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High-resolution Profiling of Newborn Metabolome to Study the Impact of Tobacco Smoke Exposure During Pregnancy on Adverse Birth Outcomes in The Atlanta African American Maternal-Child Cohort

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By

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M.D.
Weifang Medical University, China
2018

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Abstract

High-resolution Profiling of Newborn Metabolome to Study the Impact of Tobacco Smoke Exposure During Pregnancy on Adverse Birth Outcomes in The Atlanta African American Maternal-Child Cohort

By Xiajie Lyu

Background: Exposure to tobacco smoke during pregnancy is an established risk factor for early birth before full term (≥ 39 weeks), including preterm birth (PTB, <37 weeks) and early term birth (ETB, 37-38 weeks). However, the underlying molecular mechanisms are poorly understood. Here, we aimed to characterize the molecular relationships between prenatal maternal smoke exposure and early birth outcomes via the newborn metabolome.

Method: Participants were enrolled in the Atlanta African American Maternal-Child Cohort between 2016-2020 (N=269). Maternal urine samples were collected during 8-14 weeks gestation for cotinine and trans-3′-hydroxy-cotinine (3HC) measurement. Newborn dried blood spots (DBS) were collected at delivery for high-resolution metabolomics profiling and gestational age (in completed weeks) was ascertained from the medical record. Using an untargeted metabolomic workflow, newborn biological pathways and markers associated with exposure and outcome were estimated with multivariable regression, followed by pathway enrichment and chemical annotation, using the meet-in-the-middle approach.

Results: The geometric mean level of urinary maternal cotinine and 3HC was 7.44 µg/g and 14.89 µg/g, respectively. In total, 648 and 503 metabolomic signals were associated with maternal cotinine and 3HC levels, respectively ($p<0.05$). Seven pathways were enriched across all metabolome-wide association studies, including tryptophan, leukotriene, and biopterin metabolism (all $p<0.05$). Six metabolites associated with both tobacco biomarkers and early birth outcomes were confirmed with level-1 evidence, including glutathione and 17-hydroxyprogesterone, which are involved in redox reactions and vasoconstriction.

Conclusion: Our findings demonstrate that metabolism is different in newborns delivered early versus full-term due to tobacco exposure during pregnancy. Future research on focusing on targeted investigations is warranted.

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Table of Contents

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Introduction

Smoking during pregnancy is the most preventable risk factor for maternal and child health (Rogers, 2009). Observational studies conducted earlier have revealed that maternal smoking during pregnancy increases the risk of stillbirth, low birth weight, and small birth size compared to infants born to non-smoking mothers during pregnancy (Salihu & Wilson, 2007). The Centers for Disease Control and Prevention has estimated that 13.0% of American adults smoked cigarettes in 2020; among them, 11% percent are women (CDC, 2022). Particularly, pregnant African American people and children are more likely to have poor health outcomes due to a higher risk of preterm delivery and disproportionately high rates of low birth weight in the United States (Burris & Hacker, 2017). However, most previous studies relied on data gathered through self-reported surveys by the participants to ascertain the extent of tobacco exposure, which is prone to recall and reporting biases. (Florescu et al., 2013). Also, as smoking rates have decreased, more individuals are involuntarily exposed to tobacco smoke than active self-smokers. Consequently, many nonsmokers are exposed to similar toxicants as smokers (USDHHS, 2006). The direct quantification of tobacco exposure indicators in biological samples is a more accurate way to assess exposure. According to Neurath et al. (1987), trans-3′-hydroxy-cotinine (3HC) is the most prevalent metabolite found in urine, and it is a well-established indicator of nicotine consumption than cotinine (Benowitz, Hukkanen, & Jacob, 2009), particularly at lower exposure levels. Since 3HC is more abundant, it may serve as a more

reliable urine marker than cotinine. This could be particularly valuable for researching young children and vulnerable populations, who may experience adverse health effects from tobacco exposure at much lower levels than healthy adults (Matt et al., 2016).

High-resolution metabolomics (HRM) is an emerging analytical method that enhances the evaluation of internal exposure and biological reactions to intricate environmental mixtures. Compared to traditional targeted approaches, HRM provides a more comprehensive understanding of biological responses by detecting and characterizing thousands of metabolic features linked to exogenous exposures and endogenous processes (Liang et al., 2018; Tan et al., 2022; Chang et al., 2022; Tchen et al., 2022; Zhang et al., 2022). Apart from the deficiencies observed in previous evaluations of tobacco smoke exposure, there is a growing interest in understanding the connection between tobacco-related metabolic alterations and reproductive health. Nevertheless, only a limited number of studies have examined whether the biological metabolic changes caused by maternal smoking mediate adverse birth outcomes (Fischer et al., 2017). Furthermore, none of the studies have focused on pregnant African American people and their newborns who are at higher risk for adverse birth outcomes. To investigate the endogenous molecular processes underlying the associations between urine cotinine and 3HC levels and adverse birth outcome, we conducted a metabolome-wide association study (MWAS) to identify metabolic signals associated with cotinine and 3HC levels and adverse birth outcomes in the Atlanta African American Maternal-Child Cohort.

Design & Methods

Study population

The study recruited pregnant African American people from Emory Midtown Hospital and Grady Memorial Hospital in Atlanta (Brennan et al., 2019; Corwin et al., 2017). Eligible participants had singleton pregnancies and no chronic medical conditions and had their first prenatal visit between 8-14 weeks of gestation. During the 8-14 week period of pregnancy, data was collected through a combination of methods, including a questionnaire capturing basic demographic information, collection of biological samples (blood and urine), and abstraction of medical records to document clinical conditions. Birth outcome data were collected from medical records, and the newborn's dried blood spots (DBS) were collected by trained hospital nursery personnel from a heel stick within 24-48 hours of birth. For this study, we included 269 mother-child dyads who had maternal urinary cotinine concentrations measured during their 1st clinic visits between the 8th and 14th week of pregnancy and had newborn metabolomics profiling conducted. This study was granted approval by the Emory University Internal Review Board (IRB ID 1017), and all study participants gave informed consent prior to their involvement in the study.

Measurement of maternal biomarker concentrations

During the mother's 8–14week gestational visit, individual spot urine samples were collected from the pregnant women and analyzed using liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS) to determine the concentrations of maternal cotinine and 3HC level. The minimum detectable amount was 2.5 ng/mL, with precision within a range of 1.58-6.21% for the same day and 1.71-6.26% for different days. Relative recoveries ranged from 82.5-97.1%. Analysis of NIST reference materials demonstrated accuracy, which showed results between 86.8-95.3%. The method was validated by participating in the German External Quality

Assessment Scheme (G-EQUAS) proficiency testing scheme (http://gequas.de). If TCOT and 3HC concentrations were below the limitation of detection (LODs), they were imputed using LOD/√2. To account for the effect of urine dilution, the creatinine levels in the urine samples were also measured and used to adjust the urinary cotinine and 3HC levels (Thompson, Barlow, Wald& Vunakis, 1990). Specifically, we calculated the ratio of cotinine and 3HC concentration to creatinine levels in each sample and used this ratio to make the adjustments when we included creatine in the regression models.

Measure of adverse birth outcomes and other factors

The primary focus of this analysis was gestational age, which was determined based on the date of delivery in relation to the estimated date of conception established during the first clinical visit. This information was obtained from medical records that took into consideration the last menstrual period and the first visit ultrasound, as outlined by Obstetricians, Gynecologists, & Practice (2017). The study participants were divided into three categories based on gestational age at birth: preterm (>20 and < 37 weeks), early term (≥37 to ＜39 weeks), and full-term (≥39 weeks) (Lumley, 2003). We collected information on potential confounding factors using questionnaires and medical records. These factors included individual-level demographic characteristics (age and education level), behavioral risk factors (self-reported tobacco and alcohol use), and maternal characteristics (fetal sex, body mass index (BMI) at the time of the clinic visit, and nulliparous status).

Untargeted High-Resolution Metabolomics Profiling

Metabolomics profiling was conducted on dried blood spots (DBS) collected from each participant's newborn with 48 hours of birth. These DBS samples were regularly gathered for medical screening and public health observation and then kept for future biomonitoring. The standardized protocol involved cleaning the heel with 75% isopropanol, using a sterile 2.5 mm lancet to obtain the sample, and saturating each circle on a standard Guthrie card with approximately 75 uL of blood (GDoPH, 2023). The card specimens were transported to the Georgia Department of Public Health Laboratory on the same collection day and kept in a walk-in refrigerator without a desiccant for up to three months. The DBS samples were then moved to our laboratory for storage at -80°C using gas-impermeable bags with a desiccant until the assay (GDoPH, 2023). The study obtained single 15-mm punches, equivalent to around 50 uL of whole blood, from 269 children between 2016 and 2020. Additionally, an extra set of 15-mm punches was collected from adjacent filter paper portions from the same Guthrie cards to use as blanks. Untargeted high-resolution metabolomics profiling was conducted in a single run by the North Carolina HHEAR Hub (NC HHEAR Hub) in The University of North Carolina at Chapel Hill (UNC) Nutrition Research Institute, North Carolina Research Campus (Kannapolis, NC) using established methods. DBS samples were extracted with ice-cold methanol containing L-tryptophan-d5, and the extracted supernatant was transferred to low-bind microfuge tubes. Blanks were also processed using the same procedures as the study samples. All supernatant aliquots were dried by vacuum concentrator and reconstituted with water-methanol for the UHPLC-HR-MS analysis. NIST reference plasma samples were prepared as external quality control samples. The metabolites were separated via an HSS T3 C18 column, and the untargeted metabolomics data was acquired using a Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer. Progenesis QI processed the data for peak identification, alignment, and

normalization. Signals that significantly differed amongst the three running batches were excluded for further analysis. The remaining signals were filtered only to include those detected in 95% of serum samples and log2-transformed to normalize positive skewness.

Statistical analyses

To investigate the metabolic signals associated with concentrations of cotinine and 3HC, and/or birth outcomes, we conducted a set of models for metabolome-wide association studies (MWAS). Specifically, we ran generalized multivariable linear models to evaluate the associations between maternal cotinine and 3HC concentrations and metabolic feature intensities.

$$\log_2 Y_{ij} = \beta_0 + \beta_{1j}\,\text{Cotinine}_i + \gamma_{1j}\text{Sex}_i + \gamma_{2j}\text{Age}_i + \gamma_{4j}\text{BMI}_i + \gamma_{5j}\text{Education}_i + \gamma_{6j}\text{Nulliparous}_i + \gamma_{7j}\text{Alcohol}_i + \varepsilon_{ij} \quad (1)$$

$$\log_2 Y_{ij} = \beta_0 + \beta_{1j}\,3\text{HC}_i + \gamma_{1j}\text{Sex}_i + \gamma_{2j}\text{Age}_i + \gamma_{4j}\text{BMI}_i + \gamma_{5j}\text{Education}_i + \gamma_{6j}\text{Nulliparous}_i + \gamma_{7j}\text{Alcohol}_i + \varepsilon_{ij}. \quad (2)$$

The $\log_2$ of the intensity of metabolic feature j for participant i is denoted as $\log_2 Y$, while β_0 represents the intercept and Cotinine_i and 3HC_i represent the total maternal cotinine and 3HC concentration for participant i. To account for potential confounding factors and covariates, the model as adjusted for fetal sex (Sex_i), maternal age (Age_i), clinical visit prenatal BMI (BMI_i), maternal education level (Education_i, categorical), parity (Nulliparous_i, categorical), and self-reported alcohol use during pregnancy (Alcohol_i, categorical). The residual random error is represented by ε_{ij}.

We also used a similar generalized multivariable linear model to identify metabolic features that may be linked to adverse birth outcomes.

$$\log_2 Y_{ij} = \beta_0 + \beta_{1j}\,\text{Birth outcome}_i + \gamma_{1j}\text{Sex}_i + \gamma_{2j}\text{Age}_i + \gamma_{4j}\text{BMI}_i + \gamma_{5j}\text{Education}_i + \gamma_{6j}\text{Nulliparous}_i + \gamma_{7j}\text{Alcohol}_i + + \varepsilon ij \quad (3)$$

In this model, "Birth outcome" is a categorical variable representing preterm, early term, and full-term birth, with full-term birth as the reference group. Separate models were created for each of these birth outcomes to analyze their respective associations with metabolic features. Overall, we carried out and evaluated four sets of MWAS models, including examining cotinine and 3HC levels and two adverse birth outcomes. The outcomes were presented visually through Manhattan plots, with each metabolic feature's retention time plotted on the x-axis and $-\log_{10}(p)$for $\beta 1$ from each equation displayed on the y-axis. All the analyses were conducted in R version 4.2.

Metabolic pathway enrichment analysis and metabolite annotation

We used mummichog (Version 1.0.10), an innovative statistical software for pathway enrichment analyses. Mummichog predicts biological networks, pathways, and metabolites based on significant signals with tentative chemical identities (Shuzhao Li et al., 2013). We used raw p-values < 0.05 to select eligible metabolic features for pathway analysis. To minimize the chance of false positive findings, we selected pathways identified by mummichog with p-values less than 0.05. We also performed a sensitivity analysis, using p-values adjusted with the false discovery rate (FDR＜0.2), to study whether the pathway enrichment results would differ at different p-values significance levels.

To confirm the chemical identity of the significant metabolites, we used in-house experimental standards library (IESL) containing over 2,400 compounds, including endogenous and

exogenous metabolites. The study samples and standard reference compounds in the IESL were tested under the same conditions. The same set of signals was also matched to public databases like NIST and METLIN. These signals were then labeled with ontology levels (OL) based on matching criteria such as retention time (RT), exact mass (MS), MS/MS fragmentation pattern, and isotopic ion pattern. Metabolites that matched to the IESL by RT (±0.5 min), MS (<5 ppm), and MS/MS (similarity score > 30) were labeled as OL1, while those that matched by RT and MS were labeled as OL2a. OL1 and OL2a metabolites were identified with the highest confidence. OL2b label was given to signals matched by MS and MS/MS to the IESL but outside the RT window. Metabolites annotated with OL2b are isomers or conjugates that share similar moieties with the matched compound in IESL. A public database (PDa) label was provided for signals matched by MS (<5 ppm) and experimental MS/MS (similarity score > 30) to public databases. Annotations with OL2b and PDa labels were considered confident, while metabolites annotated with less confidence were not used in subsequent analyses.

Meet-in-the-middle analysis

We conducted a " meet-in-the-middle " (MITM) analysis (Chang et al., 2022; Gaskins et al., 2021; Hwang et al., 2022) to investigate whether the newborn metabolome could mediate the link between maternal cotinine and 3HC levels and adverse birth outcomes. This involved performing separate pathway enrichment and chemical annotation analyses for both the exposure and birth outcomes. We then identified the common pathways or metabolites that show significant associations with both exposure and outcome.

Results

Among the 269 included in this analysis, the average age at the clinic visit was 25.7 ± 5.2 years, the average BMI is 29.2± 7.8 31.2% participants had a college degree or above, and 42% had been pregnant before. Majority of the participants had a full-term birth (56.5%), with 30.5% early term births and 12.2% preterm deliveries (Table 1). Very few of women reported using alcohol and tobacco during the month prior to visit. Among them, 12.6% reported alcohol usage and 16.4% reported tobacco usage in their clinic visit. The percentiles of 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 0.01 μg/g to 54.63μg/g during the and adjusted 3HC concentration ranging from from 0.02 μg/g to 148.06 μg/g respectively, indicative of substantial variation in magnitude of exposure to tobacco smoke among study participants. Furthermore, the investigation revealed observable levels of cotinine and/or 3HC in the urine of pregnant females who declared no utilization of tobacco within one month, as evidenced by Table S1.

After data preprocessing, quality control procedures, and data filtering (See method: Untargeted High-Resolution Metabolomics Analysis), 7089 metabolic features in were included for final analyses. The number of statistically significant (raw and FDR-corrected) metabolic features associated with cotinine and 3HC concentrations or birth outcomes is shown in Table S2. After adjusting for covariates, 648 features were significantly associated with maternal cotinine levels and 503 features were significantly associated with maternal 3HC levels. There were 648 features associated with preterm birth and 1747 features associated with preterm birth (P-value <0.05, Figure 2), while there were 108 features were significantly associated with maternal cotinine levels and 46features were significantly associated with maternal 3HC levels. There

were 48 features associated with preterm birth and 1738 features associated with preterm birth (FDR<0.2, Figure S1).

Pathway enrichment analysis

Pathway enrichment analysis revealed a total of 11 significant pathways associated with cotinine levels and 10 significant pathways associated with 3HC levels. Meanwhile, 10 significant pathways were found to be associated with preterm birth outcome and 10 significant pathways associated with early-term birth outcome. These pathways were broadly related to amino acids, bioenergetics, bioactive lipids, providing valuable insights into the metabolic processes underlying these birth outcomes.

The meet-in-the-middle analysis revealed several overlapping metabolic pathways associated with both the exposures and birth outcomes. Specifically, when examining the relationship between cotinine levels and preterm birth outcome, we identified 7 overlapping pathways, including drug metabolism, steroid metabolism, and amino group metabolism overlapped. Additionally, 5 metabolic pathways related to amino acid and steroid metabolism were found to be associated with cotinine levels and early-term birth outcome. As for 3HC, 7 pathways related to drug and amino group metabolism were shared with preterm birth outcome and 5 amino group metabolism pathways were shared with early term birth outcome (Figure 3). Interestingly, we found that biopterin metabolism was associated with both cotinine and 3HC levels, as well as all birth outcomes (Figure 4).

Metabolite annotation and confirmation

After signal identification and annotation, a total of 6 overlapping metabolites were matched to high confidence ontology levels, including 3 labeled as OL1, 2labeled as OL2a, and 1 labeled as PDa. The confirmed metabolites included carnitine, amino acids and proteins, chemical messengers, and redox reactions (Table 3). Amino acids and proteins and chemical messengers were the largest categories with 2 metabolites respectively, which were associated with both cotinine, 3HC, and preterm and early-term birth outcomes. Carnitine and redox reactions is the smallest categories with only 1 metabolites respectively, and overlapping with only cotinine. Among them, 3-Hydroxydodecanoyl carnitine and N-Acetylleucine were associated with the same exposure and outcome (cotinine and preterm birth). In contrast, 17-hydroxyprogesterone and Hexanoyl glycine were associated with 3HC and preterm birth, and S-lactoylglutathione and L-DOPA were found to be associated with cotinine and early-term birth.

Sensitivity analysis

We conducted several sets of sensitivity analyses to test the consistency and accuracy of our metabolomics analyses and avoid false discoveries. Specifically, we reran pathway enrichment analysis using the false discovery rate adjusted p-values (FDR < 0.2). Our results revealed that similar pathways, including amino group metabolism and steroid metabolism, were consistently associated with cotinine/3HC levels and birth outcomes (Figure S2). Although fewer enriched pathways and significant metabolic features were identified compared to the primary analysis given the smaller number of significant features surviving the FDR correction (Figure S3, TableS2), it is noteworthy that biopterin metabolism was still associated with cotinine, 3HC, preterm birth, and early term birth. Overall, we observed consistent findings in the sensitivity analyses.

Discussion

In this study, we employed advanced targeted exposure assessment and state-of-art untargeted HRM to investigate the association between prenatal exposure to tobacco and adverse birth outcomes among participants in the Atlanta African American mother-child cohort. Results show that maternal urinary cotinine and 3HC levels during early pregnancy, as surrogate measures of fetal exposure to tobacco, were significantly associated with perturbations in newborn DBS metabolome, which were also associated with early birth (i.e., PTB and ETB) among pregnant African American women and newborns. This study also highlights the utility of neonatal dried blood spots (DBS) metabolomics in exploring the molecular mechanisms between prenatal tobacco exposure and birth outcomes. To our knowledge, this is the first study to use newborn DBS metabolomics to explore the molecular mechanisms between prenatal tobacco exposure and adverse birth outcomes and these findings elucidate the potential biological mechanisms underlying tobacco toxicity on birth outcomes.

Changes in pathways and metabolites in amino acid metabolism

The present analysis reveals several noteworthy results, particularly the identification of several metabolic pathways and metabolites associated with cotinine and 3HC levels and adverse birth outcomes. Most of these overlapping pathways, such as glycine, leucine, and glutathione metabolism, are closely involved in systemic inflammatory responses, cell death, and redox reactions triggered by tobacco smoke exposure. These processes may ultimately lead to shorter gestation periods (Hardison et al., 2012; Das et al., 2014). Specifically, Hardison et al. investigated and identified the acetylation of proline-glycine-proline (PGP), which is involved in

the chemotactic response of neutrophils. Das et al.'s study showed that cigarette smoke leads to the death of lung cells and macrophages by reducing the levels of leucine. In addition, glutathione concentrations are reduced by early pregnancy and exposure to tobacco smoke (Biswas & Rahman, 2009). The adverse effects of active and passive maternal smoking on the antioxidant glutathione are considerable during the first trimester of pregnancy (Bizon et al., 2021).

L-DOPA is a crucial precursor to synthesizing the catecholamines dopamine, norepinephrine, and epinephrine, requiring the dietary LNAAs phenylalanine and tyrosine for its production (Aldred & Nutt, 2010). The role of dopamine in nicotine addiction has been demonstrated, with evidence suggesting that it plays a crucial role in the reinforcing effects of nicotine and the development of nicotine dependence (Bloomfield et al., 2014). Moreover, elevated maternal urinary catecholamine levels may indicate an increased susceptibility to excessive stress and/or heightened sympathetic activation, leading to a heightened risk of spontaneous preterm birth (Holzman et al., 2009).

Perturbations in pathways enriched in biopterin metabolism

Another top pathway we observed to associated with maternal smoking exposure and adverse birth outcome is the biopterin metabolism, a biochemical process involving biopterin synthesis, recycling, and breakdown. Biopterin is an essential cofactor for various enzymes, including nitric oxide synthase, phenylalanine hydroxylase, and tyrosine hydroxylase (Werner-Felmayer, Golderer, & Werner, 2002). Alterations in biopterin metabolism can lead to various health conditions, such as cardiovascular disease, diabetes, and neurological disorders (Bendall et al.,

2014). In our study, biopterin metabolism was linked with both cotinine and 3HC levels, as well as all birth outcomes -PT and ET. These results were consistent with the previous findings, where cigarette smoking was linked to alterations in biopterin metabolism (Abdelghany et al., 2018). Specifically, the constituents of cigarette smoke can cause dysfunction and uncoupling of endothelial nitric oxide synthase by depleting tetrahydrobiopterin. Maternal biotransformation is strongly associated with intrauterine fetal growth, where tetrahydrobiopterin levels were confirmed to decrease in shorten-age pregnancies, resulting in reduced nitric oxide synthase activity and impaired vasodilation (Miklós Tóth, 2002). Additionally, our study revealed that maternal urinary cotinine concentrations and adverse birth outcomes were associated with several antioxidant metabolites, specifically S-lactoglutathione, which may be an internal response to exposure, as an increased risk of preterm birth has been positively associated with oxidative stress biomarkers (Aung et al., 2019). While these metabolomic findings require validation in other fetal and neonatal populations, they imply that oxidative stress may serve as a common link between prenatal tobacco exposure and adverse birth outcomes.

Effects in pathways and metabolites enriched in lipid and fatty acid metabolism

In addition to amino acid and biopterin metabolism, fatty acid metabolism has been identified as another prominent pathways that are influenced by cotinine and 3-hydroxyisobutyrate (3HC) levels in our research, and it has also been shown to have significant associations with adverse birth outcomes. These findings align with previous research indicating that smoking is associated with alterations in fatty acid and metabolism (Jain & Ducatman, 2018). Perturbations in maternal fatty acid metabolism is also strongly associated with intrauterine fetal growth, indicating that maternal dyslipidemia is associated with an increased risk of preterm birth (Smith et al., 2018).

The metabolic feature of lipid metabolism was validated, and 17-hydroxyprogesterone was identified as a metabolite associated with tobacco exposure levels in lipid metabolism. 17-Hydroxyprogesterone (17-OHP) in premature infants as a marker of intrauterine stress (Ersch, Beinder, Stallmach, Bucher, & Torresani, 2008). Preterm and early-term infants often have higher 17-OHP levels than healthy term infants (Ryckman et al., 2012). Additionally, the study revealed a positive correlation between acylcarnitine, involved in fatty acid oxidation, and preterm delivery. Lower levels of acylcarnitine have been observed to result in decreased energy production and reduced fat oxidation, which may contribute to the association between smoking during pregnancy and unfavorable birth outcomes (Zoula et al., 1966). Taken together, these findings suggest that monitoring maternal lipid metabolism during pregnancy and reducing smoking may be crucial for preventing preterm birth and improving birth outcomes.

Study Strength and limitation

Our study has uncovered new insights into the possible biological mechanisms that underlie the impact of maternal tobacco exposure on adverse birth outcomes. Despite the promising findings, we also acknowledge a number of limitations in the current study. Firstly, our study design was cross-sectional, and therefore we were not able to establish a causal relationship between maternal cotinine/3HC levels, metabolic disturbances, and adverse birth outcomes. Tobacco exposure may also interact with other environmental chemicals, may mask the actual health effects of tobacco, when exposure to mixtures of other environmental chemicals with lower detection rates and shorter half-lives may also contribute to the observed associations. As a result, more research is needed to fully understand the potential combined effects of environmental chemicals and non-chemical stressors on perinatal health outcomes. While we

accurately measured maternal cotinine concentrations in urine samples, we were not able to determine the exact source of nicotine exposure. We found that a significant percentage of women in our cohort who reported no tobacco use had high urinary cotinine concentrations, which could indicate potential reporting bias or alternative routes of tobacco exposure (i.e., second-hand smoking). Moreover, one-time exposure measurements may not reflect long-term exposure status, which could limit the interpretation of potential mechanisms for the effect of maternal smoking on birth outcomes. The selection of biological samples for measuring cotinine levels should also be considered. Additionally, due to limited statistical power, we did not adjust for several potential confounders, including some socioeconomic backgrounds. However, sensitivity analyses showed consistent results in the MWAS model and pathway enrichment analysis. Nevertheless, our limited sample size highlights the need for a more extensive study, which could be facilitated by neonatal DBS sampling with larger sample size. Although untargeted DBS metabolomics analysis was normalized according to DBS size and weight, we were unable to perform other potentially more optimal normalizations due to the limited sample size available.

Furthermore, we used the MITM framework, which requires fewer assumptions than formal mediation analyses. Therefore, results should be interpreted with caution. Lastly, given that we are solely focusing on African American, which may limit the generalizability of the study findings to other population.

Conclusion

Using advanced targeted exposure assessment and untargeted high-resolution metabolomics, we discovered and validated various metabolic pathways and metabolites associated with both cotinine/3HC levels and adverse birth outcomes. Our analysis of the neonatal DBS metabolome revealed that perturbations in biological pathways related to amino acids, lipids, and biopterin were part of the potential mechanisms underlying the link between cotinine/3HC and adverse birth outcomes. We also identified prominent metabolites in the neonatal circulatory system, further elucidating the molecular networks involved in tobacco-related toxicity and its impact on maternal health.

In summary, our study contributes new insights into the mechanisms underlying the link between maternal tobacco exposure and preterm birth risk while advancing our understanding of the biological response to tobacco exposure and developing reliable biomarkers. Future research is warranted to replicate our findings in diverse populations and validate the metabolic mechanisms and biomarkers using hypothesis testing approaches. Ultimately, these efforts may lead to targeted interventions that reduce adverse birth health effects from tobacco smoke exposure in pregnant people and newborns.

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Tables and figures

Table 1: Prenatal characteristic of study participants(n=269) overall, the Atlanta African-American maternal child cohort（2016-2020）

	Overall	Preterm	Early term	Full term
N	269	33	84	152
Age, years, mean (SD)	25.7 (5.2)	25.8 (4.5)	25.3 (5.3)	25.8(5.3)
Maternal BMI, kg/m², mean (SD) [a]	29.2 (7.8)	27.2 (6.7)	29.3 (8.5)	29.6 (7.6)
Education level n (%)				
Less than high school	39 (14.4)	4 (12.1)	14 (16.7)	21 (13.8)
High school	117 (43.5)	20 (60.6)	40 (47.6)	57 (37.5)
Some college or more	113 (42.0)	9 (27.3)	30 (35.7)	74 (48.7)
Nulliparous, n (%)				
Yes	113 (42.0)	10 (30.3)	33 (39.3)	70 (46.1)
No	156 (58.0)	23 (69.7)	51 (60.7)	82 (53.9)
Sex of the baby, n (%)				
Male	131 (48.7)	23 (69.7)	40 (47.6)	68 (44.7)
Female	138 (51.3)	10 (30.3)	44 (52.4)	84 (55.3)
Alcohol Use				
Not used last month	235 (87.4)	27 (81.8)	77 (91.7)	131 (86.2)
Used last month	34 (12.6)	6 (18.2)	7 (8.3)	21 (13.8)
Marijuana use				
Not used last month	169 (62.8)	19 (57.6)	56 (66.7)	94 (61.8)
Used last month	100 (37.2)	14 (42.4)	28 (33.3)	58 (38.2)
Tobacco use				
Not used last month	225 (83.6)	25 (75.8)	70 (83.3)	130 (85.5)
Used last month	44 (16.4)	8 (24.2)	14 (16.7)	22 (14.5)

Abbreviations: BMI, body mass index

a. Maternal BMI was measured at the pregnant women's clinic visit.

b. Elective abortion and spontaneous abortion were not included

Table 2: Quintiles of unadjusted and creatinine-adjusted maternal cotinine and 3HC concentrations, the Atlanta African American Maternal-Child cohort (2016-2020)

	Min	25% Quantile	Median	Geometric Mean	75% Quantile	Max
Unadjusted cotinine, ng/mL	89.00	118.38	801.13	1250.38	7656.69	1023909.20
Adjusted cotinine, µg/g Creatinine	0.23	0.98	5.03	7.44	42.48	5462.60
Unadjusted 3HC, ng/mL	89.00	330.00	1770.27	2502.64	17578.83	2775288.11
Adjusted 3HC, µg/g Creatinine	0.25	1.69	9.45	14.89	106.34	14806.27

Table 3. Chemical identity of the metabolites significantly associated with maternal cotinine and 3HC levels and adverse birth outcome (raw P < 0.05)

Metabolite	m/z	RT (min)	Matching Library ID	Class	Overlapped exposure and outcome	Associated with TCOT exposure [a]	Associated with 3HC exposure
OL1							
3-Hydroxydodecanoyl carnitine	360.2741	11.69	71464535	Carnitines	TCOT- PT	β=-0.022	/
Hexanoyl glycine	173.1052	7.05	99463	Amino acids and proteins	3HC- PT	/	β=0.004
N-Acetylleucine	173.1052	6.81	70912	Amino acids and proteins	TCOT- PT	β=0.016	/
OL2a							
S-Lactoylglutathione	380.112	2.49	25138-66-3	Redox reactions	TCOT-ET	β=-0.038	/
L-DOPA	197.0687	1.44	6047	Chemical messengers	TCOT-ET	β=0.0160	/
PDa							
17-Hydroxyprogesterone	331.2265	10.68	44235	Chemical messengers	3HC-PT	/	β=-0.007

Note: The metabolic features were verified to be chemically identical by comparing the peaks' accurate mass to charge ratio and retention time to genuine reference standards under identical conditions with tandem mass spectrometry.
Abbreviations: m/z: mass to charge ratio; RT: retention time; TCOT: total cotinine concentration in urine sample. 3HC: total trans-3'-Hydroxycotinine in urine sample; ET: early term birth (≥ 37 and < 39 weeks); PT: preterm birth (>20 and < 37 weeks).

a. The beta coefficient represents the change in log-transformed metabolite intensity per 1-unit increase in urinary cotinine level.

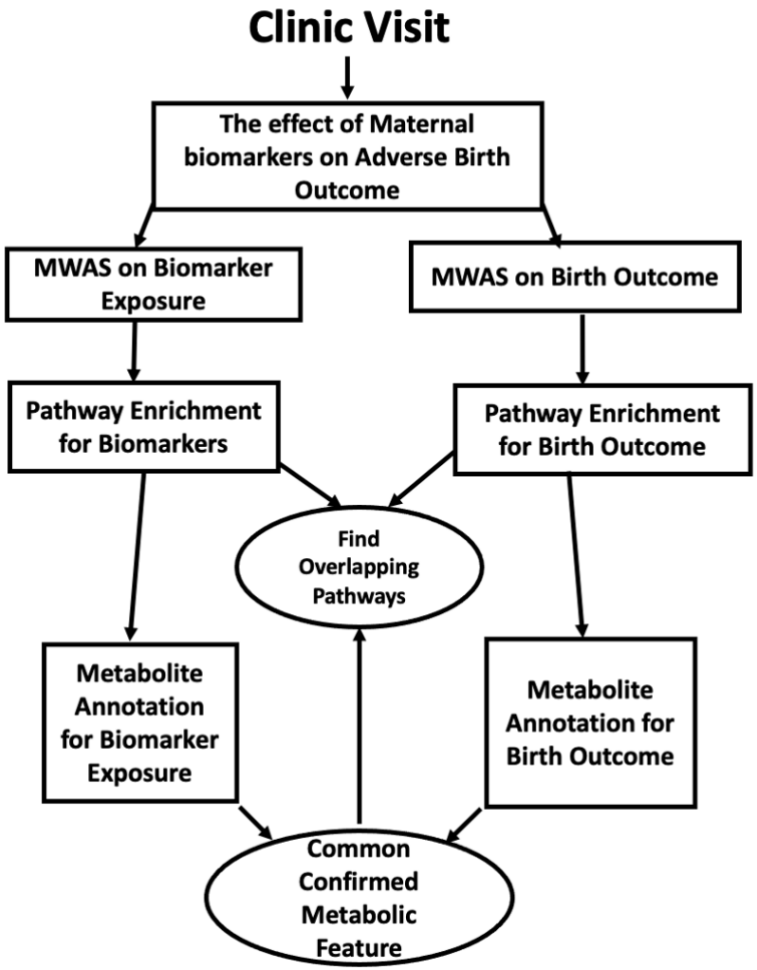

Figure 1: The flow chart of meet-in-the-middle approach in our study.

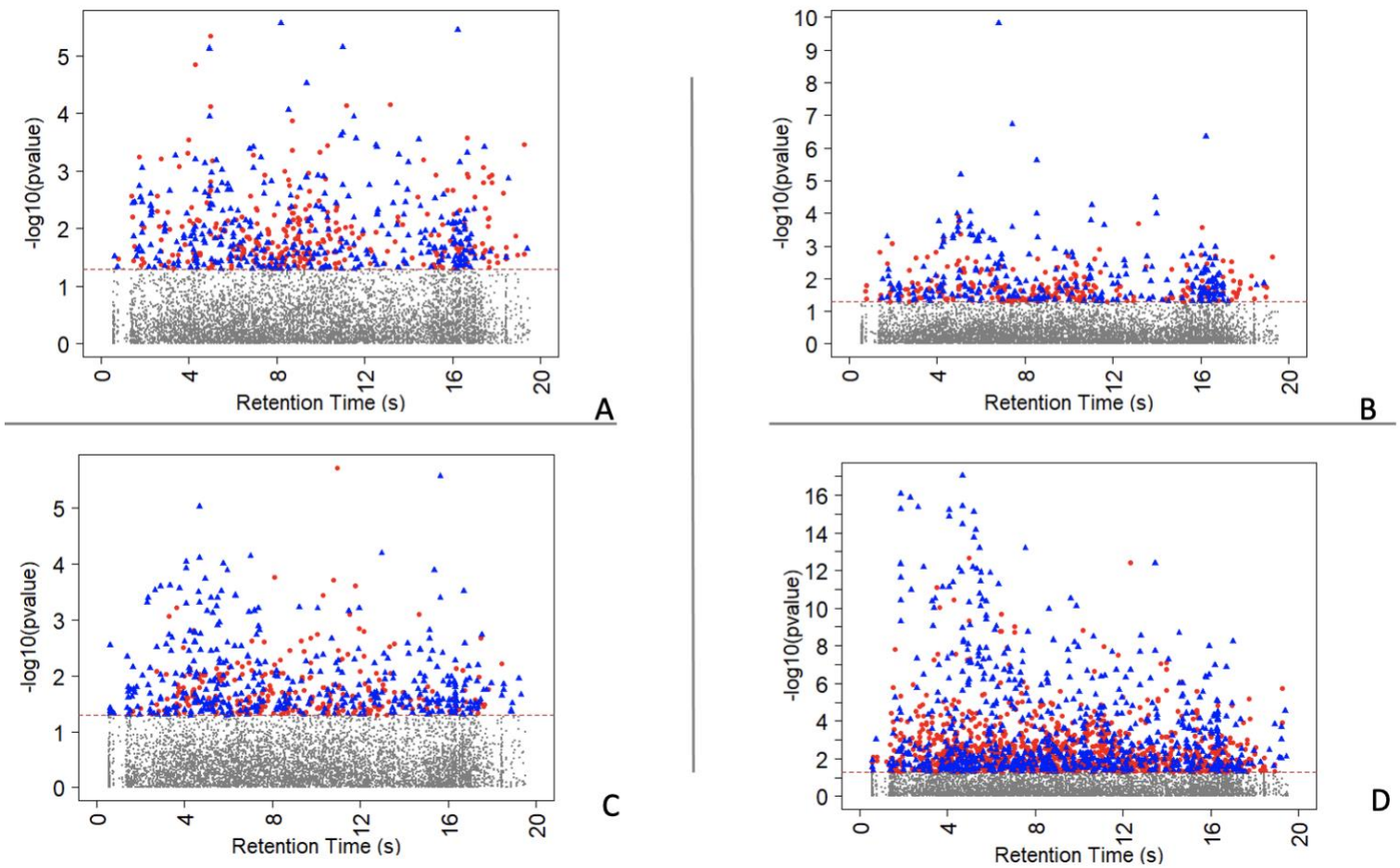

Figure 2: Manhattan plots of significant metabolic features (raw p< 0.05)
Note: X-axis denotes the retention time of the metabolic features and Y-axis denotes the negative natural log of p-value in exposure to metabolites association. The higher the dots appeared, the more significant the features were associated exposure. Red dots indicated positive association and blue indicated negative association. The dashed line indicated the threshold cut-off p-value 0.05.
A: Manhattan plots of associations between changes in metabolic feature intensities with cotinine level
B: Manhattan plots of associations between changes in metabolic feature intensities with 3HC level
C: Manhattan plots of associations between changes in metabolic feature intensities with early-term birth outcome.
D: Manhattan plots of associations between changes in metabolic feature intensities with pre-term birth outcome

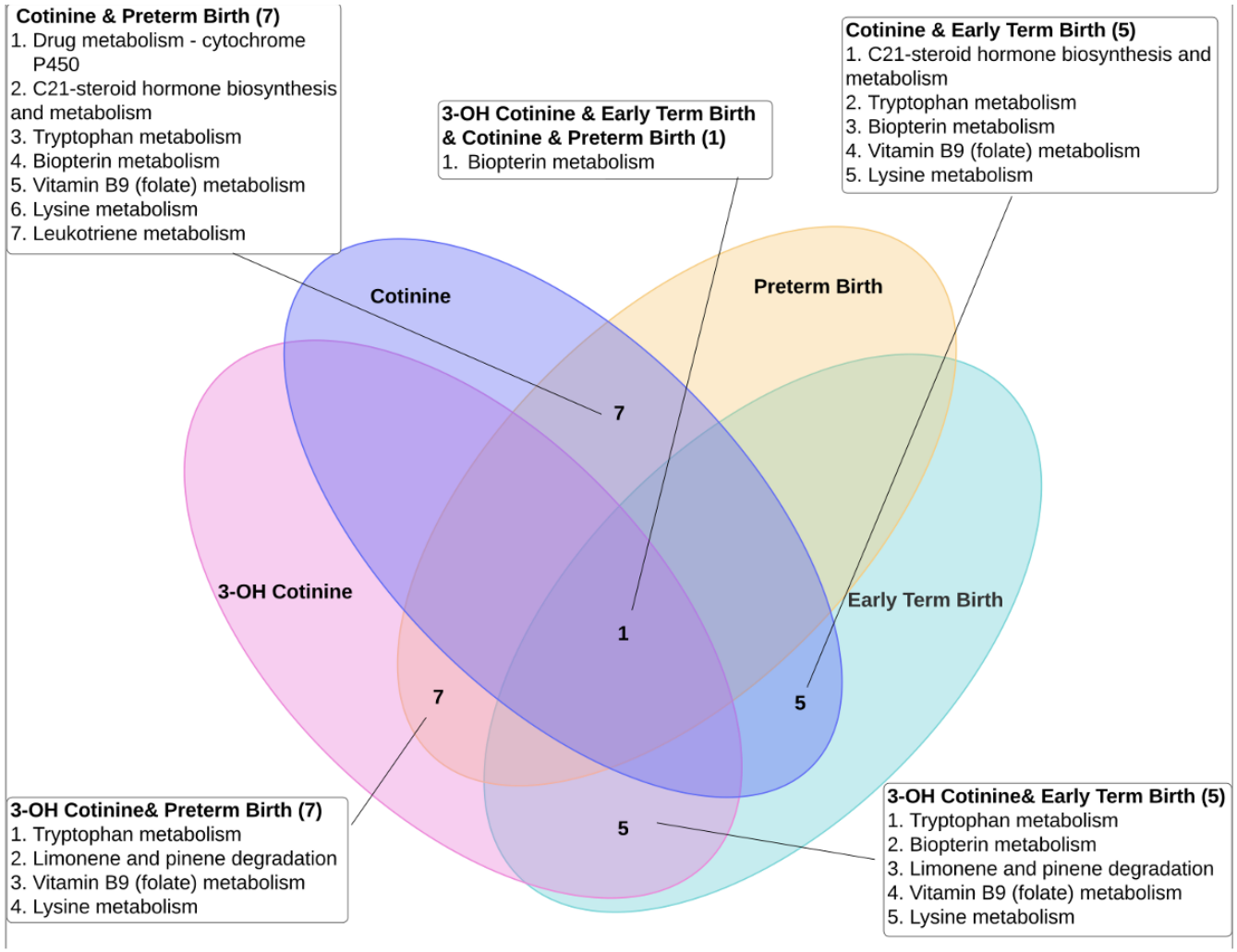

Figure 3. The Venn diagram of significant overlapped pathways

Note: The metabolic pathways associated with maternal cotinine and/or 3HC levels and adverse birth outcome were presented in Venn diagram.

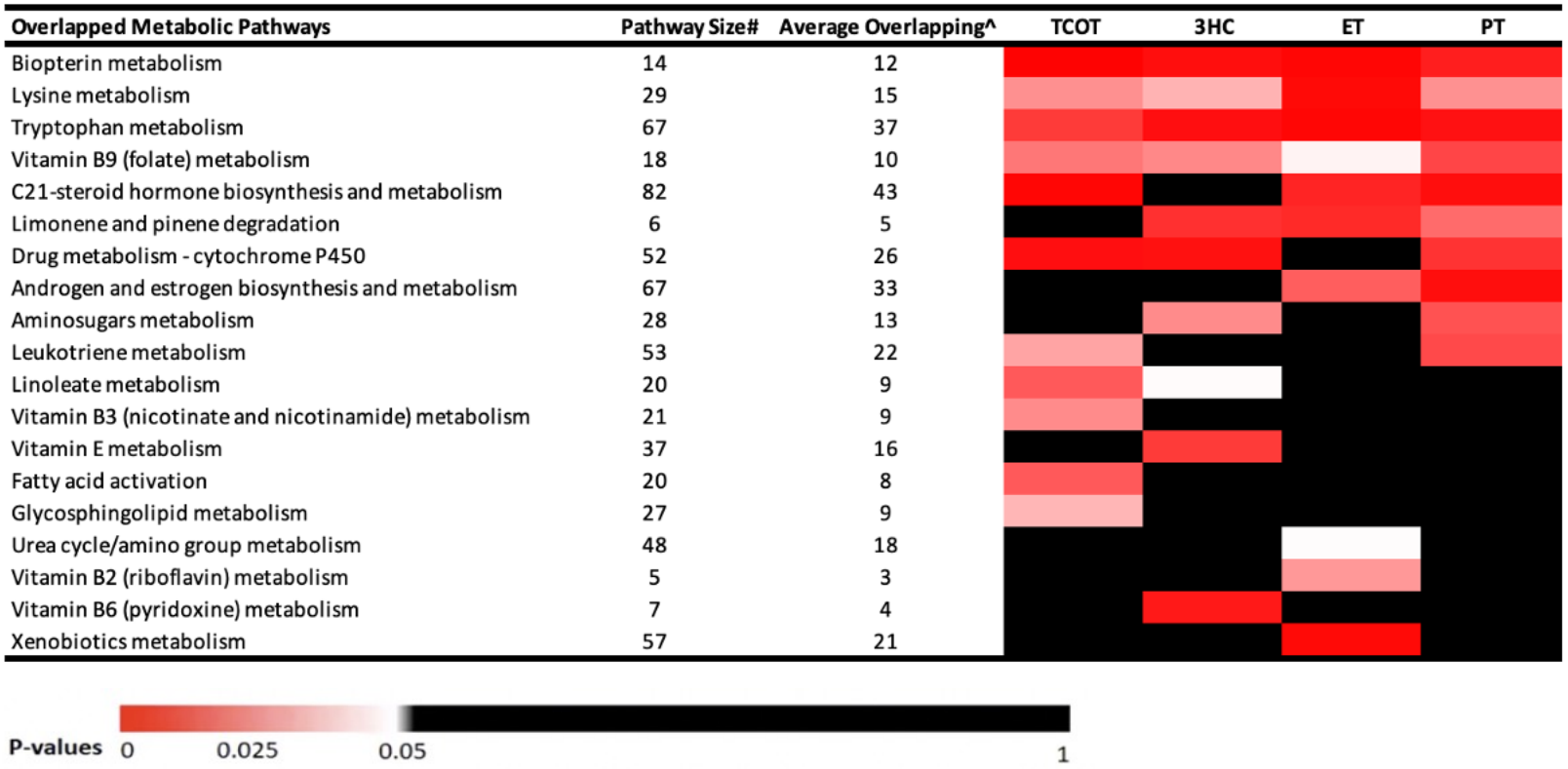

Figure 4. Heat map of overlapping metabolic pathways

Note: Cell color corresponds to the p-value for a metabolic pathway that overlaps in at cotinine and/or 3HC levels and at least one adverse birth outcome. The reference group was healthy full-term births. The average overlapping^ are the average numbers of significant putative metabolites enriched in the overlapping pathways and associated an exposure and/or outcome. The metabolic pathways are grouped by amino acids, enzymes, coenzymes, and cofactors, and bioactive lipids.

Abbreviations: TCOT: total cotinine concentration in urine samples; 3HC: total trans-3'-Hydroxycotinine in urine sample; ET: early term birth (≥ 37 and < 39 weeks); PT: preterm birth (>20 and < 37 weeks). # The pathway size is total number of features in the specific metabolic pathway across the significant metabolism-exposure or metabolism-birth outcomes associations. ^The average number of metabolic features with m/z matched within the specific metabolic pathway across the significant metabolism-exposure or metabolism-birth outcomes associations

Supplemental Tables

Table S1: Compare the self-reported tobacco use with the level of detected biomarker

Detection status / Self-reported status	Non-detected	Detected
Not used tobacco last month	9	226
Used tobacco last month (n=14)	1	44

Note: Columns are stratified by the cut off value of creatinine adjusted cotinine levels > 0 for differentiating non-smoking and smoking mother.

Table S2: Metabolic features detected in African American newborn dried blood spot samples and significantly associated with adverse birth outcomes under different cutoff p-values, the Atlanta African American Maternal-Child cohort (2016-2020)

Exposure	FDR 0.05	FDR 0.2	RAW p<0.0005	RAW p<0.005	RAW p<0.01	RAW p<0.05
Cotinine	7	108	31	147	229	648
3-hydroxycotinine	6	46	31	101	151	503
Early Term Birth	3	48	30	106	180	648
Preterm Birth	927	1738	467	846	1037	1747

Supplemental Tables

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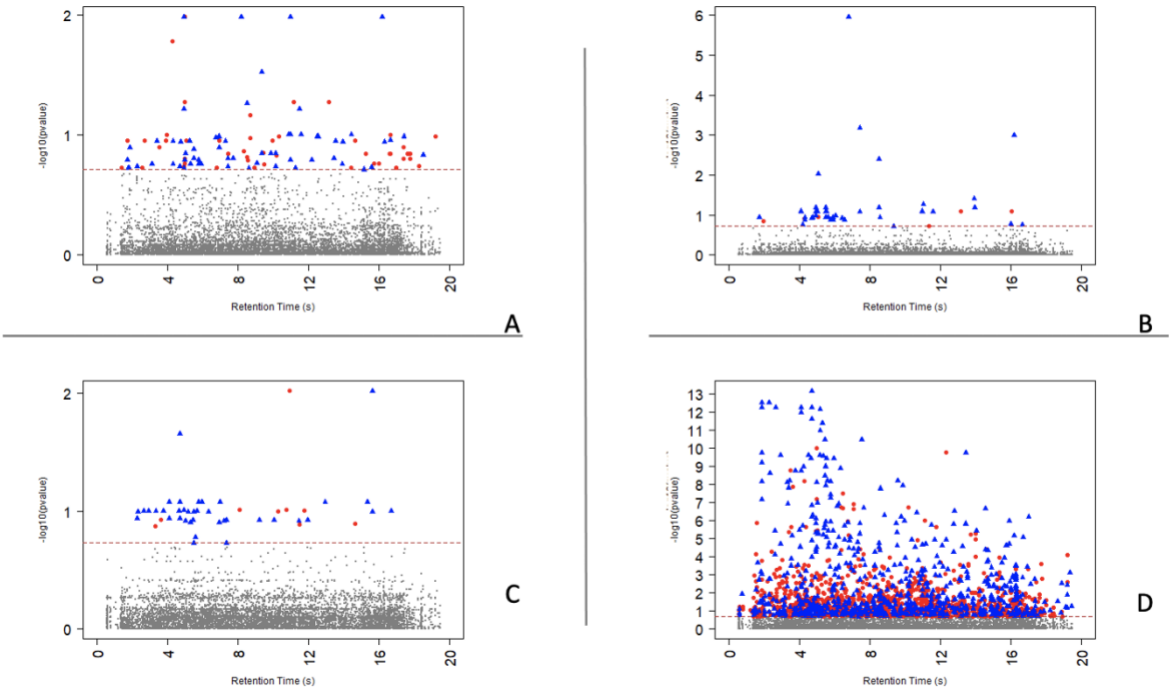
Note: Both false discovery rate (FDR) procedure and raw p-value were used to identify a reasonable number of significant metabolic features. Full-term birth is used as reference group to compare with early term and preterm birth.

Supplement Figures

Figure S1: Manhattan plots of significant metabolic features (FDR < 0.2)



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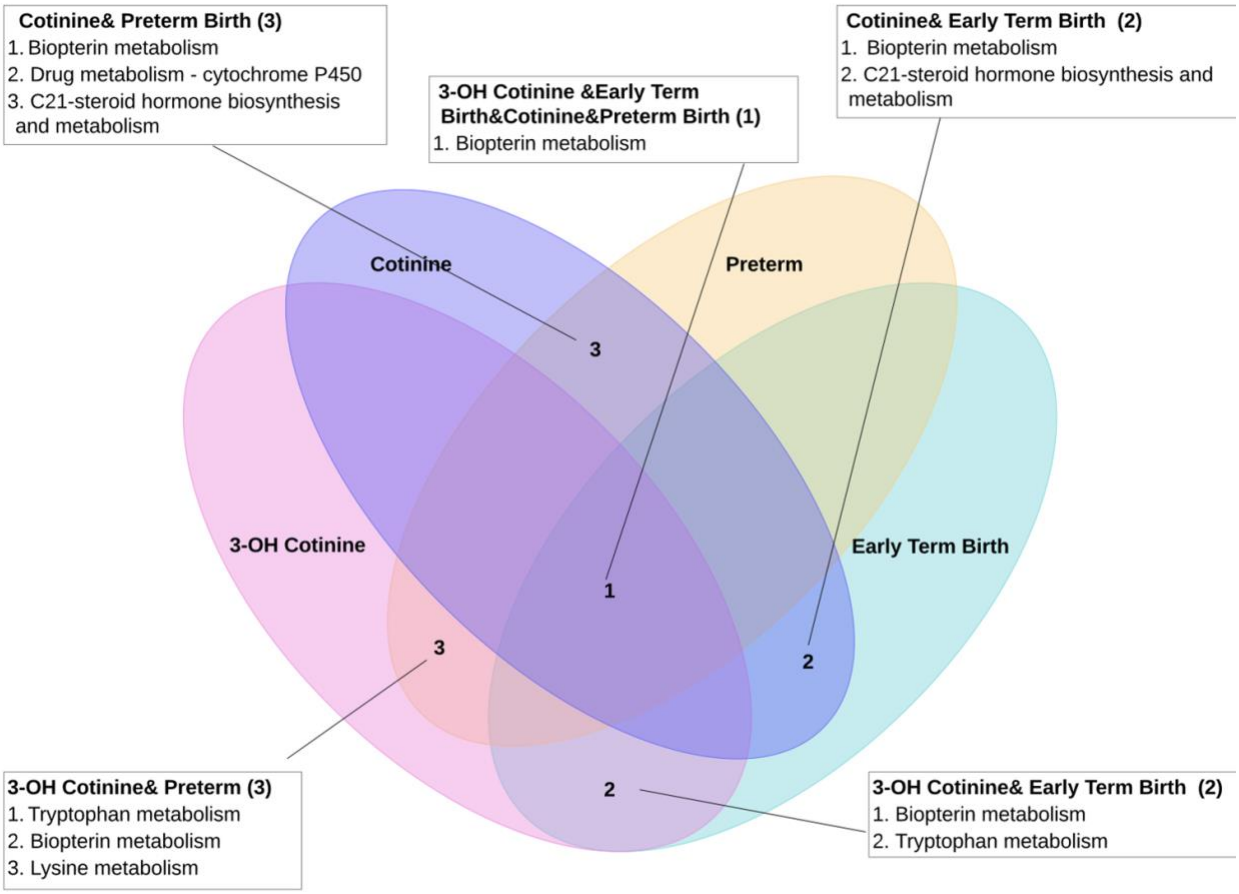
A: Manhattan plots of associations between changes in metabolic feature intensities with cotinine level

B: Manhattan plots of associations between changes in metabolic feature intensities with 3HC level

C: Manhattan plots of associations between changes in metabolic feature intensities with early-term birth outcome.

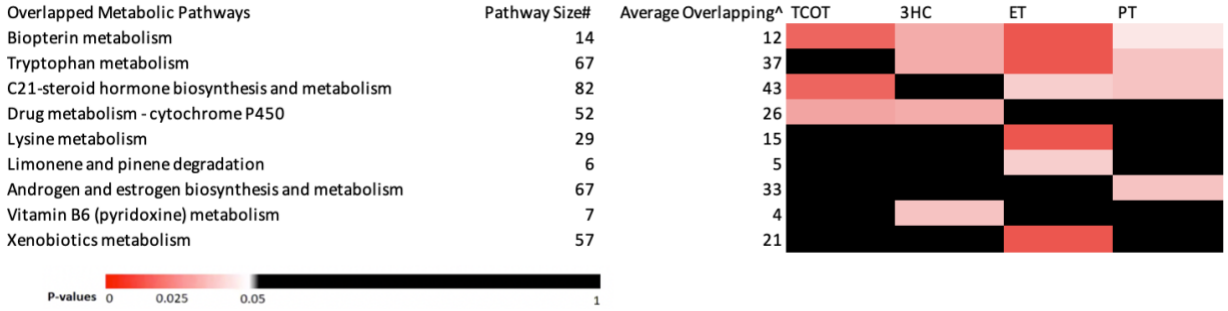
D: Manhattan plots of associations between changes in metabolic feature intensities with pre-term birth outcome

Figure S2: The Venn diagram of significant overlapped pathways (FDR < 0.2)



Note: The metabolic pathways associated with maternal cotinine and/or 3HC levels and adverse birth outcome were presented in Venn diagram.

Figure S3: Heat map of overlapping metabolic pathways (FDR<0.2)



Note: Heat map of overlapping metabolic pathways. Cell color corresponds to the p-value for a metabolic pathway that overlaps in at cotinine and/or 3HC levels and at least one adverse birth outcome. The reference group was healthy full-term births. The average overlapping^ are the average numbers of significant putative metabolites enriched in the overlapping pathways and associated an exposure and/or outcome. The metabolic pathways are grouped by amino acids, enzymes, coenzymes, and cofactors, and bioactive lipids.

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