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Investigation of biological effects from ambient temperature exposure on preterm birth in the Atlanta African American Maternal-Child Cohort using untargeted high-resolution metabolomics

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Abstract

Investigation of biological effects from ambient temperature exposure on preterm birth in the Atlanta African American Maternal-Child Cohort using untargeted high-resolution metabolomics

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Exposure to heat exposure in the era of climate change induced global warming has been associated with numerous adverse health outcomes especially birth outcomes. However, the underlying biological mechanisms leading to the adverse outcome are still unknown. In this study, we conduct metabolome- wide association studies to observe the overlapping metabolic pathways between ambient temperature exposure and preterm birth outcome in Atlanta African American cohort of 330 participants throughout their pregnancy to evaluate the maternal metabolome profile during early and late stage of pregnancy. This was assessed by utilizing untargeted HRM followed by pathway enrichment analysis, chemical annotation, and meet-in-the-middle approach to check the overlap between the pathways. A total of 13,616 and 11,600 metabolic features were identified in HILIC and C18 columns respectively. It was observed that 11 and 6 metabolic pathways were significantly associated with at least 2 temperature exposure window and preterm birth during early and late pregnancy respectively. Lysine metabolism, Methionine and cysteine metabolism, Vitamin B6 (pyridoxine) were the three pathways overlapping between Exposure during both stages of pregnancy and preterm birth. A total of 6 metabolites were associated with ambient temperature exposure were significant for at least 3 exposure windows. A total of 6 metabolites overlapped between preterm birth and ≥ 2 exposure windows including methionine and choline. The metabolites seen to have a significant association were linked with amino acid metabolism, lipid metabolism, Tryptophan metabolism, inflammatory pathways, and xenobiotics biosynthesis which can lead to adverse birth outcomes. The findings from this study will potentially be a groundwork for the future research to understand the underlying biological mechanisms of preterm birth and assess the role of ambient temperature exposure.

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Introduction

Due to climate change induced global warming, there has been a significant increase in the average heat index throughout the globe. Rising temperatures impact public health, primarily vulnerable populations, and are associated with increased mortality and morbidity. Extreme heat exposure is thought to be especially harmful in early life. A growing body of evidence suggests that heat is associated with increased risk of preterm birth, low birthweight, and stillbirth in the United States (Kuehn & McCormick, 2017). A recent systematic review conducted in the United States (Bekkar et al., 2020) concluded that a 5.6 °C increase in ambient temperature increased the rate of preterm birth by 11.6%, and among preterm births, birthweight decreased by 16 g per interquartile range increase in ambient temperature. While exposure to heat may be an important risk factor of adverse birth outcomes, the underlying biological mechanisms remain largely unknown.

With the advancement in high-throughput analytical platforms, various omics technologies have emerged as novel platforms to investigate the impact of heat exposure on animals and to elucidate the underlying mechanism. In a study of dairy cows, Min et al., used metabolomics to find that heat stress impacts lipid, carbohydrate, and protein metabolism, resulting in alternations in the endocrine and signaling proteins, while also exhibiting detrimental effects on the mammary glands, thus decreasing the milk production (Min et al., 2017). In a panel of human volunteers exposed to extreme heat in a sauna, Bouchama et al., observed heat responses in the human transcriptome, explaining deterioration in liver and renal functions due to cellular toxicity, the underlying mechanism of which include significant alteration in the cellular development, cellular growth and proliferation, cellular function, and maintenance (Bouchama et al., 2017). More recently, in a study on rainbow trout exposed to acute thermal stress, Roh et al., applied multi-

omics and found heat stress was associated with perturbations in biological pathways closely involved in protein processing in the endoplasmic reticulum and glycolysis activation (Roh et al., 2020).

A recent study conducted on rabbits reported that the changes in the hormonal secretion and microbial composition in the rabbit vagina were associated with perturbations in numerous metabolic pathways, including endocrine and other factor-regulated calcium reabsorption and Steroid biosynthesis. These perturbations may in turn lead to adverse birth outcomes. (Shi et al., 2022). Further, an earlier study of the impact of heat stress on three different species of cows revealed that under heat stress, 55 different metabolites were associated with the three breeds of cows. (Liao et al., 2018) These metabolites were involved in glycolysis, amino acid metabolism, fatty acid metabolism, the TCA cycle, and purine metabolism Additionally, serum and urine levels of citric acid, aconitic acid, and fumaric acid were significantly higher in one category of the cows. Another study by (Carter et al., 2019) demonstrated that many metabolites present in the preterm birth in humans have significant changes analogous to the metabolite changes in the cows which were impacted by heat stress. Specifically, among participants with preterm birth, alterations were found in the glycolysis pathway, lipid metabolism, amino acids pathways as well as TCA cycle. While not all the levels of metabolites are corelating, there is still a noticeable overlap.

The causes of preterm birth in humans are multifactorial, and common risk factors include physiological and pathological complications like vaginal and uterine infections, structural impairments like shortened cervix or uterine anomalies (Romero et al., 2014)alongside stress, pre-existing chronic conditions, as well as environmental exposures such as air pollution(Qian et al., 2016).

While there are studies reporting changes in the maternal plasma lipid metabolism during preterm birth outcomes(Chen et al., 2022), there is a wide knowledge gap to understand the impact of temperature exposure on the human metabolome. Hence, our study aims at investigating the changes in the maternal metabolome due to ambient temperature exposure in humans, as well as its correlation with the preterm birth outcomes. To address the current research gaps, we plan to utilize Metabolome-wide association study (MWAS) followed by untargeted high-resolution metabolomics to identify the metabolic changes associated with ambient temperature exposure and preterm birth outcomes in the Atlanta African American Maternal-Child Cohort. The results from this study provide a groundwork for future research to investigate biological mechanisms in the association of ambient temperature of preterm birth outcomes and can additionally facilitate the development the biomarkers related to ambient temperature risk on the minorities.

Methods

Study Population

The current analysis consisted of 330 pregnant people who self-identified as African American women and enrolled in the Atlanta African American Maternal-Child Cohort between 2014-2018. The inclusion criteria of the cohort are as follows: self-reported African American race, clinically verified singleton pregnancy with 8-14 weeks of gestation, age between 18-40 years, absence of any other chronic medical condition.

The details of the study cohort have been published previously (Brennan et al., 2019; Corwin et al., 2017). Briefly, the demographic details of the study participants included age, highest attained education, and socioeconomic status, which were collected during the first clinical visit followed

by an in-depth health survey. The data collected during the clinical visits had information about consumption of alcohol, tobacco and recreational drugs followed by collection of pregnancy specific data like parity, pre-pregnancy body mass index (BMI), gestational weeks, and fetal sex.

Questionnaire data

For the study we included information like maternal age, education, income-to-poverty ratio, prenatal health insurance type, marital and cohabiting status, and consumption of tobacco and/or marijuana and medical records abstraction for prevailing clinical conditions. Birth outcome data was collected from medical records post-delivery.

Exposure Assessment

Exposure to ambient temperature was assessed via daily maximum ambient temperature obtained from the monitoring station located at the Atlanta Hartsfield Jackson International Airport (station USW00013874) by extracting the data from National Oceanic and Atmospheric Administration. For the current analysis, the average temperature was calculated for each subject an(Li et al., 2013)d for 6 exposure windows: 12 weeks preconception to early/mid pregnancy (PC-V1), 12 weeks preconception to late pregnancy (PC-V2), conception to late pregnancy (C-V2), and preconception to conception (PC-C) for both the pregnancy stages. Date of conception and preconception was calculated by back tracking the date from gestation age to further estimate the season of conception and the related average daily max ambient temperature.

High-Resolution Metabolomics

The profiling of untargeted high-resolution metabolomics was conducted at the Emory Clinical Biomarker Laboratory utilizing an established protocol ((Chang et al., 2022). Precipitated proteins were obtained by mixing the serum samples with two sample volumes of ice-cold acetonitrile.

Further, the precipitated proteins were separated to obtain supernatant for which, the samples were incubated on ice for 30 minutes followed by centrifugation at 14,000g for 10 mins and was stored at 4°c for future analysis. (Johnson et al., 2010). Liquid chromatography and Fourier-transform high-resolution mass spectrometry (LC-HRMS) (Dionex Ultimate 3000, Thermo Scientific Q-Exactive HF) were used to analyze the extractants in triplicate.

The chromatography types used for the analyte separation were hydrophilic interaction liquid chromatography (HILIC) with positive electrospray ionization (ESI) and reverse phase (C18) chromatography with negative electrospray ionization. The detected signals (metabolic features) were extracted using apLCMS with modifications by xMSanalyzer. Peak detection, mass-to-charge ratio (m/z), retention time (RT) alignment, feature quantification, and data quality filtering were performed following these modifications(Uppal et al., 2013; Yu et al., 2009). For optimizing the data quality and filtering the noise signals, only metabolic features detected in more than 15% of the serum samples were included, with a median coefficient of variation (CV) among technical replicates of less than 30%, and a Pearson correlation coefficient (ρ) > 0.7. Individual features defined by m/z, retention time, and ion intensities were assessed in the resulting analytic data set. The replicated samples with at least one non-zero intensity were averaged perform, and $\log_2$ transformed to normalize the metabolomics data (Tan et al., 2022).

Statistical Analysis

Descriptive analyses were performed for the temperature data and maternal demographics data.

The associations between metabolic feature intensities and ambient temperature exposure and preterm birth outcomes (All types of preterm vs All types of full-term births) were evaluated by fitting multivariable linear regressions and logistic regressions (models 1 and 2), using a meet-in-the-middle approach. Specifically, the effects of ambient temperature on metabolomic signal

intensities were estimated with multiple linear regression models (model 1), which were adjusted for maternal sociodemographic, including maternal age of enrollment (in years), maternal education, infant sex, prenatal BMI (kg/m^2), parity, marital status, gestational age during both visits , prenatal alcohol marijuana and tobacco consumption, season of conception. Before performing MWAS for ambient temperature exposure during late pregnancy,142 occurrences where the sample collection dates during visit 2 were not recorder, were dropped.

The association between metabolic feature intensities and birth outcome was examined in model 2.

$$\log_2(\text{Intensity}) = \beta_0 + \beta_1 \log_2(\text{Average maximum ambient temperature}) + \beta_2(\text{Maternal Age at enrollment}) + \beta_3 \text{Education} + \beta_4 \text{Parity} + \beta_5 \text{BMI} + \beta_6 \text{Substance use} + \beta_7 \text{Birth weight} + \beta_8 \text{Sex} + \beta_9 \text{ Marital Status} + \beta_{10}(\text{Season of Conception}) + \beta_{11}(\text{Gestational age}) + \mathcal{E}\text{ij} \quad \ldots (1)$$

$$\text{Preterm birth} = \beta_0 + \beta_1 \log_2(\text{Intensity}) + \beta_2(\text{Maternal Age at enrollment}) + \beta_3 \text{Education} + \beta_4 \text{Parity} + \beta_5 \text{BMI} + \beta_6 \text{Substance use} + \beta_7 \text{Birth weigth} + \beta_8 \text{Sex} + \beta_9 \text{ Marital Status} + \beta_{10}(\text{Season of Conception}) + \beta_{11}(\text{Gestational age}) + \mathcal{E}\text{ij} \quad \ldots (2)$$

Here Intensity is the feature of each metabolite, β_0 is the intercept and β_{1-11} represent coefficients corresponding to each covariate that we adjusted. In this study, the covariates which could have potentially influenced the outcome like maternal age, education, parity, BMI, tobacco use, marijuana use, alcohol use, marital status, season of conception and gestational age (in weeks –

during visit 1 and visit 2) were controlled in the MWAS models. The models were performed for each metabolic feature. All analyses were performed in R.

The metabolome wide association study (MWAS) model was utilized to further evaluate the type of global metabolomics which are associated with both ambient temperature exposure and preterm birth using the meet-in-the-middle framework. The MWAS on ambient temperature exposure was conducted during early pregnancy for PC-C, C-V1, PC-V1 and late pregnancy for PC-C, C-V2 and PC-V2.

Pathway Enrichment Analysis

Mummichog (v1.0.10) was used to analyze the organization of metabolic pathways and network to predict its functionality without revealing the chemical identity(Li et al., 2013)Fischer's exact test on the null distribution was used to calculate the significance of metabolite pathways and further estimated by permutation where the features were randomly selected form the complete list of extracted metabolic features. The analysis was done for the features detected in early pregnancy as well as late pregnancy for PC-C, C-V1, PC-V1, C-V2 and PC-V2.

Metabolite Confirmation and Annotation

Metabolic features significantly associated with ambient temperature exposure and preterm birth, and enriched in a related pathway were annotated by matching mass m/z value to common adducts in METLIN, ChemSpider, Human Metabolome Database (HMDB), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, using a mass error threshold of 10 ppm. The extracted ion chromatographs (EICs) of each significant feature were visually examined to differentiate true peak from noise to minimize false positive discovery (determined by clear gaussian peak shapes and signal-to-noise ratio above 3:1) Finally, a select number of annotated metabolites were

confirmed with level-1 evidence (Sumner et al., 2007) by comparing their m/z, retention time, and ion dissociation patterns to analytical standards in an in-house library that contains a list of exogenous or endogenous metabolites analyzed under identical experimental conditions.

Meet-in-the-middle (MITM) Framework

To identify potential intermediate biological mechanisms and markers related to ambient temperature exposure and preterm birth, the MITM framework was leveraged (Chadeau-Hyam et al., 2011). For these analyses, we conducted separate pathway enrichment analysis and chemical annotation for each MWAS, and then searched if there were any common pathways or metabolites.

Results

In this study, we included 330 participants who completed metabolomics profiling during early pregnancy and 270 pregnant participants during late pregnancy. Among these participants, the average maternal age was 25 ± 4.8 years, with average prenatal BMI at 29 ± 7.6 kg/m^2. At least 15% of participants had graduate degrees and 48% of them were married. 45% of the participants reported prenatal consumption of alcohol, marijuana, or tobacco. About 27% of participants have been pregnant more than twice before. The average birth weight was 3100 ± 570 grams. About 20% (N=66) of the infants were born preterm. 21.8% (N=72) were conceived during fall, 25.2% (N=83) during spring, 32.4% (N=107) during summer and 20.6% (N=68) during winter (**Table 1**). Further, the average daily maximum temperature across 330 participants during Visit 1 from Preconception to conception period was 75±11 °F, Conception to visit 1 was 76±11 °F and 75±8.7 °F during pre-conception to visit 1. Similarly, in late pregnancy assessment, 270 participants were

exposed to 75 ±12 °F during preconception to conception period, 75±7.1 °F from conception to visit 2 and 75±3.7 °F from preconception to visit 2 (**Table 2**).

MWAS

After implementing QA/QC measures outlined in the Methods, 13,616 and 11,900 features were extracted from HILIC positive ESI and C18 negative ESI, respectively. In early pregnancy assessment, for HILIC (p value <0.05), 678 metabolic features were found to have a significant association with ambient temperature exposure during PC-C period, 727 during C-V1, and 709 features were found to be significantly associated during PC-V1. Similarly, for C-18 (p-value<0.05), PC-C period had 688 significantly associated features, 494 features during C-V1 and 898 features were significantly associated with the outcome during PC-V1 after adjusting for all the covariates. During late pregnancy, a higher number of significant metabolic features were observed. In HILIC, 1274, 1173, and 894 features were associated with temperature from PC-C, from C-V2, and from PC-V2, respectively (p value < 0.05). In C18, 962, 1088, and 836 were associated with temperature from PC-C, from C-V2, and from PC-V2, respectively (p value < 0.05).

A total of 1,126 metabolic features were identified during MWAS evaluation of the birth outcomes (early–676, late- 550; p value<0.05) for HILIC and during C18, a total of 1074 metabolic features were identified (early-605, late-469; p value<0.05) (**Table 3**)

Pathway Enrichment Analysis

In the early pregnancy metabolomic profile, there were 11 pathways, including Lysine Metabolism, Tryptophan metabolism, and Cysteine metabolism were significantly associated with at least 2 temperature exposure window and preterm birth (**Figure 1a**). Similarly, 6 pathways,

including Glycerophospholipid, Lysine metabolism, and TCA cycle were significantly associated with at least 2 exposure windows and preterm birth among features detected in the late pregnancy (**Figure 1b**). Lysine metabolism, Methionine and cysteine metabolism, Vitamin B6 (pyridoxine) were the three pathways overlapping between exposure during both visits and preterm birth (**Figure 2**).

Metabolite Confirmation and Annotation

A total of 6 metabolites were associated with ambient temperature exposure were significant for at least 3 exposure windows (p-value<0.05) (**Table 4**). These confirmed metabolites were involved in amino acid metabolism and lipid metabolism. 51 metabolites were associated with two ambient temperature exposure windows, most of which were involved in the amino acid metabolism, TCA cycle, lipid metabolism, inflammation and oxidation related pathways, biosynthesis of secondary metabolites and xenobiotics biodegradation and metabolism (**Table 5**). These results were derived from both positive and negative ESI. A total of 40 metabolites with level-1 confidence were identified as significantly associated with preterm birth (**Table 6**). These metabolites included cysteine, taurine, methionine which are closely involved in the oxidative stress-related amino acid metabolism.

A total of 6 metabolites overlapped between preterm birth and ≥ 2 exposure windows. Specifically, methionine was found to be associated with ≥ 3 exposures and preterm birth. Other common metabolites identified to be associated with 2 temperature exposure windows and preterm birth outcome were choline, phthalic anhydride, indole-3-acetate, tryptophan and itaconate.

Discussion

In the study we observed a significant number of metabolites which demonstrate an association between heat and preterm birth as well as several common over lapping pathways between early and late pregnancy in the Atlanta African American Maternal-Child Cohort, utilizing untargeted high-resolution metabolomics. In addition, a considerable number of metabolic changes were detected in relation to both premature birth and exposure to high temperatures. These metabolites were closely associated with amino acid metabolism, lipid metabolism, Tryptophan metabolism, inflammatory pathways, and xenobiotics biosynthesis. The findings from this study will be beneficial to comprehend various biological processes associated with preterm birth outcome resulting from prenatal exposure to high ambient temperatures.

Maternal Metabolomic Signatures in Early/Mid Pregnancy

Metabolic pathways significantly associated with temperature exposure identified during MWAS in early/mid pregnancy included Aspartate & Asparagine metabolism, Glycine, Serine, Alanine & Threonine metabolism, Glycoxylate & dicarboxylate, Tryptophan metabolism, Butanoate metabolism, Sialic acid metabolism, Tyrosine metabolism urea cycle and amino acid group metabolism and vitamin D3 metabolism.

Vitamin D3 is a fat-soluble vitamin which maintains calcium homeostasis and bone health in the body. Deficiency of Vitamin D3 has been linked to adverse birth outcomes especially preterm birth with and increased risk of preeclampsia(Woo et al., 2019). African American population is at a highest risk of Vitamin D3(cholecalciferol) deficiency(Ames et al., 2021).The metabolic pathways identify that there is Vitamin D3 perturbation especially during preconception phase which may further enhance the impact in our cohort which is already susceptible to deficiency. Further, in a

study by(Parris et al., 2021) shows that butyrate from butanoate metabolism plays a role in the inflammatory pathway leading to chorioamnionitis resulting in preterm birth(Prince et al., 2016).

Maternal Metabolomic Signatures in Mid/Late Pregnancy

During the late pregnancy MWAS, the identified metabolic pathways significantly associated with temperature exposure involved Glycerophospholipid metabolism, Cytochrome 450 metabolism, Histidine metabolism, TCA cycle perturbation, Glutamate, Leukotriene metabolism, and vitamin B5 biosynthesis. In a recent study conducted by(Hong et al., 2023) it was observed that lipidomics revealed a significant change in the sphingolipids metabolism in the cervicovaginal fluid during mid pregnancy resulting in preterm birth which can further draw down to the involvement of lipid metabolism in the preterm birth outcome.

Maternal Metabolomic Signatures Across Pregnancy

The pathways associated with both ambient temperature exposure and preterm birth during early as well as late pregnancy include lysine, methionine and cysteine metabolism which are constituents of the 5 essential amino acids. Methionine is a known antioxidant responsible for maintaining many physiological processes of the body.

These maternal amino acids are responsible for fetal protein synthesis and any disruption may lead to adverse birth outcomes. The cervico-vaginal fluid sampling in a cohort with preterm birth outcomes showed significant changes in the amino acid metabolism(Ghartey et al., 2015). The metabolic pathways were seen to be significantly expressed in the metabolomic profiles of maternal plasma during spontaneous birth outcome(Lizewska et al., 2018).

Another common pathway overlapping between exposure and outcome across pregnancy was lysine metabolism. Though there are not many animal or human studies reporting the impact on lysine metabolism by either temperature exposure or preterm birth outcome, some plant studies

suggest that under thermal stress the lysine levels were reduced in Soy products(Fontaine et al., 2007).

Confirmed and Annotated Metabolites

The involvement of amino acid and tryptophan metabolism was confirmed by identification and annotation of metabolites. Many animal studies show protein denaturation and the breakdown of amino acids due to presence of ambient temperature exposure. Further, increased temperature influences the hydrophobicity of the amino acid making the protein fold unstable and hence making the proteins dysfunctional(Wolfenden et al., 2015) which may in turn lead to adverse health outcomes. We also identified common metabolite such as methionine, which functions mainly as an antioxidant regulating the cellular mechanism(Levine et al., 2000) Changes in these molecules can lead to perturbations as shown in the study conducted by (Priante et al., 2022) which mentions the disruptions in the methionine-cysteine level in the infants with intra uterine growth retardation. Other perturbed metabolic pathways included lipid metabolism which was observed to be associated with both heat exposure and outcome. Similarly, a study conducted by (Virgiliou et al., 2017) states that during the second trimester, increased lipid levels in the maternal serum metabolome can be causative factor for preterm labor along with alterations in the other metabolic pathways including tryptophan and nicotinamide which are in line with our study. However, more research is needed to understand how ambient temperature exposure impacts tryptophan and nicotinamide metabolism in humans. There have been a few studies focused on the impact of thermal stress on the dietary intake of proteins, however the evaluation of the environmental ambient temperature exposure on the human protein and lipid metabolism still needs to be studied. While it’s imperative to evaluate how heat stress causes alterations in the metabolic pathway, ultimately resulting in preterm birth, there is a literature gap considering such studies in humans.

Strengths and Limitations

The scope of the study is limited due to its cross-sectional nature. Unlike outcome, the exposure is assessed for three exposure windows during both the pregnancy stages. Residual confounding could be observed in the models due to influence of potential confounders like diet, which is not assessed or controlled for in our study model. Further, the serum samples collected were non-fasting, this may potentially lead to some perturbations Though we have controlled for season, there is still a gap in the information regarding the migration of the participants raising the question if the subjects were present in their mentioned location throughout their pregnancy. Also, it would be further interesting to consider the indoor house temperature that these women were subjected to. More in depth studies are necessary to further evaluate the impact of these individual metabolites on the outcome as well as the mechanism behind these perturbations.

This study effectively serves as a bridge to address the knowledge gap surrounding the impact of heat exposure on humans, which has not been previously investigated. The study evaluates the outcome in a racially minor group, bolstering the representation of involvement of the African American community in the research. However, further research is needed to understand the impacts in the other groups of communities, as they may genetically vary which might be a potential effect mediator in these analysis (Duello et al., 2021).

Conclusion

The findings from our study suggest that hotter temperatures during the periconception period have a significant impact on perturbations in the serum metabolome of the African American cohort in Atlanta. The pathways involved mainly included Amino acid and lipid metabolism. Many

additional pathways were also identified, however additional research is needed to understand the association between the perturbations of these pathways and the preterm birth.

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Tables and Figures

Table 1. Characteristics of participants by maternal metabolome analytic sample in the Atlanta African American Maternal-Child Cohort, (2014-2018).

Characteristic	Maternal Metabolome	
	Early/Mid Pregnancy	**Mid/Late Pregnancy**
N	330	270
Maternal age at enrollment (years)		
Mean (SD)	25 (4.8)	25 (4.8)
Prenatal body mass index (kg/m^2)		
Underweight	11 (3.3%)	3 (3.3%)
Normal weight	126 (38.2%)	101 (37.4%)
Overweight	74 (22.4%)	58 (21.5%)
Obese	119 (36.1%)	102 (37.8%)
Prenatal BMI		
Mean (SD)	29 (7.6)	29 (7.7)
Maternal education		
Less than high school	54 (16.4%)	38 (14.1%)
High school	124 (37.6%)	105 (38.9%)
Some college	100 (30.3%)	82 (30.4%)
College graduate or above	52 (15.8%)	45 (16.7%)
Fetal sex		
Male	165 (50.0%)	136 (50.4%)
Female	165 (50.0%)	134 (49.6%)
Parity		
0	149 (45.2%)	114 (42.2%)
1	90 (27.3%)	81 (30.0%)
+2	91 (27.6%)	75 (27.8%)
Marital status		
Married or Living Together	158 (47.9%)	131 (48.5%)
Single	172 (52.1%)	139 (51.5%)
Prenatal alcohol, marijuana, or tobacco use		
No	178 (53.9%)	148 (54.8%)
Yes	152 (46.1%)	122 (45.2%)
Season of conception		
Fall	72 (21.8%)	53 (19.6%)
Spring	83 (25.2%)	67 (24.8%)
Summer	107 (32.4%)	89 (33.0%)
Winter	68 (20.6%)	61 (22.6%)
Gestational age at sample collection (weeks)		
Mean (SD)	11 (2.2)	27 (2.6)
Gestational age at delivery (weeks)		
Mean (SD)	38 (3.1)	39 (2.2)
Preterm birth		
No	210 (63.6%)	285 (68.5%)
Yes	66 (20.0%)	42 (15.6%)

Missing	54 (16.4%)	43 (15.9%)
Birth weight (g)		
Mean (SD)	3000 (690)	3100 (570)

Abbreviations: BMI=Body Mass Index. The total number of study participants were 330 during early pregnancy, out of which 270 were involved in the study during late pregnancy. Early pregnancy- visit 1 serum sample collection, Late pregnancy- visit 2 sample collection. Season of conception was calculated backwards from

Table 2. Average daily maximum ambient temperature at Atlanta Hartsfield Jackson Airport, 2014-2018

Temperature Exposure Window	**2014**	**2015**	**2016**	**2017**	**2018**	**Overall**
	Early/Mid Pregnancy, Mean (SD)					
N	61	94	78	78	19	330
PC-C	74 (11)	74 (12)	78 (12)	76 (9.5)	69 (11)	75 (11)
C-V1	83 (5.5)	77 (11)	75 (12)	75 (9.7)	60 (4.2)	76 (11)
PC-V1	78 (7.0)	75 (9.3)	77 (10)	75 (6.3)	64 (4.1)	75 (8.7)
	Mid/Late Pregnancy, Mean (SD)					
N	26	81	78	67	18	270
PC-C	65 (7.9)	71 (11)	77 (10)	80 (11)	72 (12)	75 (12)
C-V2	82 (2.5)	75 (7.6)	74 (7.2)	74 (5.9)	71 (6.8)	75 (7.1)
PC-V2	76 (2.2)	74 (3.7)	75 (4.2)	76 (2.9)	72 (2.7)	75 (3.7)

Abbreviations: PC- Preconception, C- Conception, V1- Visit 1 of serum sample collection, V2- Visit 2 of serum sample collection. All the temperature values are in °Fahrenheit.

Table 3. Metabolic features in the maternal metabolome by temperature exposure window, Atlanta African American Maternal-Child Cohort, 2014-2018

	HILIC				**C18**			
Maternal Metabolome	**PC-C**	**C-V**	**PC-V**	**PTB**	**PC-C**	**C-V**	**PC-V**	**PTB**
Early/mid pregnancy	678	727	709	676	688	494	898	605
Mid/late pregnancy	1274	1173	894	550	962	1088	836	469

Note: Abbreviations: PC- Preconception, C- Conception, V1- Visit 1 of serum sample collection, V2- Visit 2 of serum sample collection, PTB- preterm birth. pvalue<0.05 and Benjamini-Hochberg false discovery rate (FDR) was used to identify the significant number of metabolic features.

Table 4. Confirmed metabolites significantly associated with ≥3 temperature exposure windows.

Temperature Exposure Window	Metabolite	Pathway	Super Pathway	m/z	RT	Adduct
PC-C [a], PC-V1, C-V2, PC-V2	Chenodeoxycholate[d]	Primary bile acid biosynthesis	Lipid metabolism	375.2896	28.4	M-H2O+H
PC-C [a], PC-C1, C-V2, PC-V2	3alpha,12alpha-dihydroxy-5beta-cholanate[d]	Secondary bile acid synthesis	Lipid Metabolism	375.2896	28.4	M-H2O+H
PC-C[b], PC-V1, PC-V2	L-Methionine[d]	Cysteine and Methionine Metabolism	Amino Acid metabolism	150.05885	53	M+H
PC-C[b], PC-V1, PC-V2	Methionine[d]	Cysteine and Methionine Metabolism	Amino Acid metabolism	150.0583	53	M+H
C-V1, C-V2, PC-V2	Palmitoyl carnitine[d]	Fatty Acid metabolism	Lipid Metabolism	400.3415	22.3	M+H
PC-C[b], PC-V1, C-V2, PC-V2	3-alpha,11-beta,17-alpha,21-tetra hydroxy- 5-alpha-pregnan-20-one[e]	Steroid metabolism	Lipid metabolism	401.21	64.8	M+Cl

Abbreviations: PC- Preconception, C- Conception, V1- Visit 1 of serum sample collection, V2- Visit 2 of serum sample collection, m/z- sass to charge ratio, RT- retention time.

[a] During both the pregnancy timepoints(early and late).

[b] Only during early pregnancy.

[c] Only during late pregnancy.

The metabolic features were identified and confirmed by comparing their peaks based on accurate mass to charge ratio and retention time with the baseline reference standards utilizing tandem mass spectrometry performed under identical conditions.

pvalue<0.05 was used to identify the metabolic features.

Table 5. Metabolites associated with 2 temperature exposure windows.

Exposure window	Identified Metabolite	Pathways	Metabolism	m/z	RT(s)	Adduct
HILIC						
C-V2, PC-C[c]	Formylglycine	Unknown	Amino acid metabolism	104.0342	40	M+H
C-V2, PC-V2	Choline	Glycine, serine, alanine, and threonine metabolism & Glycerophospholipid metabolism	Amino Acid and Lipid Metabolism	104.10754	38	M+
C-V2, C-V1	Maleamate	Tropane, piperidine and pyridine alkaloid biosynthesis & Metabolism of xenobiotics by cytochrome P450	Biosynthesis of other secondary metabolites & Xenobiotics biodegradation and metabolism	116.03474	88	M+H
C-V2, PC-V2	Glycine	serine, threonine metabolism & Glycerophospholipid metabolism	Amino Acid and Lipid Metabolism	120.0032	75	M+2Na-H
C-V2, PC-V2	Nicotinamide	Tropane, piperidine and pyridine alkaloid biosynthesis & Metabolism of xenobiotics by cytochrome P450	Biosynthesis of other secondary metabolites & Xenobiotics biodegradation and metabolism	123.05581	36.5	M+H
C-V2, PC-V2	Nicotinamide(b3)	Tropane, piperidine and pyridine alkaloid biosynthesis & Metabolism of xenobiotics by cytochrome P450	Biosynthesis of other secondary metabolites & Xenobiotics biodegradation and metabolism	123.0553	37	M+H
C-V2, PC-C[c]	Creatine	Arginine and Proline metabolism	Amino acid metabolism	132.07728	71.7	M+H

C-V1, PC-V1	N,n-dimethyl-1,4-phenylenediamine	Unknown	Unknown	137.10785	31.1	M+H
C-V1, PC-V1	Dimethyl phenylenediamine	Unknown	Unknown	137.1073	31	M+H
PC-C[b], PC-V1	L-lysine	Lysine degradation	Amino acid metabolism, Lysine metabolism	147.11333	105	M+H
C-V1, PC-C[c]	Phthalic anhydride	Organic compound used by chemical industries to make polymers	Metabolism of xenobiotics by cytochrome P450	149.0233	30	M+H
PC-C[c], PC-V2	Succinate	Oxidative phosphorylation, TCA Cycle, Alanine, aspartate and glutamate metabolism, Lysine degradation	Energy metabolism, ATP synthesis	162.9979	33.7	M+2Na-H
C-V2, PC-V2	N-amidino-l-aspartate			176.06711	74	M+H
C-V2, PC-V2	Indole-3-acetate		Tryptophan metabolism	176.07113	28.8	M+H
C-V2, PC-V2	Indole-3-acetic acid		Tryptophan metabolism	176.07113	29.3	M+H
C-V2, PC-V2	Guanidinosuccinate	Arginine biosynthesis	Arginine metabolism	176.0666	74	M+H
PC-V1, PC- C[c]	Formyl-l-methionyl peptide	Methionine metabolism	Amino acid metabolism	178.05377	33.6	M+H
PC- V1, PC- C[c]	Formyl methionyl peptide	Methionine metabolism	Amino acid metabolism	178.0532	34	M+H
PC-V1, PC-C[b]	Methyl vanillate	Organic compound used for fragrances	Metabolism of xenobiotics by cytochrome P450	183.06571	29.5	M+H
PC-V1, C-V1	10-hydroxydecanoate	Fatty acid metabolism	Lipid metabolism	189.14905	28.7	M+H
C-V2, PC-C[c]	L-tryptophan	Glycine, serine and threonine metabolism/Tryptophan metabolism/Phenyl alanine, tyrosine	Amino acid metabolism & carbohydrate metabolism	205.09768	47	M+H

		and tryptophan biosynthesis				
C-V2, PC-C[c]	D-tryptophan	Glycine, serine and threonine metabolism/Tryptophan metabolism/Phenylalanine, tyrosine and tryptophan biosynthesis	Amino acid metabolism & carbohydrate metabolism	205.09768	47.9	M+H
C-V2, PC-V2	Omega-hydroxydodecanoic acid	Fatty acid metabolism	Lipid metabolism	217.1804	27.7	M+H
C-V2, PC-C c	5-hydroxy-l-tryptophan	Tryptophan metabolism	Amino acid metabolism	221.09259	58.5	M+H
C-V2, PC-C c	5-hydroxytryptophan	Tryptophan Metabolism	Amino acid metabolism	221.0921	59	M+H
C-V1, PC-V1	L-cystathionine	Cysteine and methionine metabolism	Amino acid metabolism	223.07523	197.4	M+H
C-V1, PC-V1	Cystathionine	Cysteine and methionine metabolism	Amino acid metabolism	223.0752	197	M+H
C-V2, PC-V2	N-acetyl-d-tryptophan	Tryptophan metabolism	Amino acid metabolism	247.10824	30	M+H
C-V2, PC-V2	Acetyltryptophan	Tryptophan metabolism	Amino acid metabolism	247.108	30	M+H
PC-V1, PC-C b	1-methyladenosine	Purine Metabolism		282.12021	40.5	M+H
C-V 2, PC-C c	Xanthosine	Purine Metabolism		285.08349	50.6	M+H
C-V1, PC-V1	Aldosterone	Steroid metabolism	Lipid metabolism	361.201	27	M+H
PC-C b, PC-V1	Lysopc(14:0)	Fatty Acid metabolism	Lipid metabolism	468.3085	42	M+H
C18						
C-V2, PC-V2	2-hydroxybutyric acid	Propanoate metabolism/Fatty acid metabolism	Lipid metabolism	103.03954	22.8	M-H

C-V2, PC-V2	Alpha-hydroxyisobutyric acid	Propanoate metabolism/Fatty acid metabolism	Lipid metabolism	103.03955	24.1	M-H
C-V2, PC-V2	3-hydroxybutanoic acid	Propanoate metabolism/Fatty acid metabolism	Lipid metabolism	103.03955	22.8	M-H
PC-C[b], PC-V1	2-methylmaleate	Tropane, piperidine and pyridine alkaloid biosynthesis & Metabolism of xenobiotics by cytochrome P450	Biosynthesis of other secondary metabolites & Xenobiotics biodegradation and metabolism	129.01881	21.6	M-H
PC-C[b], PC-V1	Itaconate	C5-Branched dibasic acid metabolism (TCA cycle)		129.01881	22.2	M-H
PC-C[b], C-V2	(R,r)-tartaric acid	Glyoxylate and dicarboxylate metabolism	Carbohydrate metabolism	149.00864	20.5	M-H
PC-C[b], C-V2	(S,s)-tartaric acid	Glyoxylate and dicarboxylate metabolism	Carbohydrate metabolism	149.00864	20.4	M-H
C-V2, PC-V2	N-alpha-acetyl-l-asparagine	Asparagine metabolism	Amino acid metabolism	173.05626	19.9	M-H
C-V2, PC-V2	Omega-hydroxydodecanoic acid	Fatty acid metabolism	Lipid metabolism	215.16475	59	M-H
C-V2, PC-C[c]	Biotin	Biotin metabolism	Metabolism of cofactors and vitamins	243.08037	24.6	M-H
C-V2, PC-V2	Retinoate	Retinol metabolism	Metabolism of cofactors and vitamins	299.20113	225.6	M-H

C-V2, V2	PC-	Retinoic acid	Retinol metabolism	Metabolism of cofactors and vitamins	299.2017	226	M-H
C-V2, V2	PC-	Fa20:5(eicosapentaenoic acid)	Biosynthesis of unsaturated fatty acids	Lipid Metabolism	301.2173	224	M-H
C-V2, V2	PC-	Rac-glycerol 1-myristate	Used as a surfactant	Xenobiotics biodegradation and metabolism	301.23791	226.2	M-H
C-V2, V2	PC-	Fa22:5 n-3 or n-5 (Docosapentaenoic acid)	Fatty Acid metabolism	Lipid metabolism	329.2486	240	M-H
C-V2, V2	PC-	Fa22:4 (docosatetraenoic acid)	Biosynthesis of unsaturated fatty acids	Lipid metabolism	331.2642	258	M-H
C-V2, PC-C[c]		Uridine 5'-diphosphate	Pyrimidine metabolism/Zeatin biosynthesis/ Nucleotide metabolism	Pyrimidine ribonucleotide biosynthesis	402.994924	18.6	M-H
C-V1, V1	PC-	Uridine 5'-diphosphoglucuronic acid	Nucleotide metabolism		579.026502	17.8	M-H

Note: Abbreviations: PC- Preconception, C- Conception, V1- Visit 1 of serum sample collection, V2- Visit 2 of serum sample collection, m/z- sass to charge ratio, RT- retention time.

[a] During both the pregnancy timepoints(early and late).

[b] Only during early pregnancy.

[d] identified and confirmed in HILIC(ESI+) column

[e] identified and confirmed in C18(ESI-) column

The metabolic features were identified and confirmed by comparing their peaks based on accurate mass to charge ratio and retention time with the baseline reference standards utilizing tandem mass spectrometry performed under identical conditions.

pvalue<0.05 was used to identify the metabolic features.

Table 6. Metabolites associated with Preterm Birth Outcome

Pregnancy stage	Metabolite	Pathways	m/z	RT(s)	Adduct	Column
Early	2-oxobutyrate	Glycine, serine and threonine metabolism/Cysteine and methionine metabolism/ Valine, leucine and isoleucine biosynthesis	101.0246	22.5	M-H	C18
Early	Choline	Glycerophospholipid metabolism	104.1072	37.7	M+	HILIC
Early	(L)-valine	Valine, leucine and isoleucine degradation	116.0718	24.7	M-H	C18
Early	5-aminopentanoate	Lysine degradation/Arginine and proline metabolism			M-H	C18
Early	Taurine	Primary bile acid biosynthesis/Taurine and hypotaurine metabolism	126.022	57.7	M+H	HILIC
Late	5-oxo-d-proline	D-Amino acid metabolism	128.0355	20.9	M-H	C18
Early/Late	Itaconatea	C5-Branched dibasic acid metabolism (TCA cycle)	129.0195	22.1	M-H	C18
Early	4-methyl-2-oxovaleric acid	Valine, leucine and isoleucine degradation	129.0558	21.1	M-H	C18
Early	Leucine	Valine, leucine and isoleucine degradation	130.0874	23.9	M-H	C18
Early	N-acetylalanine	Alanine, aspartate and glutamate metabolism	132.0656	65	M+H	HILIC
Early	Hydroxyproline	Arginine and proline metabolism			M+H	HILIC
Late	(L)-aspartate	Arginine biosynthesis/Alanine, aspartate and glutamate metabolism/Glycine, serine and threonine metabolism	134.0448	69.4	M+H	HILIC
Late	Hypoxanthine	Purine metabolism	135.0304	19.7	M-H	C18
Late	Caprylic acid	Fatty acid biosynthesis	143.1077	25.4	M-H	C18
Late	Phthalic anhydride	Metabolism of xenobiotics by cytochrome P450	149.0232	22.3	M+H	HILIC
Early	(L)-methionine	Cysteine and methionine metabolism	150.0583	47.1	M+H	HILIC
Early	2,3-dihydroxybenzoate	Benzoate degradation/Xenobiotics biodegradation and metabolism	153.0193	21.6	M-H	C18

Early	Xanthine	Purine metabolism	153.0396	115.1	M+H	HILIC
Late	L-phenylalanine	Phenylalanine metabolism/Phenylalanine, tyrosine and tryptophan biosynthesis	164.0716	25	M-H	C18
Early	Phenylpyruvate	Phenylalanine metabolism	165.0546	49.7	M+H	HILIC
Late	Urate	Purine metabolism	167.0208	18.8	M-H	C18
Late	Citrulline	Arginine biosynthesis	174.0885	20.9	M-H	C18
Early	Vitamin C	Ascorbate and aldarate metabolism	175.0248	18.7	M-H	C18
Early	D-glucuronolactone	Ascorbate and aldarate metabolism			M-H	C18
Early	(L)-tyrosine	Tyrosine metabolism/Phenylalanine metabolism/Ubiquinone and other terpenoid-quinone biosynthesis	180.0665	23.3	M-H	C18
Early	(L)-tyrosine	Tyrosine metabolism/Phenylalanine metabolism/Ubiquinone and other terpenoid-quinone biosynthesis	182.0812	50.3	M+H	HILIC
Early	10-hydroxydecanoate	Fatty acid metabolism	187.1338	24	M-H	C18
Early/Late	CITRIC acid	Citrate cycle (TCA cycle)/Alanine, aspartate and glutamate metabolism/Glyoxylate and dicarboxylate metabolism	191.0196	21.5	M-H	C18
Early	3-methoxy-4-hydroxymandelate	Tyrosine metabolism	197.0434	22.3	M-H	C18
Early/Late	Methyl ecgonine	Tropane, piperidine and pyridine alkaloid biosynthesis	200.1282	39.2	M+H	HILIC
Late	Hexanoylcarnitine	Fatty acid metabolism	260.1855	28.2	M+H	HILIC
Late	Inosine	Purine metabolism	267.0732	22.9	M-H	C18
Early/Late	Progesterone	Steroid hormone biosynthesis	315.2331	24.6	M+H	HILIC
Late	Decanoylcarnitine	Fatty acid degradation	316.248	24.3	M+H	HILIC
Late	Lauroylcarnitine	Fatty acid metabolism	344.2792	23.7	M+H	HILIC
Early/Late	Cortexolone	Steroid hormone biosynthesis	347.2215	24.5	M+H	HILIC
Early/Late	1-oleoyl-rac-glycerol	Fatty acid biosynthesis	357.3003	23.6	M+H	HILIC

Early	Aldosterone	Steroid hormone biosynthesis	361.201	25	M+H	HILIC
Late	25-hydroxycholesterol	Primary bile acid biosynthesis	385.3466	21.8	M-H2O+H	HILIC
Early	Bis(2-ethylhexyl)phthalate	Xenobiotics biodegradation and metabolism	391.2842	22	M+H	HILIC

Notes: The metabolic features were identified and confirmed by comparing their peaks based on accurate mass to charge ratio and retention time with the baseline reference standards utilizing tandem mass spectrometry performed under identical conditions.
These results were derived from both positive and negative ESI (HILIC=ESI+, C18=ESI-)
ESI: Electrospray ionization mode
pvalue<0.05 was used to identify the metabolic features.

Fig 1a. Heatmap of intermediate biological pathways in the maternal metabolome at early/mid pregnancy.

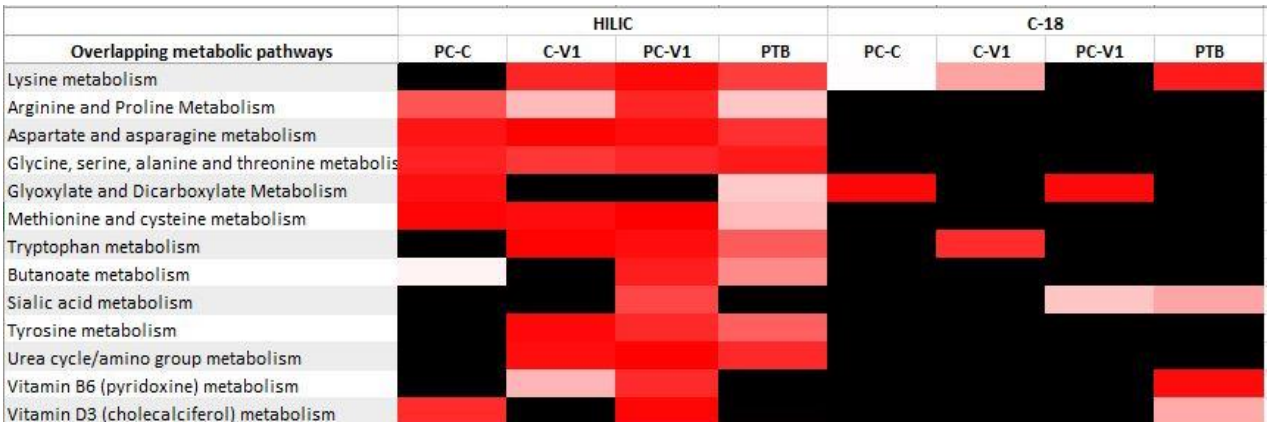
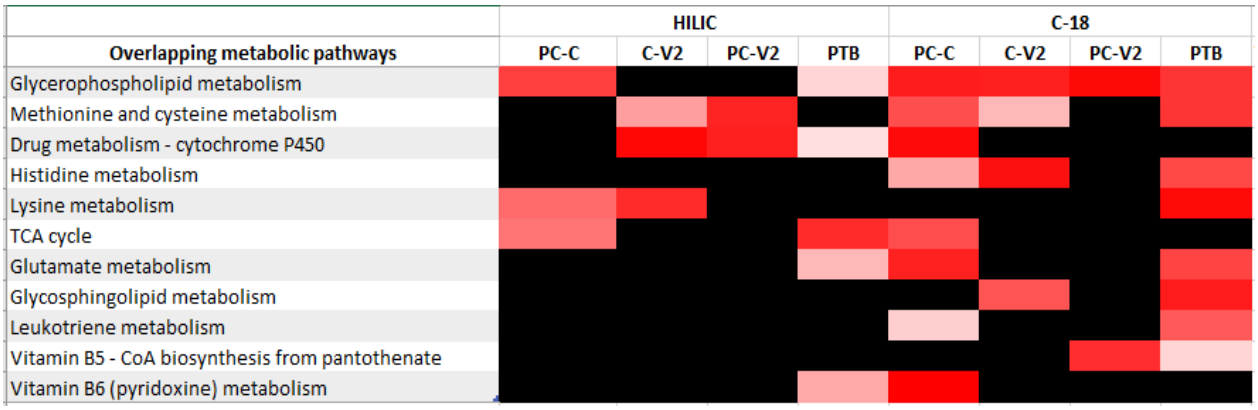


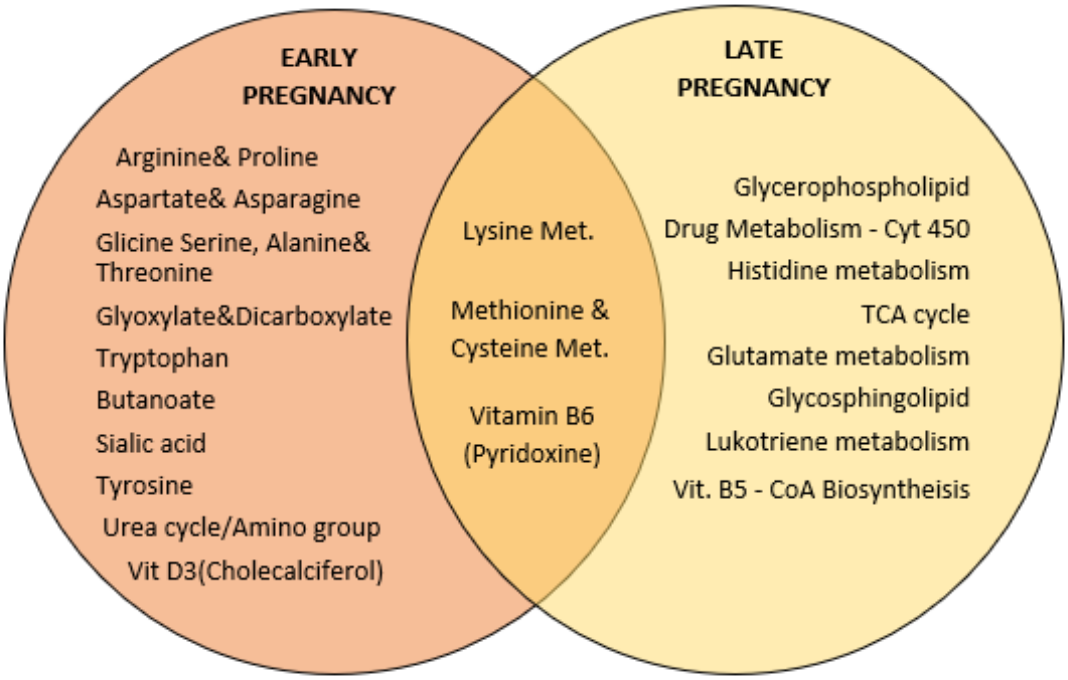
Fig 1b. Heatmap of intermediate biological pathways in the maternal metabolome at mid/late pregnancy.



Note: The heatmaps show the pathways associated with both ambient temperature exposure and the adverse birth outcomes. For each metabolic pathway associated between the exposure and outcome, the cells are shaded according to the p values. The pathways were analyzed for the three

exposure windows (preconception to conception, conception to visit 1&2, preconception to visit 1&2) and ordered in this sequence following the total number of significant associations (p value < 0.05) for both HILIC and C-18 columns.
Abbreviations: PC-C: preconception to conception, C-V1&V2: conception to visit 1 and visit 2 respectively for early and late pregnancy, PC-V1&V2: preconception to visit 1 and 2 for early and late pregnancy respectively, PTB: Preterm birth (> 20 and < 37 weeks).
pvalue<0.05 was used to identify the metabolic pathways.

Fig.2. Overlapping pathways in the maternal serum metabolome between periconceptional ambient temperature exposure and preterm birth throughout pregnancy.



Note: Metabolic pathways associated with temperature exposure at early/mid pregnancy and mid/late pregnancy in the maternal serum metabolome. The common metabolic pathways identified were Lysine metabolism, Methionine and Cysteine metabolism and Vitamin B6(Pyridoxine) metabolism.