the multivariate linear regression of birth weight on maternal antibiotic use, in which unexposed infants served as the baseline category. These models were adjusted for infant gender, race/ethnicity, maternal BMI, delivery route, gestational weight gain, gestational age at delivery, folic acid intake, physical activity, parity and maternal smoking. We observed a significant inverse association between antibiotic use during pregnancy and birth weight ($\beta=-132.99$, s.e. $=50.70$, $P=0.008$), corresponding to 138 g lower birth weight among infants born to antibiotic users (Table 2). This difference was more pronounced in newborns exposed to antibiotics other than penicillins (which included nitrofurans, metronidazoles, tetracyclines, macrolides, cephalosporins, quinolones; $\beta=-135.53$, s.e. $=57.38$, $P=0.02$). The most commonly reported antibiotic, penicillin, was not significantly associated with birth weight ($\beta=-125.91$, s.e. $=90.45$, $P=0.16$).

Because the potential effects of any drug will depend on maternal obesity, we repeated these analyses among women with pre-pregnancy BMI 30–35, BMI $\geqslant 35$ and in those with BMI <30 kg m^{-2}. We found that these associations were limited to infants born to women with BMI <30 kg m^{-2} (β-coefficient $=-204.75$, s.e. $=62.51$, $P=0.001$). However, the prevalence of BMI $\geqslant 30$ in African-American women was higher (45%) compared with Whites (25%) and Hispanics (24%). Restricting these analyses by race revealed a more pronounced difference in Whites ($\beta=-343.12$, s.e. $=101.65$, $P=0.0007$), compared with African Americans ($\beta=-137.04$, s.e. $=76.43$, $P=0.07$). The direction of the association between antibiotics and birth weight was in the opposite direction for Hispanic women ($\beta=+20.14$, s.e. $=97.13$ $P=0.84$). We stratified analyses by infant gender and found that these associations were stronger in male infants ($\beta=-197.15$, s.e. $=68.87$, $P=0.004$). These analyses were restricted to gestational age >37 weeks. These analyses were repeated in a larger sample of 1254 subjects, where 286 women reported antibiotic use, and the association between antibiotic use and birth weight was similar (all antibiotics: $\beta=-90.22$, s.e. $=35.88$, $P=0.01$; penicillins ($n=78$): $\beta=-25.00$, s.e. $=64.00$, $P=0.69$; and any other antibiotic ($n=208$): $\beta=-58.21$, s.e. $=20.43$, $P=0.004$). Furthermore, using birth weight for gestational age *z*-scores as the outcome to account for the non-linear growth *in utero,* we also found a strong inverse association between antibiotic use and birth weight ($\beta=-0.28$, s.e. $=0.11$, $P=0.01$) that was also stronger in infants exposed to any antibiotics ($\beta=-0.28$, s.e. $=0.12$, $P=0.02$) than to penicillins ($\beta=-0.27$, s.e. $=0.19$, $P=0.16$).

DNA methylation at DMRs regulating imprinted genes, prenatal antibiotic use and birth weight

We also examined whether DMR methylation of imprinted genes involved in growth and development mediated the association between antibiotic use during pregnancy and birth weight. To allow for unconstrained model entry of individual CpGs at each DMR, we used mixed models to first evaluate associations between DNA methylation levels at each of the nine DMRs and maternal antibiotic use. Using a liberal cutoff $P<0.2$, we found associations between DNA methylation and maternal antibiotic use for *MEG3* ($P=0.006$), *IGF2* ($P=0.05$), *PLAGL1* ($P=0.01$), *PEG3* ($P=0.08$) and *H19* ($P=0.15$), after adjusting for race/ethnicity, maternal education, gestational age at delivery, folic acid intake, maternal smoking and infant gender (Table 3). After accounting for multiple comparisons using Bonferroni's correction, methylation at only two DMRs, *PLAGL1* and *MEG3*, remained statistically

Table 1. Characteristics of the 397 NEST study participants by antibiotic use

Characteristic	*None* (n = *315*) N *(%)*	*All antibiotics* (n = *82*) N *(%)*	*Penicillins* (n = *21*) N *(%)*	*Any other antibiotics*[a] (n = *61*) N *(%)*
Maternal age (years)		$P = 0.50$	$P = 0.58$	$P = 0.74$
<25 ($n = 126$)	105 (33.33)	21 (25.61)	4 (19.05)	17 (27.87)
25–29 ($n = 122$)	95 (30.16)	27 (32.93)	7 (33.33)	20 (32.79)
30–34 ($n = 108$)	85 (26.98)	23 (28.05)	7 (33.33)	16 (26.23)
35+ ($n = 41$)	30 (9.52)	11 (13.41)	3 (14.29)	8 (13.11)
Maternal BMI		$P = 0.92$	$P = 0.89$	$P = 0.77$
<30 ($n = 269$)	214 (67.94)	55 (67.07)	14 (66.67)	41 (67.21)
30–35 ($n = 67$)	52 (16.15)	15 (18.29)	3 (14.29)	12 (19.67)
⩾30 ($n = 61$)	49 (15.56)	12 (14.63)	4 (19.05)	8 (13.11)
Race		$P = 0.28$	$P = 0.74$	$P = 0.39$
African American ($n = 163$)	130 (41.27)	93 (36.76)	8 (38.10)	25 (40.98)
Caucasian ($n = 83$)	60 (19.05)	88 (34.78)	6 (28.57)	17 (27.87)
Hispanic ($n = 116$)	97 (30.79)	44 (17.39)	5 (23.81)	14 (22.95)
Other ($n = 35$)	28 (8.89)	28 (11.07)	2 (9.52)	5 (8.20)
Gestational age at enrollment		$P = 0.70$	$P = 0.60$	$P = 0.82$
First trimester (0–13 weeks, $n = 222$)	173 (54.92)	49 (59.76)	14 (66.67)	35 (57.38)
Second trimester (>13 weeks, $n = 171$)	139 (44.13)	32 (39.02)	7 (33.33)	25 (40.98)
Third trimester (>26 weeks, $n = 4$)	3 (0.95)	1 (1.22)	0 (0.00)	1 (1.64)
Education		$P = 0.97$	$P = 0.85$	$P = 0.99$
<High-school graduate ($n = 72$)	57 (18.10)	15 (18.29)	4 (19.05)	11 (18.03
High school ($n = 174$)	139 (44.13)	35 (42.68)	8 (38.10)	27 (44.26)
Some college/college graduate ($n = 151$)	119 (37.38)	32 (39.02)	9 (42.86)	23 (37.70)
Gender of infants		$P = 0.22$	$P = 0.97$	$P = 0.15$
Male ($n = 194$)	149 (47.30)	45 (54.88)	10 (47.62)	35 (49.73)
Female ($n = 203$)	166 (52.70)	37 (45.12)	11 (52.38)	26 (50.27)
Delivery route		$P = 0.02$	$P = 0.08$	$P = 0.07$
Vaginal ($n = 270$)	223 (70.79)	47 (57.32)	11 (52.38)	36 (59.02)
Caesarean section ($n = 127$)	92 (29.21)	35 (42.68)	10 (47.62)	25 (40.98)
Folate intake		$P = 0.007$	$P = 0.25$	$P = 0.009$
Yes ($n = 37$)	23 (7.30)	14 (17.07)	3 (14.29)	11 (18.03)
No ($n = 360$)	292 (92.70)	68 (82.93)	18 (85.71)	50 (81.97)
Maternal smoking		$P = 0.83$	$P = 0.44$	$P = 0.42$
Yes ($n = 34$)	27 (8.57)	7 (8.54)	3 (14.29)	4 (6.56)
Quit during pregnancy ($n = 78$)	60 (19.05)	18 (21.95)	2 (9.52)	16 (26.23)
No ($n = 285$)	228 (72.38)	57 (69.51)	16 (76.19)	41 (67.21)
Environmental smoke		$P = 0.73$	$P = 0.99$	$P = 0.68$
Yes ($n = 77$)	60 (20.73)	17 (20.73)	4 (19.05)	13 (21.31)
No ($n = 320$)	255 (80.95)	65 (79.27)	17 (80.95)	48 (78.69)
Physical activity		$P = 0.93$	$P = 0.70$	$P = 0.76$
Yes ($n = 110$)	87 (27.62)	23 (28.05)	5 (23.81)	18 (29.51)
No ($n = 287$)	228 (72.38)	59 (71.95)	16 (76.19)	43 (70.49)
Parity		$P = 0.83$	$P = 0.21$	$P = 0.68$
Primiparous ($n = 115$)	92 (29.21)	23 (28.05)	8 (38.10)	15 (24.59)
Non-primiparous ($n = 282$)	223 (70.79)	59 (71.95)	13 (61.90)	46 (75.41)
Gestational weight gain (kg)		$P = 0.42$	$P = 0.61$	$P = 0.20$
(mean, s.d.)	13.39 (8.14)	12.53 (8.57)	14.34 (5.12)	11.92 (9.43)
Birth weight (g)		$P = 0.02$	$P = 0.50$	$P = 0.02$
(mean, s.d.)	3374 (479.63)	3236 (461.1)	3299 (595.42)	3214 (408.60)
Birth weight		$P = 0.47$	$P = 0.15$	$P = 0.29$
Normal BW 2500–4000 g ($n = 356$)	282 (89.52)	74 (90.24)	16 (76.19)	58 (95.08)
LBW <2500 g ($n = 13$)	9 (2.86)	4 (4.88)	2 (9.52)	2 (3.28)
HBW >4000 g ($n = 28$)	24 (7.62)	4 (4.88)	3 (14.29)	1 (1.64)

Abbreviations: BMI, body mass index; BW, birth weight; HBW, high birth weight; LBW, low birth weight; NEST, Newborn Epigenetic STudy. Numbers not necessarily add up due to missing values. [a]Any antibiotics include: nitrofurans, metronidazoles, tetracyclines, macrolides, cephalosporins, quinolones and any other antibiotic.

significant. We then examined whether DNA methylation at these DMRs was also associated with birth weight and whether inclusion of *PLAGL1* or *MEG3* DMRs in the base model altered the association between antibiotic use and birth weight. We found that a 1% increase in DNA methylation at the *PLAGL1* ($P = 0.04$) but not at the *MEG3* ($P = 0.42$) DMR was significantly associated with a 10-g increase in birth weight (Table 4). After adjusting for infant gender, race/ethnicity, maternal BMI, delivery route, gestational weight gain, gestational age at delivery, folic acid intake, physical activity, parity and maternal smoking (Table 5), including *PLAGL1* DMR methylation into the base model depicting the association between antibiotic use and birth weight revealed a somewhat stronger inverse association between antibiotic use and birth weight ($\beta = -151.38$, s.e. = 71.59, $P = 0.03$ in all antibiotic users, $\beta = -216.42$, s.e. = 76.36, $P = 0.005$, in users of antibiotics other than penicillins). Because DMR methylation may be less malleable in late pregnancy, we also repeated these analyses among 222 women who were enrolled in the first trimester, and these findings remained unaltered (data not shown).

Table 2. Adjusted regression coefficients and s.es. for the associations between antibiotic use during pregnancy and birth weight[a]

	Any antibiotics (n = *82*)	*Penicillins* (n = *21*)	*Any other antibiotics* (n = *61*)
	β-*coefficient, s.e.,* P-*value*	β-*coefficient, s.e.,* P-*value*	β-*coefficient, s.e.,* P-*value*
All (*n* = 396)	− 132.99, 50.70, 0.008	− 125.91, 90.45, 0.16	− 135.53, 57.38, 0.02
Blacks (*n* = 162)	− 137.04, 76.43, 0.07	− 292.11, 139.37, 0.04	− 86.09, 85.16, 0.31
Whites (*n* = 83)	− 343.12, 101.65, 0.0007	− 253.16, 179.32, 0.16	− 379.28, 117.55, 0.001
Hispanics (*n* = 116)	+ 20.14, 97.13, 0.84	+ 11.33, 183.02, 0.95	+ 23.02, 109.53, 0.83
Maternal BMI < 30 (*n* = 268)	− 204.75, 62.51, 0.001	− 138.24, 122.14, 0.22	− 227.76, 70.27, 0.001
Maternal BMI 30–35 (*n* = 67)	− 20.82, 134.17, 0.84	− 390.25, 265.55, 0.14	+ 50.68, 140.63, 0.72
Maternal BMI > 35 (*n* = 353)	− 14.64, 91.28, 0.87	− 156.68, 160.99, 0.33	35.03, 102.07, 0.73
Male infants (*n* = 193)	− 197.15, 68.87, 0.004	− 141.33, 130.54, 0.28	− 214.23, 76.74, 0.005
Female infants (*n* = 203)	− 70.27, 74.66, 0.35	− 138.92, 125.85, 0.27	− 41.71, 85.68, 0.62

Abbreviation: BMI, body mass index. [a]Adjusted for infant gender, race, maternal BMI, delivery route, gestational weight gain, gestational age at delivery, folic acid intake, physical activity, parity and maternal smoking.

Table 3. Mean DNA methylation percentages, regression coefficients (β, s.e.) and *P*-values for the association between antibiotic exposure and DMR methylation among 396 infants[a]

DMR	*Mean methylation % (s.d.)*	*Any antibiotics* (n = *82*) β-*coefficient, s.e.,* P-*value*	*Penicillins* (n = *21*) β-*coefficient, s.e.,* P-*value*	*Any other antibiotics* (n = *61*) β-*coefficient, s.e.,* P-*value*
IGF2	51.30 (4.37)	+ 0.11, 0.32, 0.73	+ 1.24, 0.56, 0.05	− 0.32, 0.36, 0.40
H19	47.85 (3.86)	+ 0.35, 0.23, 0.15	+ 0.18, 0.40, 0.65	+ 0.42, 0.27, 0.15
PLAGL1	57.34 (6.53)	+ 0.85, 0.32, 0.01	+ 1.84, 0.56, 0.003	+ 0.47, 0.36, 0.21
MEG3	72.37 (5.51)	+ 0.17, 0.43, 0.69	+ 2.43, 0.79, 0.006	− 0.57, 0.48, 0.25
MEG3_IG	49.45 (3.33)	+ 0.17, 0.46, 0.72	+ 0.06, 0.85, 0.94	+ 0.20, 0.52, 0.70
NNAT	55.08 (5.84)	+ 0.14, 0.52, 0.79	+ 0.83, 0.85, 0.35	− 0.17, 0.60, 0.77
PEG3	36.09 (3.34)	+ 0.31, 0.17, 0.08	+ 0.41, 0.32, 0.21	+ 0.28, 0.19, 0.15
SGCE/PEG10	45.07 (5.44)	+ 0.17, 0.27, 0.55	+ 0.01, 0.49, 0.98	+ 0.22, 0.31, 0.48
MEST	43.41 (4.55)	− 0.21, 0.30, 0.49	+ 0.22, 0.51, 0.67	− 0.38, 0.34, 0.28

Abbreviations: DMR, differentially methylated region; *IGF2, insulin-like growth factor 2; MEG3, maternally expressed gene 3; MEST, mesoderm-specific transcript; NNAT, neuronatin; PEG3, paternally expressed gene 3; PLAGL1, pleomorphic adenoma gene-like 1; SGCE, epsilon sarcoglycan.* [a]Adjusted for infant gender, race/ethnicity, maternal education, maternal smoking, folic acid intake and gestational age at delivery.

Table 4. Adjusted regression coefficients and s.es. for the associations between DMR methylation and birth weight among 396 infants[a]

DMR	*Any antibiotics* (n = *82*) β-*coefficient, s.e.,* P-*value*
MEG3	− 4.7, 5.85, 0.42
PLAGL1	+ 10.47, 5.22, 0.04

Abbreviations: DMR, differentially methylated region; *MEG3, maternally expressed gene 3*; *PLAGL1, pleomorphic adenoma gene-like 1.* [a]Adjusted for infant gender, race/ethnicity, maternal education, maternal smoking, folic acid intake and gestational age at delivery.

DISCUSSION

Antibiotics are the most commonly prescribed medication during pregnancy,[4] and in livestock, antibiotic use has been associated with rapid growth.[35] We examined whether antibiotic use in pregnancy up to 30 weeks gestation was associated with birth weight in a cohort of 397 newborns. Our key finding was that newborns exposed to antibiotics during pregnancy had a lower birth weight when compared with infants not prenatally exposed to antibiotics. These differences were most apparent in males and white infants and in newborns born to women with BMIs $< 30\,kg\,m^{-2}$. We also found that *PLAGL1* DMR methylation was associated with both antibiotic exposure and birth weight. Previous studies have found that infants with lower birth weights (even within normal birth weights) have more rapid postnatal catch-up growth and overweight in early infancy, a condition likely to persist into adulthood.[36] These findings support imprinted gene plasticity in response to antibiotic exposure that may affect intrauterine growth.[37]

Our findings are consistent with other studies showing that antibiotic exposure during pregnancy is associated with reduced birth weight.[5] Czeizel's group[5] reported an average decrease in birth weight of 40 g for infants exposed to penicillins, compared with unexposed infants—similar to the 75-g difference reported here. However, these findings contrast with a report where a 57-g increase in birth weight was reported among infants exposed to antibiotics during pregnancy, compared with infants not exposed to antibiotics,[6] although antibiotic use for most women in this study was after the third trimester of pregnancy.[6] Our finding is consistent with the US trend in reduced birth weight observed between 1990 and 2005 (− 52 g in the overall population and − 79 g in a homogeneous low-risk subgroup),[18] as antibiotic use in pregnant women increased.[4] In our study, even strong predictors of HBW, such as maternal overweight and obesity, did not influence the inverse association between antibiotic exposure during pregnancy and birth weight.

Our finding that antibiotic exposure in non-obese mothers was inversely associated with birth weight is intriguing, as antibiotics are growth stimulants in livestock[38] and have been suggested as contributors to the obesity epidemic.[38] However, in the womb, the

Table 5. [a]Adjusted regression coefficients and s.e.s. for the associations between antibiotic use during pregnancy and birth weight when DMR methylation is introduced into the model

DMR	*Any antibiotics* (n = *82*)	*Penicillins* (n = *21*)	*Any other antibiotics* (n = *61*)
All (n = 396)	β-*coefficient, s.e.,* P-*value*	β-*coefficient, s.e.,* P-*value*	β-*coefficient, s.e.,* P-*value*
Base model	− 132.99, 50.70, 0.008	− 125.91, 90.45, 0.16	− 135.53, 57.38, 0.02
PLAGL1	− 151.38, 71.59, 0.03	+ 128.99, 142.99, 0.36	− 216.42, 76.36, 0.005

Abbreviations: DMR, differential methylated region; *PLAGL1*, *pleomorphic adenoma gene-like 1*. [a]Adjusted for infant gender, race/ethnicity, maternal body mass index, delivery route, gestational weight gain, gestational age at delivery, folic acid intake, physical activity, maternal smoking and parity.

infant gut is relatively sterile[39] and thus antibiotics could not be exerting the same effect as in fully colonized guts. In addition, our findings of reduced birth weight also have implications for postnatal development, particularly catch-up growth in early life. Children who are SGA and exposed to antibiotics early in life have a higher risk of overweight later in life, by age 7 years.[40,41] Antibiotics may alter the diversity and composition of the maternal gut microbiota and possibly reducing the population of obesogenic microbes. The latter include the *Bacteroides*, *Clostridium*, *and Staphylococcus* genuses, which are found in higher concentrations in overweight pregnant women and high gestational-weight-gain women, as well as in overweight children.[42] According to this hypothesis, newborns of mothers exposed to antibiotics would inherit a less obesogenic maternal microbiota, which is consistent with evidence of a reduced risk of overweight for infants born to obese mothers who were administered antibiotics in the early postnatal period.[43] Taken together, our findings and other studies support the hypothesis that early exposure to antibiotics may prematurely alter the diversity of microbiota in the fetus. It has been suggested that the cumulative effect of pre- and postnatal antibiotic exposure may increase the risk of chronic diseases in adulthood, as gut microbiota composition is intimately linked to a healthy immune system,[44] via epigenetic adjustment evident at some loci.

Reasons for the present increase in SGA births in the United States are not known, and although racial and ethnic disparities and variation by geography and familial aggregation have been suggested as potential contributors,[45] the effect of prescription drug intake during pregnancy on birth weight has not been thoroughly investigated. Although associations between antibiotic use during pregnancy and birth weight outcomes have been described recently in a handful of studies,[5,6] and epigenetics has been hypothesized to mediate these relationships, to our knowledge, this is the first study to examine a potential epigenetic mediation to birth weight outcomes when infants are exposed to antibiotics *in utero*. Clarifying the relationship between antibiotic use during pregnancy and birth weight outcomes has the potential to further our understanding of whether frequently prescribed medications taken during fetal development permanently alter the epigenome and thus increase the risk of chronic diseases in childhood and adulthood. Methylation differences between antibiotic exposed and unexposed infants were found at *MEG3*, *PEG3*, *PLAGL1*, *IGF2* and *H19* DMRs; however, only methylation at *PLAGL1* DMR was statistically significantly associated with birth weight, suggesting that DNA methylation alterations at this DMR may be potentially mediating the association between antibiotic use and birth weight. The effect of *PLAGL1* DMR has implications for follow-up studies in larger populations to determine whether these epigenetic signatures become permanent and influence these imprinted genes' expression and thus infant development in early postnatal life.

Our findings should be interpreted in the context of their limitations. Even though the NEST cohort includes a large number of pregnant women, antibiotic use was self-reported with no follow-up metabolite analysis. Less than half of participants who used antibiotics recalled the name of the antibiotic prescribed. However, it is unlikely that women would report antibiotic use when none were used. In addition, although we did not measure antibiotic dosage nor duration, or metabolites, most women reported antibiotic use in the 24 weeks previous to enrollment, a time frame that included periconception and the first 12 weeks of pregnancy, when fetal vulnerability is highest during organogenesis. Another limitation is the possibility that there could have been confounding by indication. However, sub-analysis of reported morbidity did not suggest that morbidity was associated with birth weight. The small numbers of infants with DNA methylation data did not allow us to present data further stratified by sex and race/ethnicity, although these findings did not diverge from what is reported here. Moreover, because this is an ongoing prospective study, we plan to confirm these initial results in larger samples. Another limitation is that we were not able to measure gut microbiota composition in antibiotic exposed versus unexposed infants, nor did we analyze transcript levels of the imprinted genes, although such studies are planned.

Although small numbers limit our ability to make inferences, our results suggest that prenatal exposure to antibiotics is associated with lower birth weight, an association that may be mediated, at least in part, by alterations in the differential methylation at regulatory regions of imprinted genes. Larger studies using agnostic approaches to epigenetic targets are needed to confirm these findings.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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