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High-resolution metabolomics of exposure to tobacco smoke during pregnancy and adverse birth outcomes in the Atlanta African American Maternal-Child cohort

By

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Master of Science in Public Health

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2019

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An abstract of
a thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
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Abstract

High-resolution metabolomics of exposure to tobacco smoke during pregnancy and adverse birth outcomes in the Atlanta African American Maternal-Child cohort

By Youran Tan

Background: Exposure to tobacco smoke during pregnancy has been associated with a series of adverse reproductive outcomes; however, the underlying molecular mechanisms are not well-established. To help close this gap, we conducted a metabolome-wide association study (MWAS) to identify the metabolic perturbations and potential biomarkers underlying the association between cotinine, a widely used biomarker of tobacco exposure, and adverse birth outcomes.

Methods: We collected early and late pregnancy urine samples for cotinine measurement and serum samples for high-resolution metabolomics (HRM) profiling from 105 pregnant women from the Atlanta African American Maternal-Child cohort (2014-2016). Maternal metabolome perturbations mediating prenatal tobacco smoke exposure and adverse birth outcomes (preterm birth, early term birth *vs*. full term birth; gestational age at birth) were assessed by an untargeted HRM workflow using generalized linear models, followed by pathway enrichment analysis and chemical annotation, with a *meet-in-the-middle* approach.

Results: The median maternal urinary cotinine concentrations were 5.93 ug/g creatinine and 3.69 ug/g creatinine in early and late pregnancy, respectively, with a total of 29 women having higher than 100 ug/g creatinine concentration. In total, 16,481 and 13,043 metabolic features were identified in serum samples at each visit using liquid chromatography-high resolution mass spectrometry with positive and negative electrospray ionization (ESI) modes, respectively. Thirteen metabolic pathways were found to be associated with cotinine concentrations and adverse birth outcomes during early and late pregnancy, including tryptophan, histidine, urea cycle, arginine, and proline metabolism. We confirmed 47 metabolites associated with cotinine exposure, preterm birth, and shorter gestational length, including glutamate, serine, choline, and taurine. The identified metabolites are closely involved in endogenous inflammation, vascular reactivity, and lipid peroxidation processes.

Conclusions: The metabolic perturbations associated with cotinine exposure were related to inflammation, oxidative stress, placental vascularization, and insulin action, which could contribute to shorter gestations. These findings support the future development of targeted interventions to reduce adverse birth outcomes associated with tobacco smoke exposure, especially among African American women who are disproportionately exposed to high tobacco smoke and experience higher rates of adverse birth outcomes.

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BACKGROUND

Burden of Adverse Birth Outcomes among Pregnant Women

Adverse birth outcomes usually refer to preterm births (medically defined as less than 37 weeks of gestation) and low birth weight (less than 5.5 pounds), which could lead to higher rates of illness and infection of newborns, as well as long-term neurological and health problems.[1] In 2009, approximately 12% of infants born in the U.S. were premature and around 8% of infants were classified as low birth weight.[2] Preterm births account for 75% of prenatal mortality and more than half of the long-term morbidity. Although most preterm babies survive, they are at increased risk of neurodevelopmental impairments and respiratory and gastrointestinal complications.[3] Low birth weight accounts for most deaths in the first year of life, and low birth weight rates remained consistently elevated, as has the black-white disparity in birth outcomes.[4] Despite the tremendous efforts in maternal and child health over the past century, socioeconomic and racial/ethnic disparities in preterm birth and low birth weight have been relatively intractable in the U.S.[5] It has been shown that the rates of preterm birth are always higher among women living in poverty compared to higher-income women.[6] African American (AA) women were about twice as likely as non-Hispanic white women (14.0% *vs.* 7.3%) to have a LBW infant in 2006,[7] and their risk of infant death was 2.4 times the white rate in 2005, as they were reported to shoulder the greatest burden of preterm birth, low birth weight and even infant death of all racial/ethnic groups in the country.[8] Moreover, AA women's reproductive vulnerability is evident across all of the leading causes of infant death,[8] and pregnant AA women have greater accrued risk and fewer protections against poor birth outcomes due to their potential social inequities.

Risk Factors for Adverse Birth Outcomes

The importance of preterm birth and low birth weight not only comes from its capacity to predict increased risk of mortality and morbidity among infants with these adverse birth outcomes, it also reflects the maternal exposures to other risk factors such as socio-economic conditions, malnutrition, behavioral factors and potential diseases. It has been reported many maternal or fetal characteristics

that have been associated with preterm birth, including maternal demographic characteristics (socioeconomic and education status, maternal ages and marital status),[9] nutritional status (body-mass index, nutritional intake and serum assessment of various analytes),[10] mental health, adverse behaviors (smoke, drug and alcohol use), infection uterine contractions and cervical length.[11] Several similar variables as preterm birth related have been identified in the literature as being associated with low birth weight and the most often cited are maternal age, maternal education level, number of prenatal care visits, sex of the neonate and the duration of gestation.[12] Abuse, unhealth diet, poor maternal weight gain, psychological morbidity, stress as well as lack of social support have also been identified as risk factors for low birth weight.[13]

One of the most studied potential risk factors for adverse birth outcomes involves racial and socioeconomic disparity. The disparity in preterm birth rates between black and white women has remained largely unchanged and unexplained, and contributes to a cycle of reproductive disadvantage with far-reaching social and medical consequences. It was reported that U.S.-born Blacks had a 3.2 (95% confidence interval, CI: 3.0-3.5) and 4.4 (95% CI: 4.3-4.5) percentage point higher risk of preterm birth than foreign-born Blacks and U.S.-born Whites, respectively.[14] Meanwhile, African American women have reported more racial discrimination in education, getting a job, housing and medical care, and they were 3 times more likely to have preterm birth.[15] The odds ratio (OR) for the birth of a child weighing less than 1500 gm was doubled for women who reported having experienced racial discrimination,[16] and the OR for preterm birth were 1.3 (95% CI: 1.1-1.6) for women who reported unfair treatment on the job and 1.4 (95% CI: 1.0–1.9) for women who reported that people acted afraid of them at least once a week.[17]

Other maternal risk factors for adverse birth outcomes mentioned above have been also largely reported. Working long hours and undertaking hard physical labor under stressful conditions are probably associated with an increase in preterm birth that women who worked at a high-strain job full-time (OR=1.4, 95% CI: 0.9-2.0) or for 30 or more weeks (OR=1.4, 95% CI: 1.0-2.2) had a modestly increased risk,[18] and 50% elevation in the risk of preterm delivery among women who reported working at night (RR=1.5, 95% CI: 1.0-2.0).[19] Another analysis revealed that stress was significantly associated with spontaneous preterm birth and with low birth weight with ORs of 1.16 (*p*-value=*0.003*)

and 1.08 (*p*-value=*0.02*), respectively.[20] Maternal nutrition has been also examined in some studies that folic acid supplementation had favorable effects on birth weight and reduced prevalence of fetal growth retardation and maternal infections, [21] and obese women had fewer spontaneous preterm births at < 37 weeks of gestation (6.2% *vs*. 11.2%; *p*-value< *0.001*) and at < 34 weeks of gestation (1.5% *vs* 3.5%; *p*-value = *0.012*) compared to women with normal BMI.[10]

Tobacco Smoke Exposure and Adverse Birth Outcomes

It has been well established that exposure to tobacco smoke during pregnancy is associated with a series of adverse reproductive outcomes, including preterm birth, various adverse placental conditions, and intrauterine growth restriction.[22] Both active and passive smoking have been found to alter expression of key mediators of placental development,[23] which could illustrate the potential mechanism for the adverse birth outcomes associated with tobacco smoke exposure. Active tobacco smoke exposure was shown to be associated with a nearly two-fold higher risk of preterm birth,[24] and a lower birth weight.[25] Specifically, cigarette smoking was associated with higher risk of preterm delivery among both African American (OR=1.77, 95% CI: 1.12-2.79) and White women (OR=1.25, 95% CI: 1.01-1.55).[26] Moreover, a dose effect of tobacco smoke exposure on preterm birth and neonatal body mass composition has been reported in many studies,[27-30] with higher frequency of smoking associated with higher rate of preterm birth and lower neonatal body mass. The highest impact of smoking was seen on risk of very preterm birth among women who smoked at least 10 cigarettes/d (OR=1.7, 95% CI:1.4-2.0) compared to non-smokers.[31] Another study showed consistency on the dose effect of tobacco smoke exposure that the ORs of very preterm birth among moderate smokers (1-9 cigarettes per day) and heavy smokers (≥10 cigarettes per day) were 1.4 (95% CI 0.8-2.4) and 2.9 (95% CI 1.5-5.7), respectively, compared to non-smokers.[32]

The tobacco smoke exposure during, but not before, pregnancy has been reported to be associated with high risk of adverse birth outcomes. Active smoking until pregnancy was not associated with low birthweight and preterm birth, and continued active smoking after pregnancy was also recorded and was associated with low birthweight (OR=1.75, 95% CI: 1.20-2.56) and preterm birth (OR=1.36, 95% CI:1.04-1.78).[33] Other observational studies also showed a consistent association of

maternal smoking during pregnancy with adverse birth outcomes but no or little association for maternal smoking before pregnancy. Mean birth weight, adjusted for maternal age, parity, parents' occupation and neonates' sex and nationality, was lower by 190 g (95% CI: 150-220) in babies of smokers (mothers reported smoking during pregnancy) than those of non-smokers and the multivariable-adjusted OR was 1.4 (95% CI: 1.1-1.9) for preterm birth.[34] Some researchers showed that past smoking was not associated with the outcomes, and compared to nonsmokers, any maternal smoking during the three months prior to conception and continued into the first trimester of pregnancy was associated with increased preterm birth (OR=1.17, 95% CI: 1.16–1.19). This risk was higher if maternal smoking continued during the second trimester (OR=1.45, 95% CI: 1.45–1.46).[35] Other adverse reproductive outcomes reported to be associated with tobacco smoke exposure included lower fetal growth,[36] higher risk of infant morbidity and mortality, and sudden infant death syndrome (SIDS).[37, 38] It was reported that maternal smoking during pregnancy is associated with a significantly higher risk of SIDS (adjusted odds ratio (AOR)=2.7, 95% CI: 2.4-3.0) and preterm-related deaths (AOR=1.5, 95% CI: 1.4-1.6), respectively.[39]

Although there is a marked drop in the rate of active maternal smoking in the US due to the increased public awareness,[40] the prevalence of exposure to passive or secondhand tobacco smoke among pregnant women remains high. It is estimated to be up to 30% in a population-based case-control study in United States.[41] Specifically, passive maternal smoking has been associated with a lower birthweight and higher risk of small for gestation age and stillbirth infants.[42, 43] While active maternal tobacco smoking has been well established to be associated with adverse perinatal outcomes, the effects of passive tobacco exposure have been less well studied and are less consistent in the literature.[44] A recent meta-analysis, which summarized the results of studies published prior to May 2009, found no effect of passive maternal smoking on preterm birth (pooled risk estimate = 1.07, 95% CI: 0.93–1.22),[44] while another meta-analysis study reported that the summary ORs of preterm birth for women who were ever exposed to passive smoking *vs.* women who had never been exposed to passive smoking at any place and at home were 1.20 (95% CI: 1.07–1.34) and 1.16 (95% CI: 1.04–1.30), respectively.[45] Such inconsistency could be due to the self-reported environmental tobacco smoke exposure or other uncontrolled confounding factors. High passive tobacco smoke exposure (7<

hours/day in non-smokers) was moderately associated with low birth weight (AOR =1.8, 95% CI: 0.82-4.1) and particularly to very preterm birth (AOR=1.6, 95% CI: 0.87-2.9), but some studies of environmental tobacco smoke exposure and low birth weights have found varying results from no effect up to about a doubling of risk.[46] It was also reported that passive tobacco smoke and low birth weight were more strongly associated in non-whites than in whites.[47]

However, most of the studies on the reproductive consequences of tobacco smoke exposure have relied on subjects' self-report rather than biochemical markers to represent the actual amount of tobacco exposure, which could be affected by underreporting or variation in smoking efficiency.[48] A more objective approach now available involves quantifying biomarkers of tobacco exposure directly in biological samples. Nicotine, the primary addictive component in tobacco smoke, is metabolized into cotinine, which is the most widely used biomarker for evaluating tobacco exposure due to its higher stability and long half-life in body fluids.[49-51] Maternal cotinine concentrations have been shown to be associated with adverse birth outcomes including low birthweight [52] and preterm birth.[53], with ORs for fetal death, preterm delivery, and term-low birth weight being 3.4, 1.8, and 1.8, respectively, in the highest cotinine quintile (0.236–10 ng/mL), compared with the lowest quintile (<0.026 ng/mL).[54] However, the endogenous biological mechanisms underlying the association between cotinine exposure and adverse birth outcomes remained unclear.[55]

High-resolution Metabolomics of Tobacco Smoke and Birth Outcomes

High-resolution metabolomics (HRM) has emerged as an innovative analytical platform for identification of internal metabolites (e.g., present in a given biological media, such as blood or saliva). This method provides new opportunities for epidemiologists to investigate the association of external exposures with endogenous processes at the molecular level.[56, 57] In most of prior work, targeted methods have been used to identify and quantify the metabolites of interest (e.g., identified biomarkers for inflammation or oxidative stress) in a single sample. Recently, untargeted HRM have been used to improve internal exposure estimation to complex environmental mixtures by providing identification and quantification of thousands of metabolic features in biological samples associated with endogenous and exogenous process.[57-59] Metabolomics can be applied to reproductive and prenatal medicine and

promote the identification of non-invasive biomarkers for diagnostic and prognostic purposes.[60] In the context of adverse birth outcomes, metabolomics may be useful to assess the actual number of pregnant women and to highlight the pathophysiological mechanisms that lead to the potential adverse birth outcomes.

There is growing interest in identifying the metabolic alterations associated with tobacco exposure and adverse birth outcomes. It has been reported that the potential perturbed metabolic pathways by cigarette smoking could involve in the benzoate, caffeine, vitamin groups (vitamin A, vitamin E and vitamin B6), steroid, amino acid and carbohydrate pathways.[61] Maternal serum cotinine exposure was shown to be associated with significant metabolic perturbation in amniotic fluid, with the most affected pathways involving aspartate and asparagine metabolism, arginine and proline metabolism, and methionine metabolism.[55] Some of those pathways include polyamines, a class of compounds recognized as critical in fetal development.[62] The metabolic signals associated with adverse birth outcomes have also been examined. The changes in level of vitamin A, progesterone, and molecules, involved in pathways related to tryptophan metabolism, carnitine shuttle, fatty acid and glycerophospholipid metabolism, could be associated with birth weight.[63] However, only few of these studies examined whether the effects of maternal smoke exposure on adverse birth outcomes are mediated by metabolic perturbations, and none of have focused on pregnant African American women, who are at higher risk for adverse birth outcomes.

Thus, to address the current research gaps and uncertainties, we performed a metabolome-wide association study (MWAS) using untargeted high-resolution mass spectrometry to identify metabolic alterations associated with maternal cotinine level and perturbed birth outcomes among African American pregnant women within the Atlanta ECHO cohort. The main objectives of this study are: 1). Examine the association between maternal cotinine levels and perturbation in the maternal metabolome in both early and late pregnant stages; 2). Assess the potential metabolic signals/pathways associated with adverse birth outcomes; 3). Explore whether the perturbed metabolic pathways associated with maternal cotinine levels are also associated with adverse birth outcomes under a *meet-in-the middle* framework. We expect this study could help us further understand the biological responses to internal

cotinine exposure, and ultimately inform targeted interventions to reduce health disparity in this minority population.

METHODS

Study Population

The current analysis included a subset of 320 pregnant women within the Atlanta African American Maternal-Child Cohort.[64, 65] Pregnant women who self-identified as African American were recruited to participate in the study from two prenatal care clinics, the Emory Midtown Hospital (private) and the Grady Hospital (public funded). In this study, prenatal enrollment was restricted to those with singleton pregnancies without chronic medical conditions and between 8- and 14-week gestation for first prenatal clinic visit. Data collection on questionnaire for basic demographic information, biological samples (blood and urine), and medical records for clinical condition were conducted at two time points: 1) prenatal care visit 1-between 8-14 weeks gestation; and 2) prenatal care visit 2-between 24-30 weeks gestation. Collection of birth outcome data from medical records was done post-delivery. In this analysis, we included a total of 105 participants with both urinary cotinine concentrations and metabolic profiling. Of them, 97 participants attended the first clinical visit and 81 attended the second visit (73 women attended both visits). This study was approved by the Emory University Internal Review Board and informed consent was obtained from all study participants.

Measurement of Maternal Cotinine Concentrations

Maternal cotinine concentrations were measured from individual urine sample collected during the first and second clinical visits, separately, and quantified using liquid chromatography combined with mass spectrometry (LC-MS-MS). Here, we used total cotinine concentration (the sum of cotinine and its glucuronide; TCOT) in urine samples to represent the exposure level to tobacco smoking. To correct the dilution effect of urinary cotinine concentrations, we also quantified the creatinine level in the urine samples and adjusted urinary cotinine level via two normalization approaches,[66] the standardization approach using the ratio of cotinine concentration to the creatinine levels measured in the same sample and the covariate adjustment approach with creatinine concentration as a covariate included in the

regression model. We chose to use the standardization approach for our main analysis and a covariate adjustment approach for later sensitivity analysis. Unadjusted analyte concentrations were provided in the unit of ng/ml and μg/g for creatinine-adjusted analyte concentrations.

Measure of Adverse Birth Outcomes and Other Factors

The birth outcomes variables in this analysis included: 1) birth outcome (categorical) with the following 3 subcategories: preterm birth (>20 and <37 weeks), early term birth (≥37 and <39 weeks), and full term birth (≥39 weeks); and 2) gestational age at birth (continuous), which is based upon the date of delivery in relation to the estimated date of pregnancy established during the first clinical visit, obtained from medical records that considers last menstrual period and first visit ultrasound. We also collected information on potential confounders including individual-level demographic characteristics (age, marital status, education, and income level), behavioral risk factors (alcohol drinking and marijuana use during pregnancy), and prenatal characteristic [gestational age at two prenatal care visits, parity, and body mass index (BMI) at first prenatal visit].

Untargeted High-Resolution Metabolomics Analysis

Metabolic profiling was completed using maternal serum samples collected at the first and second visits from each participant using previously established protocols.[67] Each sample was analyzed in triplicate using liquid chromatography with high-resolution mass spectrometry (LC-HRMS) techniques (Thermo Scientific™ Q- Exactive™ HF). To enhance the coverage of metabolic feature detection, the sample was performed in polar and nonpolar analytical columns and analysis modes, hydrophilic interaction liquid chromatography (HILIC) with positive electrospray ionization (ESI) and C18 hydrophobic reversed-phase chromatography with negative ESI. Data extraction was conducted using apLCMS and xMSanalyzer.[57, 68] Only metabolic feature detected in >15% of serum samples with median coefficient of variation (CV) among technical replicates <30% and Pearson correlation $\rho > 0.7$ were included in further analyses. The resulting analytical data contained individual features defined

by mass-to-charge ratio (m/z), retention time (RT) and ion intensities. Then, we averaged the replicate samples that have at least one non-zero intensity and performed log 2 transformation.

Statistical Analyses

To compare the baseline demographic information and prenatal characteristics among participants in two study sites, Emory Midtown hospital and Grady hospital, we used t-test for continuous variables (age, gestational age at two visits, maternal BMI) and χ^2 test for categorical variables (sex of baby). Specially, we used fisher test to compare the categorical variables if the sample size in some categories is less than 5 (birth outcomes, marital status, educational attainment, income, nulliparous, insurance type, alcohol and tobacco use in two visits). The difference of maternal cotinine concentration in two visits was compared using Mann-Whitney U test after examining the distribution of cotinine concentration in two visits.

To evaluate the association of maternal cotinine concentrations with metabolic features, generalized linear model was conducted for the first and second prenatal visit separately, respectively, using the following form :

$$log_2 Y_{ij} = \mu + \beta_{1j} Cotinine_i + \gamma_{1j} Sex_i + \gamma_{2j} Age_i + \gamma_{3j} Gestational\ Age_i + \gamma_{4j} Nulliparous_i + \gamma_{5j} BMI_i + \gamma_{6j} Alcohol_i + \gamma_{7j} Income_i + \varepsilon_{ij} \quad (1)$$

where $log_2 Y_{ij}$ refers to the log2 intensity of metabolic feature j for participant i, μ is the intercept, and $Cotinine_i$ is total maternal cotinine concentration for participant i . We also included covariates in the model to control for potential confounding, including maternal age (Age_i), corresponding gestational week at each of the two clinical visits ($Gestational\ Age_i$), parity ($Nulliparous_i$), first visit prenatal BMI (BMI_i), alcohol use during pregnancy($Alcohol_i$) and measures of socioeconomic status (individual income ($Income_i$)). Finally, we included Sex_i, sex of fetus as an *a priori* biological variable. ε_{ij} represents residual random normal error. Separate models were conducted for each column (HILIC positive ESI and C18 negative ESI) at each clinical visit.

To identify metabolic features associated with adverse birth outcomes, we ran the analysis using a similar approach, with generalized linear model for each visit point, controlling for similar covariates except gestational age at prenatal visits (*Gestational Age*$_i$).

$$log_2 Y_{ij} = \mu + \beta_{1j} Birthoutcome_i + \gamma_{1j} Sex_i + \gamma_{2j} Age_i + \gamma_{3j} Nulliparous_i + \gamma_{4j} BMI_i + \gamma_{5j} Alcohol_i + \gamma_{6j} Income_i + \varepsilon_{ij} \quad (2)$$

Where *Birthoutcome*$_i$ refers to the birth outcome including the gestational age at birth (continuous) and preterm/early term/full term birth (categorical), with full term birth as reference group, and separate models were conducted for two different types of birth outcomes.

In total, we conducted and analyzed 12 sets of models (3 for cotinine exposure plus adverse birth outcomes, with two prenatal clinic visits using two chromatograph columns). Results were presented using Manhattan plots, with the retention time of each metabolic feature on the x-axis against the $-\log10(p)$for β_1 from each equation above on the y-axis. All analyses were completed in R (version 3.6.1).

Metabolic Pathway Enrichment Analysis and Metabolite Annotation

To predict the functional activity of metabolic features from LC-HRMS output, we conducted the pathway enrichment and metabolite annotation analyses. Pathway enrichment was performed using mummichog (v. 1.0.9), a novel bioinformatics platform to predict functional biological activities of metabolites without prior identification.[69] We used two strategies to select eligible metabolic features for pathway analysis: raw p-value at 0.05 and multiple testing corrected p-value at 0.05 using Benjamini-Hochberg method for multiple comparison correction. In the first approach, to minimize the chance of false positive discovery, we excluded pathways with pathway size less than 4 of the number of features matched in pathway enrichment and identified by mummichog with p-value greater than 0.05. We also conducted a sensitivity analysis by using 0.5^{th} and 1^{th} percentile of raw p-values to perform pathway enrichment and examine whether the significant pathways would be largely different under different raw p-values. Furthermore, the metabolic features significantly associated with both

maternal cotinine level and adverse birth outcomes, and also enriched in a relevant pathway were annotated by matching mass m/z value to common adducts using METLIN, ChemSpider, Human Metabolome Database (HMDB), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, using a mass error threshold of 10 ppm. To further minimize false positive match, each matched feature was further screened on their retention time, isotope patterns, and spectrum peak quality by examining the extracted ion chromatographs (EICs). Finally, a select number of annotated metabolites were confirmed with level one evidence [70] by comparing their m/z, retention time and ion dissociation patterns to reference standards.

Moreover, we conducted hierarchical clustering heatmap analysis to characterize the statistically significant untargeted metabolic features and targeted metabolic features identified from metabolite annotation among pregnant women with high cotinine exposure (> 100 μg/g) and low cotinine exposure (<1 μg/g). Statistically significant differences were evaluated using a one-way analysis of variance (ANOVA) to identify if metabolites were differentially expressed among two groups. Visualization of metabolic expression through heatmap was performed using R packages for biomarker discovery, network, and data exploratory analysis in web portal (https://rdrr.io/github/kuppal2/xmsPANDA/man/get_hca.html).[71]

Meet-in-the-middle Analysis

We conducted meet-in-the-middle analysis to explore the role of maternal metabolome in mediating the association between maternal cotinine level and adverse birth outcomes. We separately conducted pathway enrichment analysis and chemical annotation for exposure and birth outcomes, and then we searched if there are any common pathways or metabolites significantly associated with both exposure and the outcome. The analytical flow is summarized and presented in Figure 1.

RESULTS

Among 105 pregnant women who participated in at least one prenatal clinical visit, 43 (41%) were enrolled from the Emory University (private) prenatal clinics and 62 (59%) were enrolled at the Grady Hospital (publicly funded) prenatal clinics (Table 1). Overall, the average age of pregnant women at their first visit (n =105) was 25.9±4.6 years, 16% of study participants were married, 44% had a college degree or above, and 40% have been pregnant before. Only a small proportion of women have reported using alcohol during one month before visits. Among them, 7% reported usage in their initial visit and none reported usage in their second visit. Significant differences in participant demographic were found by recruitment site (p-value<*0.05*)(Table 1). The maternal cotinine concentrations of study participants are presented in Table 2. Both unadjusted and creatinine adjusted cotinine level varied considerably, with adjusted cotinine concentration ranging from 0.113 μg/g to 3260 μg/g during the first visit and from 0.140 μg/g to 2650 μg/g in the second visit, respectively, indicative of substantial difference in the potential exposure to tobacco smoke among study participants.

MWAS Model

After data quality filtering, 16,481 and 13,043 metabolic features in HILIC positive ESI and C18 negative ESI, respectively, were left for final analyses. The number of statistically significant (raw and FDR-corrected) metabolic features associated with cotinine concentrations or birth outcomes are shown in Table 3. There were 701 features significantly associated with maternal cotinine exposure for positive ion mode, and 478 features for negative ion mode at the 1st clinic visit, after adjusting for covariates (p-value<*0.05*). A slightly larger number of significant features (HILIC: 903 and C18: 619, p-value<*0.05*) were found to be associated with cotinine exposure at the 2nd visit. The number of significant metabolic features associated with birth outcomes in each column are similar to each other in two clinic visits, although there were less significant features (N=451, p-value<*0.05*) associated with early term birth in positive ion mode in the second clinic visit compared to preterm birth (N=1522, p-value<*0.05*) and gestational age at birth (N=1113, p-value<*0.05*). For further illustrating the exposure and adverse birth outcomes to metabolites association, we presented MWAS model results in the Manhattan plots (Figure

S1a-S1b). Hierarchical clustering heatmap analysis (Figure 2) showed the characteristic patterns of expression for these significant metabolic features (FDR<*0.05*) among the high cotinine exposure groups and low cotinine exposure groups in each prenatal clinic visits separately, indicating most of significant features were differentially expressed among pregnant women with different level of tobacco smoke exposure.

Pathway Enrichment Analysis

Using the significant metabolic features from two ionization modes (raw *p*-value<*0.05*) in each clinic visit as input in the pathway enrichment analysis, total of 27 and 36 significant pathways were identified in two ionization modes from two visits to be associated with cotinine exposure and birth outcomes, respectively. The metabolic features from two analytical columns significantly associated with cotinine concentrations at the first and second visits mapped to three and 23 unique pathways, respectively (Figure S2). Urea cycle and amino group metabolism appeared as the only common pathway for both clinical visits. It is an important pathway that detoxifies ammonia produced during amino acid catabolism. In particular, some related pathways found in the second clinic visit like aspartate and asparagine metabolism, cytochrome P450, tryptophan metabolism and vitamin metabolism (vitamin C and vitamin B5) are predominantly associated with xenobiotic-mediated oxidative stress and acute inflammatory response in human body. For birth outcome associated metabolic profiling, 9 and 20 unique pathways were associated with at least two birth outcomes at clinic visits 1 and 2, respectively (Figure S3). Amino acid metabolism (arginine and proline metabolism, lysine metabolism and urea cycle and amino group metabolism), nucleic acids damage and repair pathways (pyrimidine metabolism) and lipid metabolism (linoleate metabolism and glycosphingolipid biosynthesis) were found as common for two clinic visits.

We used the *meet-in-the-middle* approach to identify overlapping metabolic pathways associated with both cotinine concentrations and at least one adverse birth outcome in two clinic visits (Figure 3). Urea cycle and amino group metabolism was found as the overlapping pathway. Twelve overlapping pathways were identified for the second clinic visit and included pathways related to amino

acid, lipid, carbohydrate and xenobiotics metabolism. Specifically, butanoate, lysine and urea cycle and amino group metabolism were found to be associated with cotinine exposure and all of birth outcomes.

Metabolite Annotation

Finally, we used the authentic reference standards, verified by tandem mass spectrometry, to confirm the chemical identity of metabolic features that were associated with maternal cotinine exposure or birth outcomes in two clinic visits. In total, we confirmed 47 metabolites with level 1 evidence, with 28 and 20 significantly associated with cotinine exposure and maternal gestation, respectively (Table 4 and Table S1). Generally, we validated more metabolites associated with cotinine exposure in late pregnancy and most of them are involved in the common amino acid metabolism and lipid metabolism. Specifically, inflammation and oxidation related amino acids were identified, including tyramine, serine, glutamate, choline and taurine, and they were all positively associated with cotinine exposure. Other oxidative stress related metabolites associated with gestation outcomes were also identified such as valine, serine and pantothenic acid. All of these endogenous metabolites showed important roles in numerous physiological pathways and metabolic functions with potential perturbation by smoke exposure or related to gestational health responses. Additionally, hierarchical clustering analysis was also performed on the 47 identified statistically significant metabolites, resulting in the illustration of clear differences among two exposure groups (Figure 4).

However, there was no common metabolic feature associated with both exposure and outcome after applying meet-in-the-middle approach to the data from the same clinic visit. Only one feature, serine, was found to have a positive association with cotinine exposure in the 2nd clinic visit and negative association with preterm birth compared to full term birth in the 1st clinic visit, which was reported to be a part of metabolic network that support oxidant/antioxidant balance and cell proliferation.[72] Although no overlapping metabolites have been confirmed, most of identified metabolites were closely linked and connected within some metabolic pathways such as tyrosine metabolism, glycine, serine, alanine and threonine metabolism and fatty acid metabolism, which can modulate physiological processes such as inflammation, vascular reactivity and lipid peroxidation with the potentially toxicant impact of cotinine exposure, especially among pregnant women.

Sensitivity Analysis

To reduce the possibility for false discoveries and test the consistency of the metabolomics analyses, we performed several sets of sensitivity analyses. First, we used different percentile of p-values from each MWAS result as a cut-off point to perform pathway enrichment analysis. The related pathways such as urea cycle and amino group metabolism, aspartate and asparagine metabolism and glycosphingolipid metabolism shown in the main analysis remained to be the overlapping pathways linking cotinine exposure and birth outcomes when we changed p-values to *0.01* and *0.005* (Figure S4a-S4b). Furthermore, we used two different methods to adjust for creatinine concentration and compare the results under exposure pathway enrichment. All significant metabolic pathways found in the first clinic visit and most of pathways found in the second visit in original analysis were also identified in the sensitivity analysis, indicating our normalization approaches were appropriate to use.

DISCUSSION

High resolution metabolomic, an analytical tool of identifying and quantifying thousands of metabolites in biological samples associated with endogenous and exogenous processes, has emerged as a powerful platform to improve internal exposure estimation to environmental exposures. In the Atlanta African American Mother-Child cohort, we applied both targeted exposure assessment and untargeted high-resolution metabolomics to explore the association between maternal cotinine exposure and adverse birth outcomes in this high-risk population. We found that maternal urinary cotinine concentrations were associated with a series of significant metabolic perturbations in maternal metabolome. Most of these metabolic perturbations, including amino acid metabolism (e.g., tryptophan metabolism, alanine, aspartate and glutamate metabolism, arginine and proline metabolism), lipid metabolism (e.g., fatty acid metabolism, glycerophospholipid metabolism) and carbohydrate metabolism, were also associated with adverse birth outcomes, especially during late pregnancy. Taken together, these findings may contribute to uncovering the potential biological mechanisms of environmental tobacco smoke exposure on potential birth outcomes.

The most pronounced finding from our current analysis was the identification of metabolic pathways and metabolites associated with cotinine exposure and adverse birth outcomes. Among the exposure associated metabolic profiling, fatty acid metabolism was considered an important pathway perturbed by cotinine exposure during pregnancy, especially in early pregnancy, and fatty acid metabolism was also shown to be significantly associated with gestational age at birth in late pregnancy in birth outcome associated metabolic profiling. These results were consistent with the previous findings, where cigarette smoking was linked to alterations in fatty acid and glycerophospholipid metabolism.[61, 63, 73, 74] On the other hand, it has been reported that maternal fatty acid metabolism is closely associated with fetal intrauterine growth that fatty acid deficiency could result in malnutrition of fetus, shorter gestational age and preterm birth,[75, 76] indicating the potential effect of maternal smoking on endogenous metabolites and birth outcomes through lipid metabolism. Cigarette smoking is known to be a significant source of oxidative stress,[77, 78] and tobacco smoke exposure during pregnancy was reported to be linked to increased production of reactive oxygen species, resulting in

oxidative damage to certain selected lipids.[79-81] In turn, the abnormality in lipid metabolism during pregnancy can also increase oxidative stress [82] to further disturb the balance of lipid peroxidation and antioxidation process. This could lead to the decomposition of some types of fatty acids that damages the function and structure of capillary endothelial cells,[83] as capillary endothelial cells are related to certain placental vascular functions, and dyslipidemia-associated oxidative stress was shown to be associated with advancing gestational age.[84]

Our results for metabolic feature annotation and validation in lipid metabolism further showed coherence to the pathway enrichment. For the confirmed metabolites significantly associated with cotinine exposure in early pregnancy, most of them were involved in lipid metabolism. Lysophosphatidylcholines (LysoPCs), groups of bioactive pro-inflammatory lipids, were found to be positively associated with maternal cotinine exposure, and have been reported to be linked with oxidative stress and inflammation.[85] In particular, early gestational maternal lipid concentrations of LysoPC and LysoPE were positively associated with birthweight,[86-88] and birthweight and gestational length are positively correlated.[89] 17/21-hydroxyprogesterone is another identified lipid metabolism-related metabolite associated with cotinine exposure, It was previously found to be one of the metabolic clocks of blood metabolites that could accurately predict gestational age approaching the labor event.[90] We also observed a positive association between acyl-carnitines, which is involved in fatty acid oxidation, and gestational age as well as preterm birth. The reduced level of acyl-carnitines might lead to a reduced energy production and fat oxidation, which could be the linkage in the mechanism leading to adverse birth outcomes in smoking mothers.[91]

A wide range of amino acid groups was found to be associated with maternal cotinine in late pregnancy and gestational length in both early and late pregnancy in the pathway enrichment analysis and chemical annotation. These results are consistent with previous findings that maternal serum cotinine concentrations induce perturbations in amniotic fluids, including aspartate and asparagine metabolism, arginine and proline metabolism, and methionine metabolism, especially in the late pregnancy. These pathways are also recognized as critical pathways in determining the potential intrauterine growth retardation.[55, 62, 92] We found multiple positive associations between maternal cotinine exposure and a variety of amino acid metabolites in those pathways that are involved in

physiological functions including inflammation, oxidative stress, angiogenesis and apoptosis. Glutamate was shown to have a positive correlation with gestational age [93, 94] and to be associated with intra uterine growth restriction (IUGR).[95, 96] It could reduce susceptibility to oxidative stress control lipid peroxidation in pregnancy and prevent free radical-linked deleterious effects among pregnant women.[94, 97-99] The positive association between cotinine exposure and glutamate is also supported by the fact that nicotine receptor activation could lead to the release of glutamate,[100] as to compensate for the oxidative effect caused by smoke exposure.

Other validated amino acids perturbed by maternal tobacco smoke exposure were mainly involved in glycine, serine and threonine metabolism, tyrosine metabolism, alanine, aspartate and glutamate metabolism, arginine and proline metabolism and methionine and cysteine metabolism, which were also found be to significant pathways associated with gestational length in our pathway analysis results. Serine, which was found to link the one-carbon cycle to glycolysis to support oxidant/antioxidant balance and cell proliferation,[72] was observed to be positively associated with cotinine exposure in late pregnancy and negatively associated with preterm birth in early pregnancy in our study. A significant decrease in plasma serine turnover was previously observed in late gestation,[72] indicating that serine concentration could potentially reflect the gestational length. We also identified homoserine in both ESI modes in relation to cotinine exposure, which is also an indicator of proinflammatory state.[101] Another confirmed metabolite related to cotinine in late pregnancy is choline. Choline was reported to be closely associated to placental vascular function through modulating inflammation, angiogenesis and apoptosis and further downregulation of the placental pro-inflammatory cytokines at several gestational time points.[102] Specifically, in late pregnancy, the increased concentration of choline can decrease the placental production and circulating concentration of placental oxidative stress factors.[103] Taurine is also a placental vascular related metabolite we identified to be associated with maternal cotinine concentration. Numerous physiological functions have been described for taurine, including defense against oxygen free radical,[104, 105] inhibiting apoptosis, inflammation, cell death while increasing NO generation in endothelial cells,[105] and ameliorating impairment of vascular reactivity. Importantly, previous studies showed that taurine was primarily associated with nicotine-induced vascular adverse events,[106] and the one of current

proposed mechanisms for how maternal smoke exposure may affect preterm birth is through nicotine-induced vasoconstriction.[107]

We also identified several inflammatory metabolites in amino acid metabolism related to our birth outcome of interest. 2-methylhippurate in phenylalanine metabolism was found to be positively associated with early term in early pregnancy, and it is closely related to tyrosine which was reported to be associated with increased risk of fetal growth restriction,[108] augmented placental oxidative stress and subsequent small for gestational age newborn.[109] This adverse effect could be due to the potential impact of maternal cotinine exposure as we observed the positive association between tyramine, derived from tyrosine, and maternal cotinine in our exposure profiling in early pregnancy. The reduction of another identified metabolite, pantothenic acid, could be related to significant increase in oxidative stress and lipid peroxidation,[110] which may help us explain the observed negative association between pantothenic acid and gestational age at birth. Another confirmed amino acid in birth outcome profiling was valine, functioned in the regulation of cell proliferation and signaling pathway activation.[94, 111] It was also shown to be important players in gluconeogenesis and glucose tolerance related birth outcomes,[112] which might help to build the linkage between the amino acid metabolism and carbohydrate metabolism in the gestation related metabolic network.

Although there were no overlapping metabolites identified in the same stage of pregnancy, our pathway analysis results consistently indicated the urea cycle and amino group metabolism as the common response pathway in both early and late pregnant profiling. Based on those collective results, we proposed that those identified molecules may be related to systemic changes under an interrelated metabolic network centered at urea cycle and TCA cycle that linked the amino acid, lipid, carbohydrate and nucleotide metabolism together to response to external cotinine exposure during the pregnancy (Figure 5). Maternal tobacco smoke exposure during pregnancy is related to oxidative stress, lipid hydroperoxides, DNA damage, inflammation, and it can also affect the endothelial l-arginine NO synthase pathway, resulting in reducing NO production and elevated oxidative stress.[113-120] The high levels of pro-inflammatory molecules could cause endothelial cell dysfunction and adversely affect placental vascularization,[121] resulting in higher risk of various adverse birth outcomes.[84, 122] Importantly, most of identified metabolites including glutamate, homoserine, taurine and choline in

amino acid metabolism are positively associated with cotinine level, and those slight increases in these antioxidant biomarkers may represent a compensatory mechanism to reduce the adverse oxidative effect caused by cotinine exposure. Additionally, we also identified inosine and hypoxanthine in nucleotide metabolism to be related to gestational age at delivery under this exposure-response network, both of which are considered to intermediate energy production and chronic inflammation process that are important in fetal development in pregnancy.[123]

On the other hand, tobacco smoke exposure during pregnancy was reported to be associated with decreased glucose tolerance and an increased risk of gestational diabetes mellitus which could result in lower gestational age at delivery.[124, 125] In our results, D-mannose level was proved to have a positive association with gestational length in early pregnancy, which was also shown to be related to spontaneous preterm labor in a previous study [126]. While our results demonstrated a negative association for glucose 6-phosphate, indicating that different carbohydrate metabolites functioned collectively to regulate the glucose metabolic pattern on birth outcomes, with the possible mechanisms on increased contribution to oxidative metabolism [127] and decreased insulin sensitivity during pregnancy.[128] Notably, it was reported that young pregnant AA women have higher insulin and lower glucose concentrations, highlighting that this vulnerable group might be more likely to experience the adverse birth outcome caused by cotinine exposure.[129]

Additionally, we also identified several exogenous toxicants and organic chemicals such as nicotine, methyl ecgonine and diisopropyl phthalate associated with maternal cotinine exposure. Nicotine and methyl ecgonine, biomarkers of tobacco smoke and crack cocaine, both were found to be positively associated with cotinine exposure in our MWAS results. Diisobutyl phthalate is widely used in household products and reported to have negative toxicant effect on reproductive health, liver, kidney and to associated with cancer.[130] Thus, we may infer that pregnant AA women exposed to tobacco smoke are also likely to be exposed to other environmental pollutants due to their potential unfavorable socioeconomic status and have a higher chance to use other drugs.

Collectively, our study revealed the underlying molecular mechanisms mediating the association between prenatal tobacco smoke exposure and potential adverse birth outcomes. We used a

high-throughput analysis method involving the identification and quantification of thousands of metabolic features to characterize the novel pathways and biomarkers in relation to the metabolic perturbation in both early and late pregnancy. We also combined the repeated targeted exposure assessment and untargeted metabolomics together, which provide the comprehensive mechanistic information on biological responses related to tobacco smoke exposure and birth outcomes. While the identified pathways are all biologically plausible, our study had a number of important limitations. First, the two cross-sectional study designs could not establish the causal relationship between metabolic features and maternal cotinine exposure as well as later gestational outcomes. Nevertheless, mapping out the endogenous metabolic networks could help us further understand the potential biological mechanisms perturbed by cotinine that affect the birth outcomes. Meanwhile, it is possible that other exogenous exposure could also result in the similar metabolic perturbation as cotinine does, and thus further research could be required to test these potential effects. Another limitation is that while we can accurately quantify the maternal cotinine concentration in urine sample, we may only infer the true source of nicotine exposure. Our data showed that large proportion of women in our cohort who reported no use of the tobacco was found to have high cotinine concentration (Table S2),[131] indicating the potential alternative routes to tobacco exposure. The one-time exposure measurement could not reflect the long-term exposure status, and thus limits our interpretation of underlying mechanisms for effect of maternal smoke on birth outcomes. Finally, our study population of pregnant African Americans women may have a different nicotine metabolism pattern compared to Caucasians,[132] and are more likely to experience adverse birth outcomes, so our findings might not be applicable to other racial groups.

In summary, we identified and validated several metabolic pathways and metabolites perturbed by cotinine exposure and associated with birth outcomes in both early and late pregnancy, and presented a potential disturbed metabolic network centered on urea cycle and TCA cycle that connects amino acid, lipid, carbohydrate and nucleotide metabolism together to response to cotinine exposure. Specifically, lipid metabolism and a series of amino acid metabolites such as glutamate, serine, choline, and taurine play a crucial role in molecular mechanisms underlying cotinine toxicity and its maternal health effect in the early and late pregnancy, respectively. And the birth outcome associated metabolic profiling

further established the connection between those pathways and metabolites and potential adverse birth outcomes. Collectively, the results could build the foundation for further development of targeted interventions to reduce the adverse birth health effect caused by maternal cotinine exposure in this minor population.

FUTURE WORK

This thesis demonstrates the association between tobacco smoke exposure during pregnancy, metabolic perturbations and potential adverse birth outcomes among African American women, suggesting that metabolomics could be employed as a powerful tool to identify potential biomarkers and biological pathways to uncover the molecular mechanisms that mediate the association between tobacco smoke exposure and adverse birth outcomes. We identified several pathways related to endocrine disruption and oxidative-stress associated with higher exposure to tobacco smoking. These promising initial findings will help us to further understand the biological mechanisms underlying the tobacco smoking – adverse birth outcomes association and will aid in detecting preclinical dysfunction, which will shift focus from overt disease endpoints to upstream stages amenable both to prevention and to targeted interventions. Our findings could also be a critical step in the development of targeted interventions aimed at reducing the birth health burden and health disparities associated with tobacco smoke exposure, particularly among pregnant women and newborns in this minority population. Importantly, our study offers little to substantiate the possibility of the effect of complex environmental mixtures that correlates with tobacco smoke exposure. The future work should take the effect of other internal environmental pollutant exposures into consideration, and explore the potential metabolic network under the multiple and correlated pollutants mixture. Moreover, future research work could also consider the fetus-sex specific analysis by testing an interaction between sex of fetus and exposure level as sex of fetus is an *a priori* biological variable and could have a potential effect on maternal metabolome. Future work on HRM and prenatal tobacco smoke exposure in this African American cohort may utilize the information on newborn blood sample we have collected to further explore how the tobacco smoke exposure during

pregnancy affect the maternal and newborn metabolome and how those perturbed pathways in maternal and newborn metabolome linked together.

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TABLES AND FIGURES

Table 1. Prenatal characteristics of study participants overall (n=105) and by recruitment site, the Atlanta African American Maternal-Child cohort (2014-2016)

Characteristic	Emory Midtown Hospital	Grady Hospital	Overall	*p*-value*
N (%)	43 (41)	62 (59)	105 (100)	
Age, years, mean (SD)	27.6 (4.8)	24.6 (4.1)	25.8 (4.6)	*0.001**
Sex of the baby, n (%)[+]				
Female	22 (51.2)	27 (45.8)	49 (48.0)	*0.417*
Male	19 (44.2)	27 (45.8)	46 (45.1)	
Gestational age at 1st visit, wks, mean (SD)	12.1 (2.1)	11.5 (2.2)	11.7 (2.2)	*0.199*
Gestational age at 2nd visit, wks, mean (SD)	26.0 (1.7)	25.6 (1.5)	25.8 (1.6)	*0.226*
Maternal BMI, kg/m^2, mean (SD)[^]	29.3 (6.9)	29.6 (8.3)	29.5 (7.7)	*0.859*
Birth outcomes, wks, n (%)[+,#]				
Full Term (⩾39)	24 (55.8)	26 (41.9)	50 (47.6)	*0.060*
Early Term (⩾37 and <39)	16 (37.2)	16 (25.8)	32 (30.5)	
Preterm (>20 and <37)	1 (2.3)	12 (19.4)	13 (12.4)	
Marital status, n (%)				
Single	30 (69.8)	58 (93.5)	88 (83.8)	*0.003**
Married	13 (30.2)	4 (6.5)	17 (16.2)	
Educational attainment, n (%)				
Less than high school	1 (2.3)	16 (25.8)	17 (16.2)	*<0.001**
High school graduate	10 (23.3)	34 (54.8)	44 (41.9)	
College or higher	32 (74.4)	12 (19.4)	44 (41.9)	
Income, n (%)				
<$26,000	14 (32.6)	53 (85.5)	67 (63.8)	*<0.001**
$26,000-$60,000	11 (25.6)	7 (11.3)	18 (17.1)	
>$60,000	18 (41.9)	2 (3.2)	20 (19.0)	
Nulliparous, n (%)[+]				
Yes	17 (39.5)	25 (40.3)	42 (40.0)	*0.221*
No	26 (60.5)	33 (53.2)	59 (56.2)	
Insurance type, n (%)				
Medicaid	20 (46.5)	61 (98.4)	81 (77.1)	*<0.001**
Private	23 (53.5)	1 (1.6)	24 (22.9)	
Alcohol use at 1st visit, n (%)[+]				
Not used last month	28 (77.8)	53 (86.9)	81 (83.5)	*0.149*
Used last month	2 (5.6)	5 (8.2)	7 (7.2)	
Alcohol use at 2nd visit, n (%)[+]				
Not used last month	32 (94.1)	45 (95.7)	77 (95.1)	*1.000*
Tobacco use at 1st visit, n (%)[+]				
Not used last month	28 (77.8)	48 (78.8)	76 (78.4)	*0.006**
Used last month	2 (5.6)	12 (19.7)	14 (14.4)	
Tobacco use at 2nd visit, n (%)[+]				
Not used last month	32 (94.1)	42 (89.4)	74 (91.4)	*0.313*
Used last month	0 (0.0)	3 (6.4)	3 (3.7)	

[*] t-test, $\chi 2$ test and fisher test were used to calculate the p-value for continuous variables and categorical variables, and the statistically significant results were labelled (*p*-value<*0.05*).

[+] The total percentage doesn't sum to 100 as missing information on these variables.

[^] Maternal BMI was measured at the pregnant women's first prenatal clinic visit.

[#] Elective abortion and spontaneous abortion were not included here.

Abbreviations: BMI, body mass index; wks, weeks.

Table 2. Unadjusted and creatinine-adjusted maternal cotinine concentrations by clinic visits, the Atlanta African American Maternal-Child cohort (2014-2016).

	First visit (N=97)	Second visit (N=81)	*P*-value[+]
Unadjusted cotinine, ng/ml			
Geometric mean (SD)	11.78 (12.57)	6.50 (15.28)	*0.089*
Median [Min, Max]	11.0 [0.354, 6050]	4.80 [0.354, 5310]	
Adjusted cotinine, μg/g			
Geometric mean (SD)	7.15 (11.95)	4.79 (12.62)	*0.275*
Median [Min, Max]	5.93 [0.113, 3260]	3.69 [0.140, 2650]	

[+] Mann-Whitney U test was conducted to test the difference of cotinine levels between two visiting stages.

Table 3. Number of statistically significant metabolic features associated with cotinine concentrations and adverse birth outcomes under different cutoff p-values,* the Atlanta African American Maternal-Child cohort (2014-2016)

Visit		HILIC+						C18-					
	Cutoff	FDR	FDR	RAW	RAW	RAW	RAW	FDR	FDR	RAW	RAW	RAW	RAW
	P-value	*0.05*	*0.2*	*p-0.0005*	*p-0.005*	*p-0.01*	*p-0.05*	*0.05*	*0.2*	*p-0.0005*	*p-0.005*	*p-0.01*	*p-0.05*
First	TCOT	22	64	54	140	225	701	21	44	36	95	144	478
	BGA	1	2	10	76	136	651	0	0	9	44	88	446
	ET#	0	0	8	96	190	993	0	0	6	47	100	612
	PT#	0	0	5	63	140	694	0	0	3	42	96	558
Second	TCOT	52	136	75	238	336	903	14	39	31	129	199	619
	BGA	0	0	6	94	221	1113	0	0	0	32	82	570
	ET#	0	0	4	35	63	451	0	0	4	48	98	579
	PT#	5	59	43	275	529	1522	1	1	4	37	68	468

*Both Benjamini-Hochberg false discovery rate (FDR) procedure and raw p-value were used to identify a reasonable number of significant metabolic features.
Full-term birth is used as reference group to compare with early term and preterm birth.
Abbreviations: TCOT: total cotinine concentration in urine samples; BGA: gestational age at birth; ET: early term birth; PT: preterm birth.

Table 4. Chemical identity^ of the metabolites significantly associated with maternal cotinine exposure (p-value<0.05).

m/z	RT(s)	Identified Metabolite	Adduct Form	Associated with TCOT exposure	Pathways	Metabolism	Visit Stage	ESI
138.0914	22.5	TYRAMINE	M+H	β=0.063	Tyrosine metabolism & Methane metabolism	Amino acid metabolism & Energy metabolism	Visit 1	ESI+
496.3397	29.9	LYSOPC(16:0)	M+H	β=0.025	Fatty Acid Metabolism	Lipid metabolism	Visit 1	ESI+
482.3243	31	LYSOPE(18:0)	M+H	β=0.030	Fatty Acid Metabolism	Lipid metabolism	Visit 1	ESI+
524.3708	33.1	LYSOPC(18:0)	M+H	β=0.037	Fatty Acid Metabolism	Lipid metabolism	Visit 1	ESI+
480.3436	30.7	LYSOPC(O/P-16:1)	M+H	β=0.030	Fatty Acid Metabolism	Lipid metabolism	Visit 1	ESI+
142.0262	109.9	ETHANOLAMINE PHOSPHATE	M+H	β=0.148	Glycerophospholipid metabolism; Glycosphingolipid metabolism	Lipid metabolism	Visit 1	ESI+
331.2272	26.9	17/21-HYDROXYPROGESTERONE	M+H	β=-0.084	C21-steroid hormone biosynthesis and metabolism	Lipid metabolism	Visit 1	ESI+
239.1487	29.6	PIRIMICARB	M+H	β=-0.124	An environmental contaminant, a xenobiotic and an insecticide	Xenobiotics biodegradation and metabolism	Visit 1	ESI+
163.1231	39.3	NICOTINE	M+H	β1=0.122; β2=0.081	Tropane, piperidine and pyridine alkaloid biosynthesis & Metabolism of xenobiotics by cytochrome P450	Biosynthesis of other secondary metabolites & Xenobiotics biodegradation and metabolism	Visit 1/Visit 2	ESI+
200.1282	37.1	METHYL ECGONINE	M+H	β1=0.097; β2=0.088	Tropane, piperidine and pyridine alkaloid biosynthesis	Biosynthesis of other secondary metabolites	Visit 1/Visit 2	ESI+
168.1019	26.1	3-METHOXYTYRAMINE	M+H	β=0.065	Tyrosine metabolism	Amino acid metabolism	Visit 2	ESI+
104.0353	24.8	L-SERINE	M-H	β=0.034	Glycine, serine and threonine metabolism & Cysteine and methionine metabolism	Amino acid metabolism	Visit 2	ESI-
118.0509	26.4	HOMOSERINE*	M-H	β=0.033	Glycine, serine and threonine metabolism & Cysteine and methionine metabolism& Lysine biosynthesis	Amino acid metabolism	Visit 2	ESI-
120.0656	67.7	HOMOSERINE*	M+H	β=0.031	Glycine, serine, alanine and threonine metabolism & Lysine biosynthesis & Methionine and cysteine metabolism	Amino acid metabolism	Visit 2	ESI+
148.0604	69.4	L-GLUTAMIC ACID	M+H	β=0.073	Tryptophan metabolism & Alanine, aspartate and glutamate metabolism & Arginine and proline metabolism & Histidine metabolism & D-Glutamine and D-glutamate metabolism & Glutathione metabolism & Butanoate metabolism	Amino acid metabolism & Carbohydrate metabolism	Visit 2	ESI+
146.046	20.9	GLUTAMATE	M-H	β=0.059	Alanine, aspartate and glutamate metabolism & Arginine and proline metabolism & Histidine metabolism & D-Glutamine and D-glutamate metabolism & Glutathione metabolism & Butanoate metabolism & Nitrogen metabolism	Amino acid metabolism & Carbohydrate metabolism & Energy metabolism	Visit 2	ESI-
134.0448	74.7	ASPARTATE	M+H	β=0.046	Arginine biosynthesis & Alanine, aspartate and glutamate metabolism& Glycine, serine and threonine metabolism & Cysteine and methionine metabolism & Histidine metabolism& beta-Alanine metabolism	Amino acid metabolism	Visit 2	ESI+
102.055	66.1	1-AMINOCYCLOPROPANE-1-CARBOXYLATE	M+H	β=0.031	Cysteine and methionine metabolism	Amino acid metabolism	Visit 2	ESI+
106.0497	76.7	SERINE	M+H	β=0.032	Glycine, serine, alanine and threonine metabolism & Tyrosine metabolism & Methionine and cysteine metabolism	Amino acid metabolism	Visit 2	ESI+
104.1071	46.5	CHOLINE	M+	β=0.033	Glycine, serine, alanine and threonine metabolism & Glycerophospholipid metabolism	Amino acid metabolism & Lipid metabolism	Visit 2	ESI+
126.022	67.8	TAURINE	M+H	β=0.038	Methionine and cysteine metabolism & Primary bile acid biosynthesis	Amino acid metabolism & Lipid metabolism	Visit 2	ESI+
581.2418	25.4	BILIVERDIN	M-H	β=-0.140	Porphyrin and chlorophyll metabolism	Metabolism of cofactors and vitamins	Visit 2	ESI-
258.1094	76.2	SN-GLYCERO-3-PHOSPHOCHOLINE	M+H	β=0.106	Glycerophospholipid metabolism	Lipid metabolism	Visit 2	ESI+
171.0061	20.5	GLYCEROL 2-PHOSPHATE	M-H	β=0.055	Glycerophospholipid metabolism	Lipid metabolism	Visit 2	ESI-
308.0996	19.5	N-ACETYLNEURAMINATE	M-H	β=0.087	Amino sugar and nucleotide sugar metabolism	Carbohydrate metabolism	Visit 2	ESI-
375.2892	26	CHENODEOXYCHOLATE	M-H2O+H	β=0.111	Primary bile acid biosynthesis	Lipid metabolism	Visit 2	ESI+

377.0867	20.8	MALTOSE	M+Cl	β=0.079	Starch and Sucrose Metabolism	Carbohydrate metabolism	Visit 2	ESI-
251.1272	29.3	DIISOPROPYLPHTHALATE	M+H	β=0.043	Role as a plasticizer	Xenobiotics biodegradation and metabolism	Visit 2	ESI+

^ Chemical identity of metabolic features was confirmed by matching peaks via accurate mass to charge ratio and retention time to authentic reference standards under the same conditions using tandem mass spectrometry.

* Metabolites were identified in both HILIC+ with positive electrospray ionization mode and C18- with negative electrospray ionization mode.

Abbreviations: m/z: mass to charge ratio; RT: retention time; ESI+: positive electrospray ionization mode; ESI-: negative electrospray ionization mode; TCOT: total cotinine concentration in urine sample.

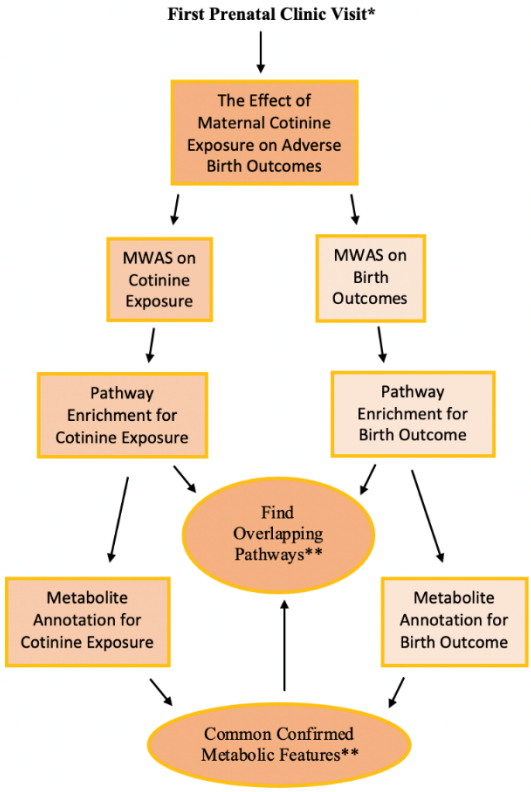

Figure 1. The flow chart of meet in the middle approach in our study
*The second prenatal clinic visit was also conducted in the same process, and at last, we compare the metabolic perturbation in both visits.
**Meet in the middle approach: If no identified features on common, then switched to pathways identification.

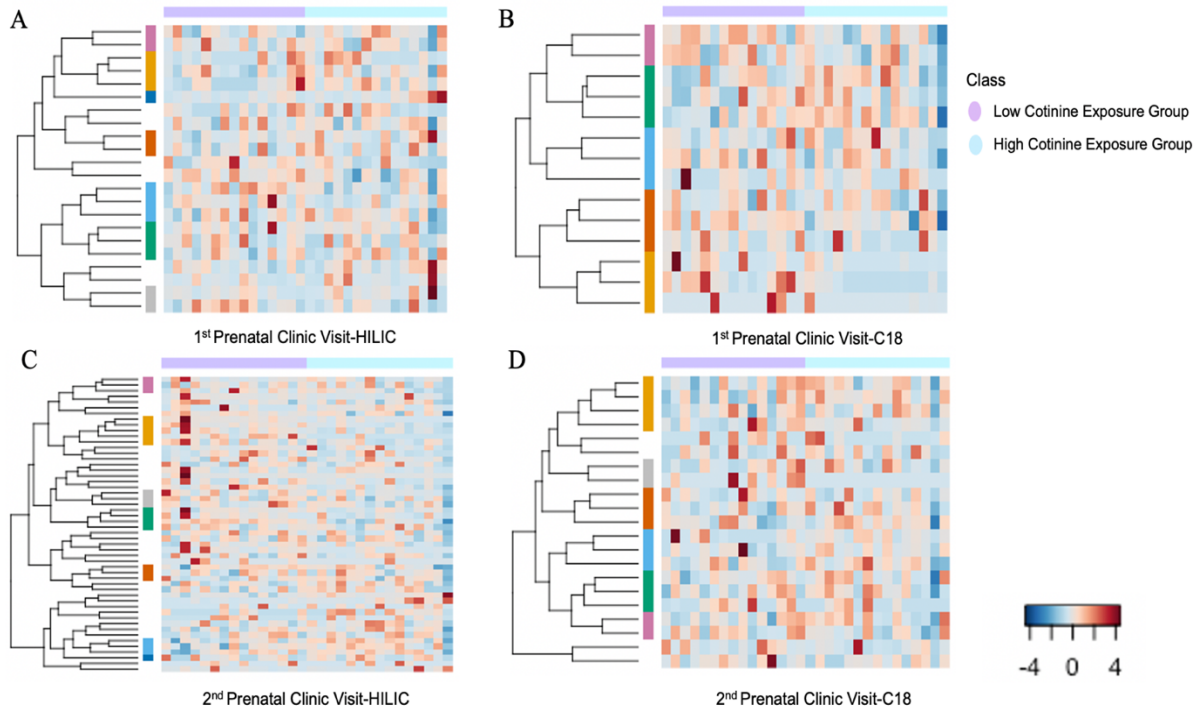

Figure 2. Two-way hierarchical clustering heatmap of significant serum metabolic features associated with cotinine concentrations (FDR<0.05) in two prenatal clinic visits in two analytical columns (A-D). Two strata of columns show the high cotinine exposure level groups (n=15) and the low cotinine exposure level groups (n=15), respectively. The row of heatmap shows the significant features from exposure associated MWAS model (FDR<0.05). The amount of each metabolite in pregnant women is expressed as relative value obtained by the auto-scaling method and represented by the color scheme in which red and blue indicates high and low concentrations of metabolites, respectively.

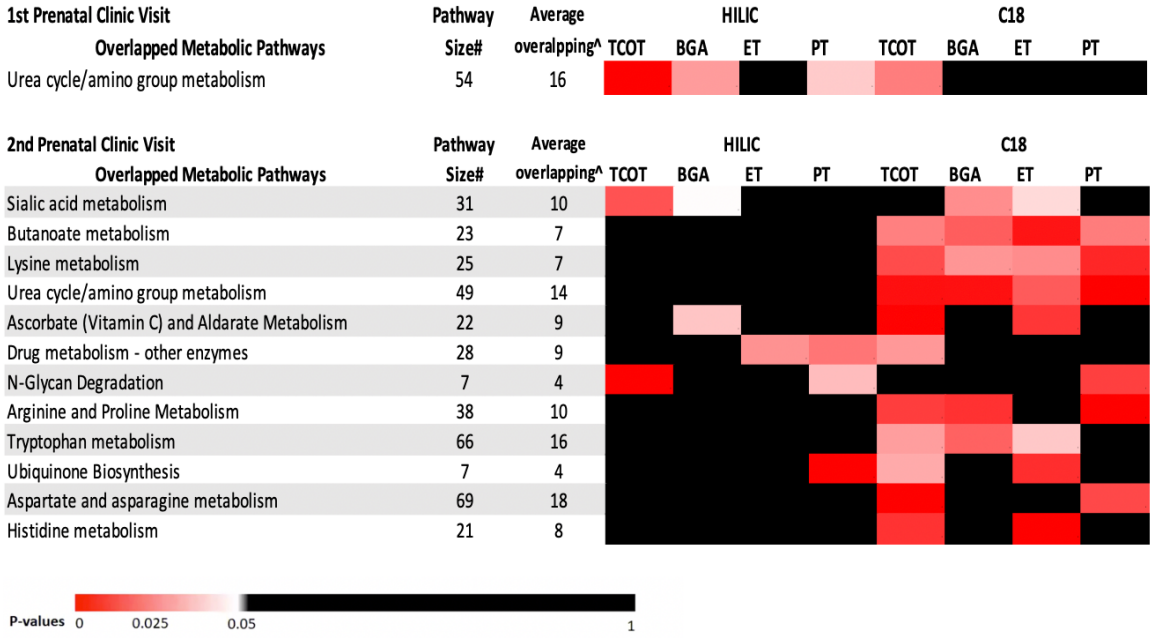

Figure 3. The heatmap of overlapping metabolic pathways in two prenatal clinic visits. Those pathways are associated with both maternal cotinine exposure and at least one adverse birth outcome. Each cell represents the association between each metabolic pathway and each exposure/birth outcome and the colors were shaded according to the p-value of this association. The pathways were ordered according to the total number of the significant associations (p-value<0.05) in HILIC mode and C18 mode.

* For HILIC positive ion mode, only the following adducts were considered: M[1+], M+H[1+], M-H2O+H[1+], M+Na[1+], M+K[1+], M+2H[2+], and M(C13)+2H[2+].

For C18 negative ion mode, only the following adducts were considered: M-H[-], M+Cl[-], M+ACN-H[-], M+HCOO[-], M(C13)-H[-], M-H2O-H[-], and M+Na-2H[-].

Total number of features in the specific metabolic pathway.

^ Total number of features in the samples with m/z matched in the specific metabolic pathway.

Abbreviations: TCOT: total cotinine concentration in urine samples; BGA: gestational age at birth; ET: early term birth; PT: preterm birth.

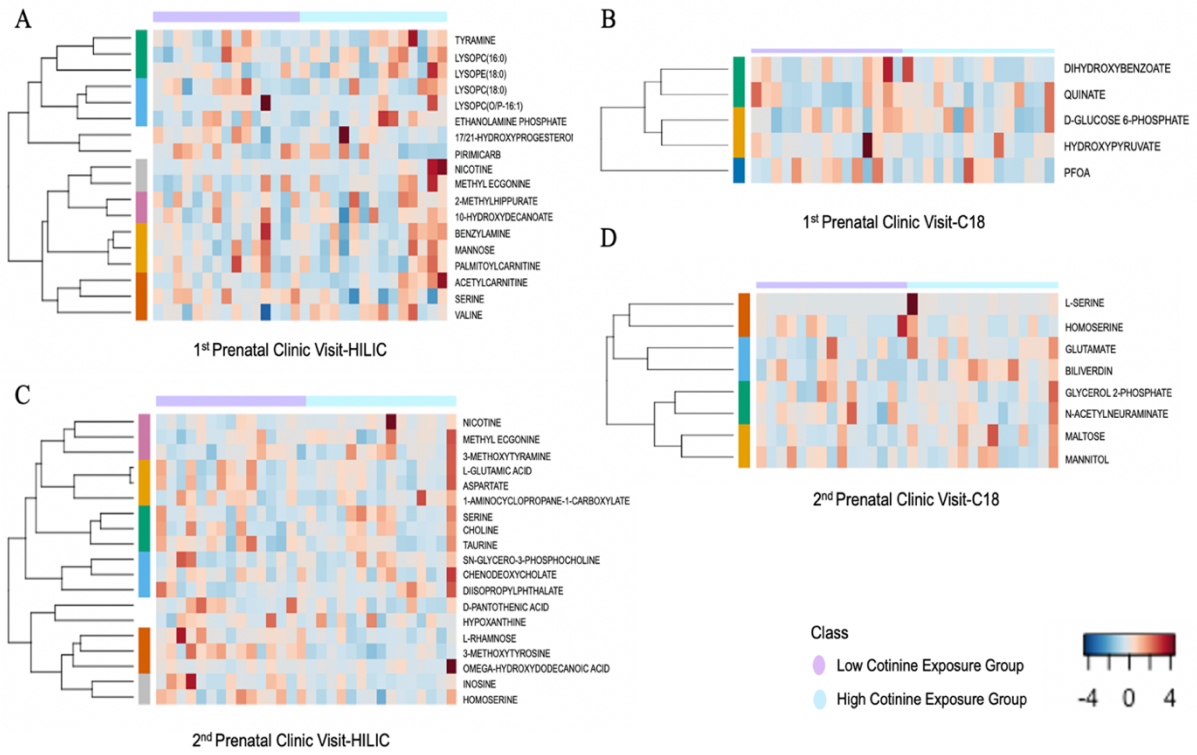

Figure 4. Two-way hierarchical clustering heatmap of identified metabolic features in two prenatal clinic visits (A-D). Two strata of columns show the high cotinine exposure level groups (n=15) and the low cotinine exposure level groups (n=15), respectively. The row of heatmap shows the identified chemicals from chemical annotation. The amount of each metabolite in pregnant women is expressed as relative value obtained by the auto-scaling method and represented by the color scheme in which red and blue indicates high and low concentrations of metabolites, respectively.

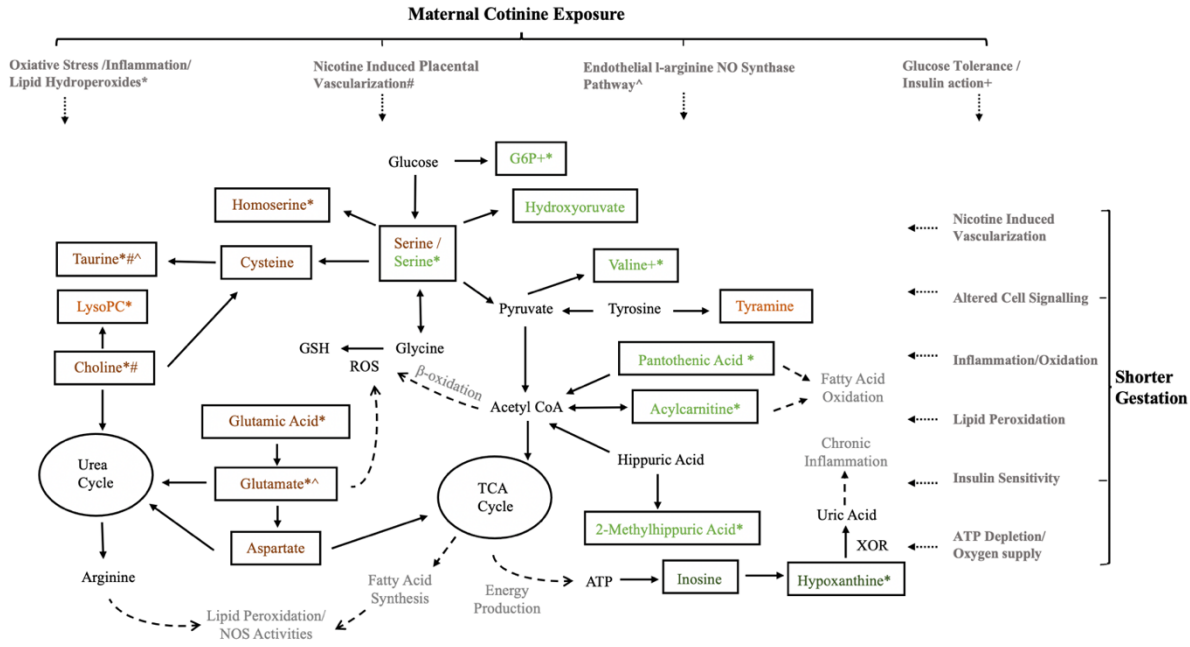

Figure 5. The potential molecular mechanisms under metabolic network for the effect of maternal cotinine exposure on the gestational birth outcomes among pregnant AA women cohort. Molecules in light orange and dark orange denote the confirmed metabolites significantly associated with cotinine exposure in the first and second clinic visit, respectively. Molecules in light green and dark green denote the confirmed metabolites significantly associated with birth outcomes in the first and second clinic visit, respectively.

*#^+: The maternal cotinine exposure could be perturbed the metabolic response and further affect the gestation mainly through four mechanisms. The confirmed metabolites and related metabolism could be involved in one or several mechanisms indicated by those symbols.

Abbreviations : ROS: reactive oxygen species; XOR: xanthine oxidoreductase; NOS: nitric oxide synthases; G6P: glucose 6-phosphate; ATP: adenosine triphosphate; LysoPC: Lysophosphatidylcholines; GSH: Glutathione.

APPENDIX

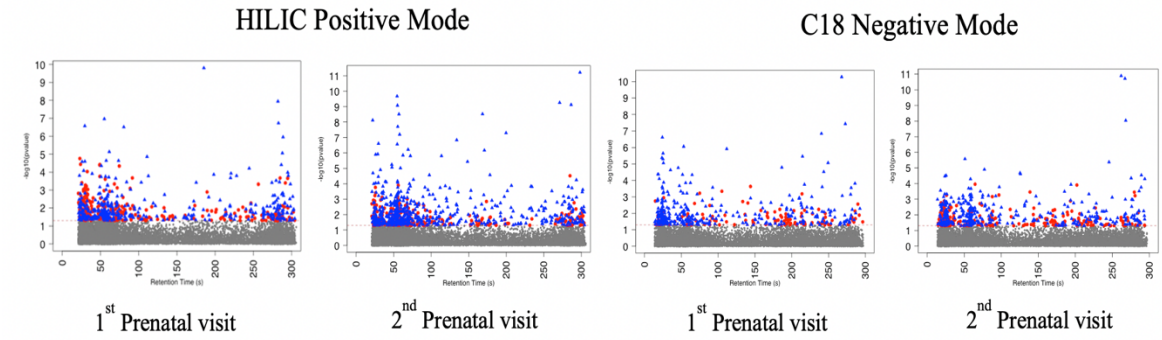

Figure S1a. Manhattan plots of associations between changes in metabolic feature intensities with cotinine exposure level in two prenatal clinic visits. X-axis denotes the retention time of the metabolic features and Y-axis denotes the negative natural log of p-value in exposure to metabolites association. The higher the dots appeared, the more significant the features were associated exposure Red dots indicated positive association and blue indicated negative association. The dashed line indicated the threshold cut-off p-value 0.05

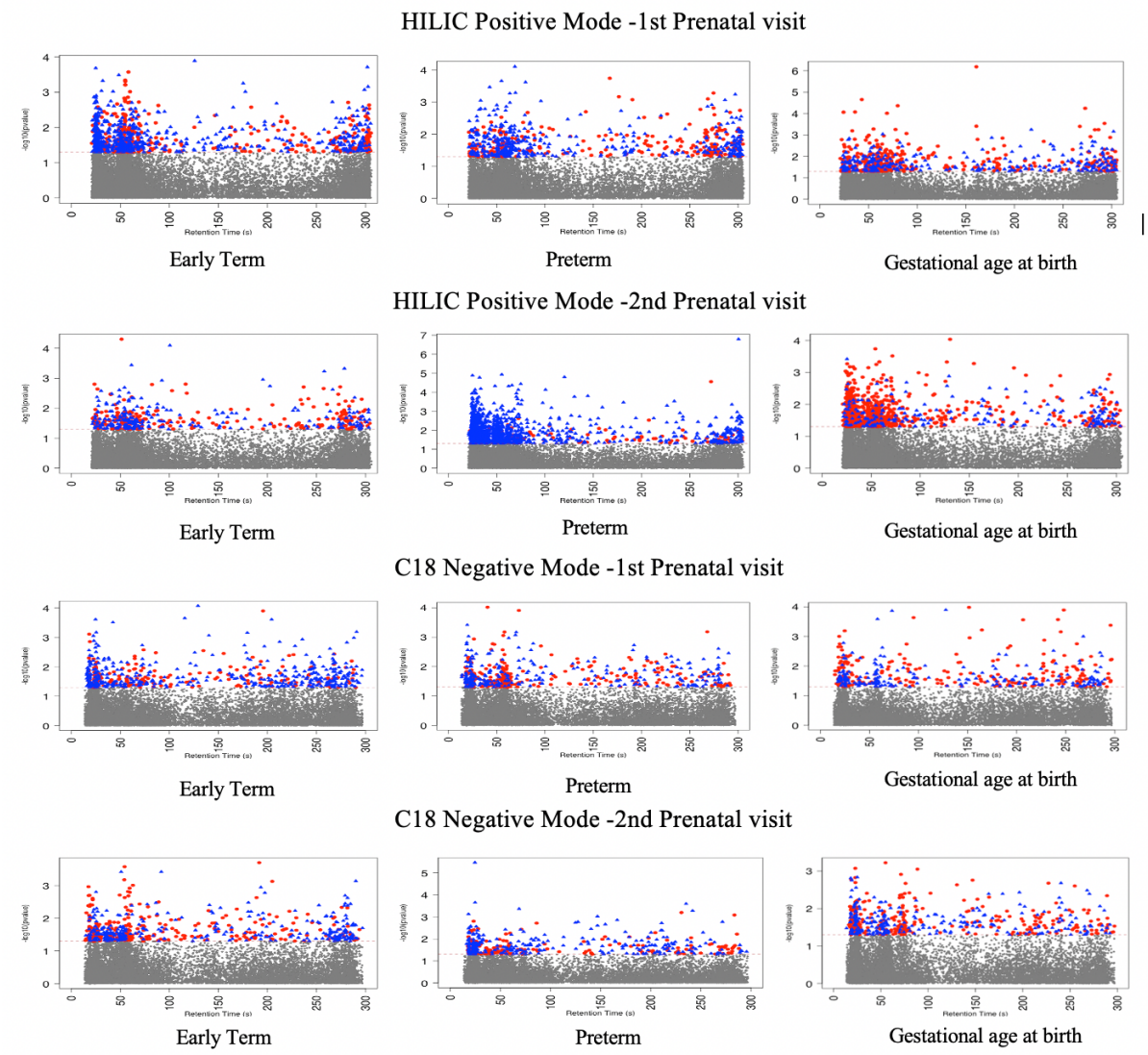

Figure S1b. Manhattan plots of associations between changes in metabolic feature intensities with adverse birth outcomes in two prenatal clinic visits. X-axis denotes the retention time of the metabolic features and Y-axis denotes the negative natural log of p-value in exposure to metabolites association. The higher the dots appeared,

the more significant the features were associated exposure Red dots indicated positive association and blue indicated negative association. The dashed line indicated the threshold cut-off p-value 0.05.

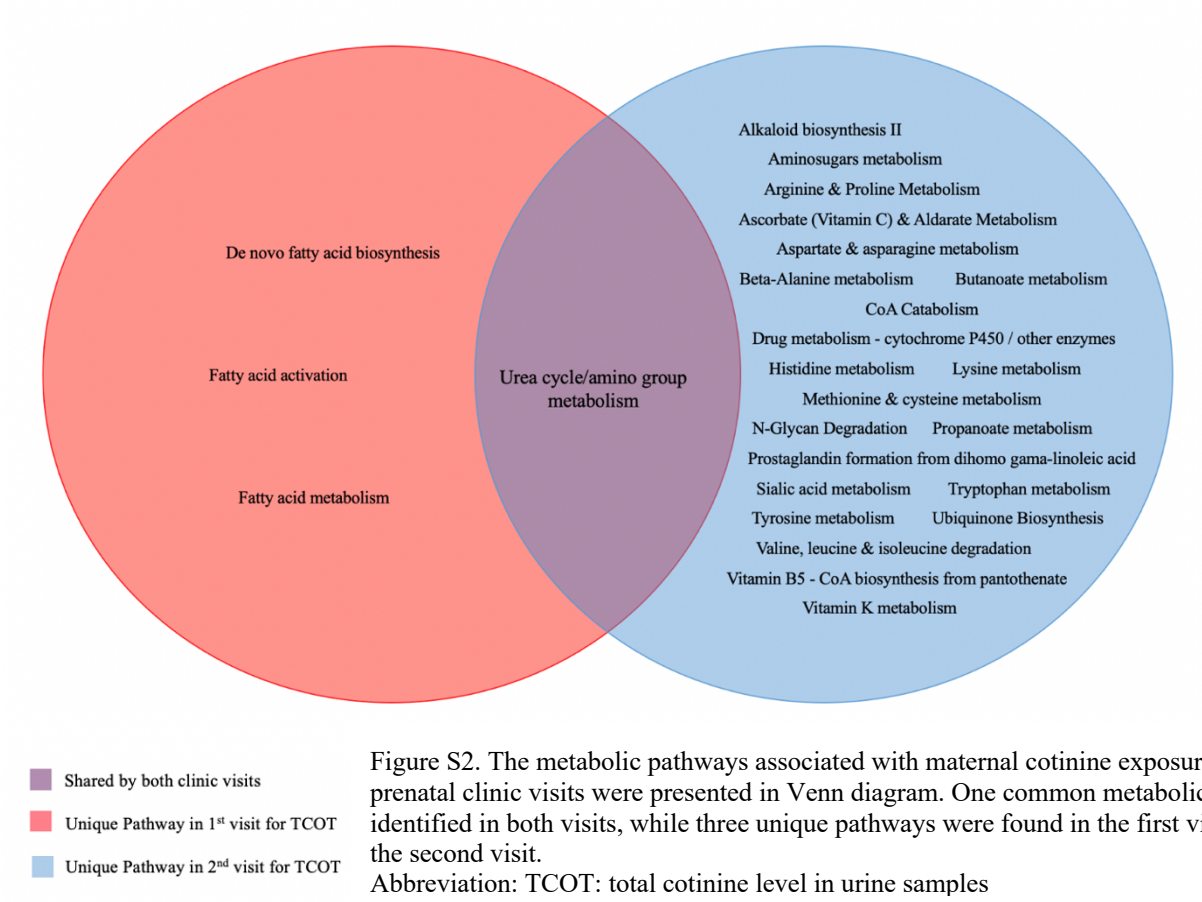

Figure S2. The metabolic pathways associated with maternal cotinine exposure in two prenatal clinic visits were presented in Venn diagram. One common metabolic pathway was identified in both visits, while three unique pathways were found in the first visit and 23 in the second visit.
Abbreviation: TCOT: total cotinine level in urine samples

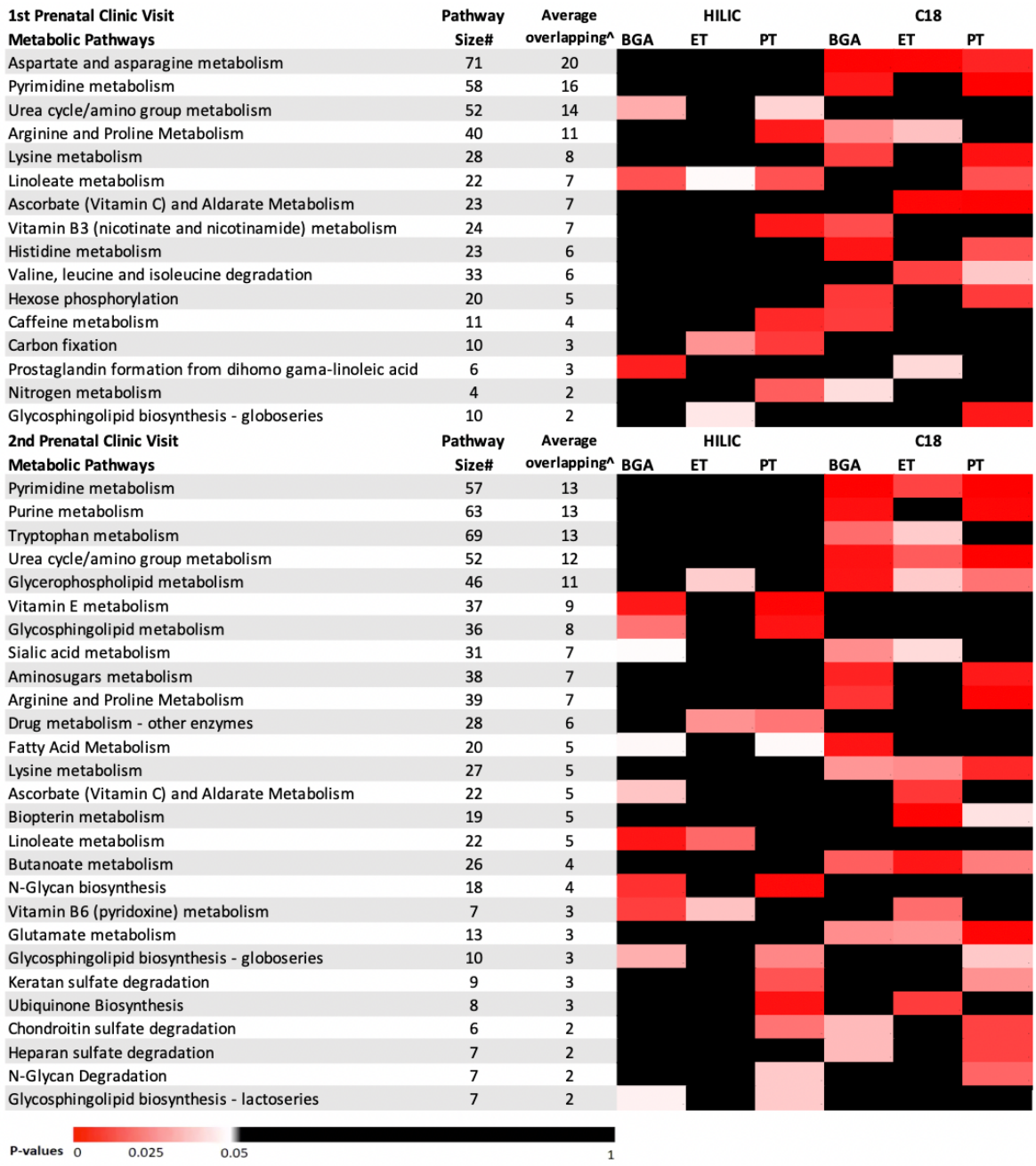

Figure S3. The metabolic pathways associated with at least two adverse birth outcomes in two prenatal clinic visits were presented in Heatmap. Each cell represents the association between each metabolic pathway and each birth outcome and the colors were shaded according to the p-value of the association. The pathways were ordered according to the total number of the significant associations (p-value<0.05) in HILIC mode and C18 mode.

* For HILIC positive ion mode, only the following adducts were considered: M[1+], M+H[1+], M-H2O+H[1+], M+Na[1+], M+K[1+], M+2H[2+], and M(C13)+2H[2+]

For C18 negative ion mode, only the following adducts were considered: M-H[-], M+Cl[-], M+ACN-H[-], M+HCOO[-], M(C13)-H[-], M-H2O-H[-], and M+Na-2H[-]

Total number of features in the specific metabolic pathway

^ Total number of features in the samples with m/z matched in the specific metabolic pathway

Abbreviation: BGA: gestational age at birth; ET: early term birth; PT: preterm birth

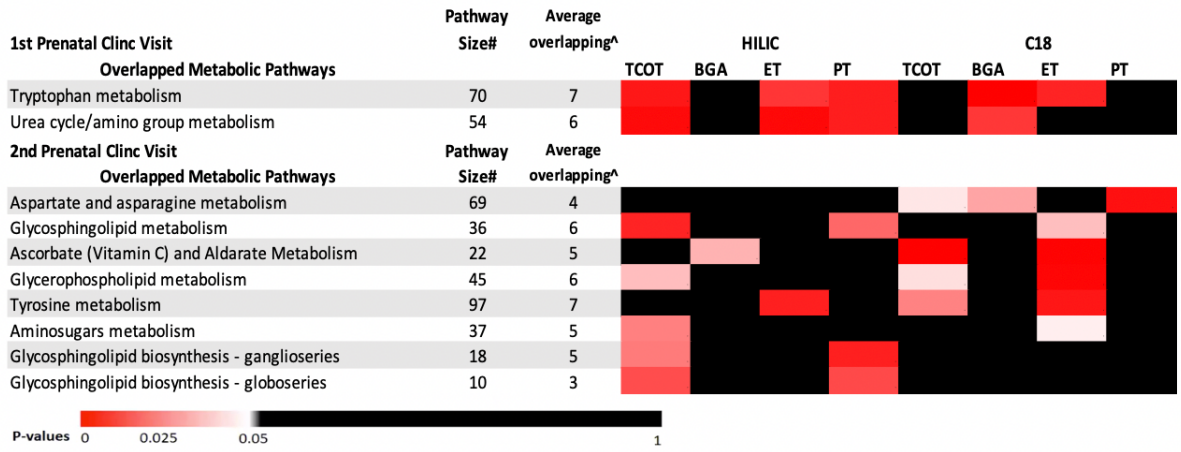

Figure S4a. The overlapped metabolic pathways in two prenatal clinic visits were presented in Heatmap. Those pathways are associated with both maternal cotinine exposure and at least one adverse birth outcome. Each cell represents the association between each metabolic pathway and each exposure/birth outcome and the colors were shaded according to the p-value of this association. The pathways were ordered according to the total number of the significant associations (p-value<0.01) in HILIC mode and C18 mode.
* For HILIC positive ion mode, only the following adducts were considered: M[1+], M+H[1+], M-H2O+H[1+], M+Na[1+], M+K[1+], M+2H[2+], and M(C13)+2H[2+]
For C18 negative ion mode, only the following adducts were considered: M-H[-], M+Cl[-], M+ACN-H[-], M+HCOO[-], M(C13)-H[-], M-H2O-H[-], and M+Na-2H[-]
Total number of features in the specific metabolic pathway
^ Total number of features in the samples with m/z matched in the specific metabolic pathway
Abbreviation: TCOT: total cotinine level in urine samples; BGA: gestational age at birth; ET: early term birth; PT: preterm birth

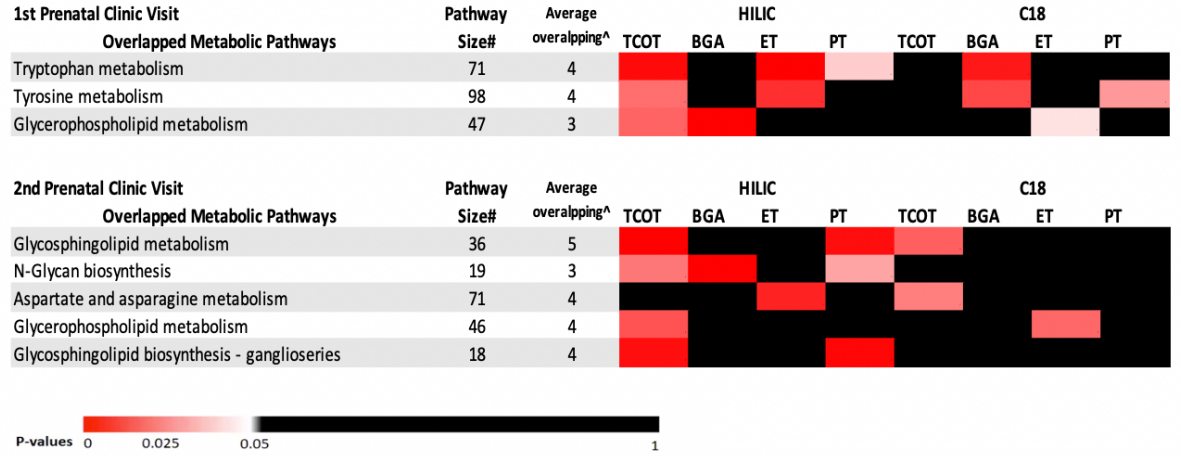

Figure S4b. The overlapped metabolic pathways in two prenatal clinic visits were presented in Heatmap. Those pathways are associated with both maternal cotinine exposure and at least one adverse birth outcome. Each cell represents the association between each metabolic pathway and each exposure/birth outcome and the colors were shaded according to the p-value of this association. The pathways were ordered according to the total number of the significant associations (p-value<0.005) in HILIC mode and C18 mode.
* For HILIC positive ion mode, only the following adducts were considered: M[1+], M+H[1+], M-H2O+H[1+], M+Na[1+], M+K[1+], M+2H[2+], and M(C13)+2H[2+]
For C18 negative ion mode, only the following adducts were considered: M-H[-], M+Cl[-], M+ACN-H[-], M+HCOO[-], M(C13)-H[-], M-H2O-H[-], and M+Na-2H[-]
Total number of features in the specific metabolic pathway
^ Total number of features in the samples with m/z matched in the specific metabolic pathway
Abbreviation: TCOT: total cotinine level in urine samples; BGA: gestational age at birth; ET: early term birth; PT: preterm birth

Table S1. Chemical identify^ of the metabolites significantly associated with adverse birth outcomes (p-value<0.05).

m/z	RT(s)	Identified Metabolite	Adduct Form	Associated with birth outcomes	Pathways	Metabolism	Visit Stage	ESI
194.0812	23.9	2-METHYLHIPPURATE	M+H	ET (β=0.387)	Phenylalanine metabolism	Amino acid metabolism	Visit 1	ESI+
189.1484	31.4	10-HYDROXYDECANOATE	M+H	ET (β=-0.244)	Fatty Acid Metabolism	Lipid metabolism	Visit 1	ESI+
108.0809	27.1	BENZYLAMINE	M+H	ET (β=-0.325)	Metabolism of xenobiotics by cytochrome P450	Xenobiotics biodegradation and metabolism	Visit 1	ESI+
203.0526	60	MANNOSE	M+Na	ET (β=-0.117)	Fructose and mannose metabolism&Galactose metabolism&Amino sugar and nucleotide sugar metabolism	Carbohydrate metabolism	Visit 1	ESI+
400.3419	30.2	PALMITOYLCARNITINE	M+H	ET (β=-0.155)	Fatty Acid Metabolism	Lipid metabolism	Visit 1	ESI+
153.0194	23.9	DIHYDROXYBENZOATE	M-H	ET (β=0.509)	Benzoate degradation	Xenobiotics biodegradation and metabolism	Visit 1	ESI-
191.0565	21	QUINATE	M-H	BGA (β=-0.097)	Phenylalanine, tyrosine and tryptophan biosynthesis	Amino acid metabolism	Visit 1	ESI-
259.0231	18	D-GLUCOSE 6-PHOSPHATE	M-H	BGA (β=-0.124)	Starch and sucrose metabolism	Carbohydrate metabolism	Visit 1	ESI-
204.123	36.5	ACETYLCARNITINE	M+H	BGA (β=0.026) PT (β=-0.198)	Insulin resistance	Endocrine and metabolic disease	Visit 1	ESI+
103.0037	18.4	HYDROXYPYRUVATE	M-H	PT (β=0.371)	Glycine, serine and threonine metabolism&Glyoxylate and dicarboxylate metabolism	Amino acid metabolism&Carbohydrate metabolism	Visit 1	ESI-
106.0497	76.7	SERINE	M+H	PT (β=-0.157)	Glycine, serine, alanine and threonine metabolism&Tyrosine metabolism&Methionine and cysteine metabolism	Amino acid metabolism	Visit 1	ESI+
118.0863	45.6	VALINE	M+H	PT (β=-0.121)	Valine, leucine and isoleucine degradation/biosynthesis&Pantothenate and CoA biosynthesis	Amino acid metabolism&Metabolism of cofactors and vitamins	Visit 1	ESI+
412.9673	57.8	PFOA	M-H	PT (β =0.219)	An industrial surfactant in chemical processes	Xenobiotics biodegradation and metabolism	Visit 1	ESI-
220.1182	26.5	D-PANTOTHENIC ACID	M+H	BGA (β=-0.142) PT (β=0.616)	beta-Alanine metabolism&Pantothenate and CoA biosynthesis	Amino acid metabolism&Metabolism of cofactors and vitamins	Visit 2	ESI+
137.0458	39	HYPOXANTHINE	M+H	BGA (β=0.072)	Purine metabolism	Nucleotide metabolism	Visit 2	ESI+
206.1008	40.2	L-RHAMNOSE	M+ACN+H	BGA (β=0.046) PT (β=-0.204)	Fructose and mannose metabolism	Carbohydrate metabolism	Visit 2	ESI+
217.0483	23.1	MANNITOL	M+Cl	ET (β=-1.946)	Fructose and mannose metabolism	Carbohydrate metabolism	Visit 2	ESI-
269.0881	42.3	INOSINE	M+H	ET (β=-0.798)	Purine metabolism	Nucleotide metabolism	Visit 2	ESI+
217.1796	31.9	OMEGA-HYDROXYDODECANOIC ACID	M+H	PT (β=-0.501)	Fatty Acid Metabolism	Lipid metabolism	Visit 2	ESI+
212.0918	42.2	3-METHOXYTYROSINE	M+H	PT (β=-0.438)	Calcium signaling pathway (Parkinson disease)	Neurodegenerative disease	Visit 2	ESI+

^ Chemical identity of metabolic features was confirmed by matching peaks via accurate mass to charge ratio and retention time to authentic reference standards under the same conditions using tandem mass spectrometry.
Abbreviation: m/z: mass to charge ratio; RT: retention time; ESI+: positive electrospray ionization mode; ESI-: negative electrospray ionization mode; BGA: gestational age at birth; ET: early term birth; PT: preterm birth

Table S2. Compare the self-reported tobacco use with the level of true cotinine biomarker*

Visit Stage \ Adjusted Cotinine		<30 μg/g	>=30 μg/g
1st clinic visit	Not used tobacco last month (n=76)	62	14
	Used tobacco last month (n=14)	2	12
2nd clinic visit	Not used tobacco last month (n=74)	58	16
	Used tobacco last month (n=3)	0	3

*Stratified by the cut off value of creatinine adjusted cotinine levels for differentiating non-smoking and smoking mother.[131]