



Household air pollution exposure is associated with perturbations in metabolic pathways in human milk: An observational untargeted metabolomics analysis from Guatemala

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ABSTRACT

Background: Exposure to air pollutants including particulate matter (PM_{2.5}), black carbon (BC), and carbon monoxide (CO), is linked to alterations in the human blood metabolome. We examined relationships between these exposures and the human milk metabolome, which may have implications for infant health.

Methods: Human milk samples from 75 women participating in the Household Air Pollution Intervention Network trial in Jalapa, Guatemala were obtained during household visits at six months postpartum. Samples were analyzed for metabolomic profiling using liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS). 24-hour personal exposure to PM_{2.5}, BC, and CO was measured during pregnancy at 9–19 weeks, 24–28 weeks, and 34–36 weeks gestation. Perturbations in the human milk metabolome associated with exposure to each pollutant during pregnancy were identified using an established untargeted metabolome-wide association study workflow.

Results: We extracted 571, 492, and 690 metabolic features in the C18 and 93, 1381, and 909 features in the hydrophilic interaction liquid chromatography (HILIC) columns, for PM_{2.5}, BC, and CO respectively ($p < 0.05$). 17 major metabolic pathways were observed to be associated with at least one of the pollutants, including pathways related to degradation of aromatic compounds, amino sugar and nucleotide sugar metabolism, and retinol metabolism. We annotated a total of 204 metabolites (level 1 confidence), of which 63 were associated with PM_{2.5}, 84 were associated with BC, and 57 associated with CO.

Conclusions: Household air pollution exposure from PM_{2.5}, BC, and CO was associated with perturbations in metabolic pathways in human milk, including pathways related to inflammation and cellular signaling and repair.

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1. Introduction

Human milk is the optimal food source for newborns, and the World Health Organization recommends that infants be exclusively breastfed for the first six months of life with continued breastfeeding up to two years of age and beyond (World Health Organization, 2023). Human milk is a complex system, composed of 87 % water; 12 % carbohydrates, lipids, proteins, and vitamins; and other bioactive components, such as growth hormones and immune factors (Casavale et al., 2019). These components play essential roles in infant growth, development, and specific biological functions such as hormone production and enzyme cofactor activity (Kim and Yi, 2020), especially during the first 6 months of life. Human milk microbiota also provide the foundation for an infant's gut microbial consortium (Jost et al., 2012). The composition of human milk changes over time, matching the infant's nutrient requirements (Ballard and Morrow, 2013), and can also be influenced by the mother's lifestyle and environment.

Multiple factors such as diet, medications, stress, environment, and personal care routines can affect the composition of human milk, possibly influencing infant outcomes (Waitt et al., 2015; Gomez-Gallego et al., 2016; Adhikari et al., 2022; Thomas et al., 2022; Gridneva et al., 2022). Observational studies have identified geographical variations in human milk composition, which may be due to environmental and lifestyle factors. For example, in one study that compared samples from China, Northern Europe, and Spain, differences were seen in the human milk microbiota and lipid composition (Kumar et al., 2016). Other observational studies have identified high levels of persistent organic pollutants such as polychlorinated biphenyls in human milk in the Czech Republic, Slovakia, and Spain, with higher concentrations being found in urban and industrial areas (Pajewska-Szmyt et al., 2019). These chemicals are of particular concern because they may bioaccumulate in the body and persist for extended periods (Kampa and Castanas, 2008). However, evidence on linkages between environmental influences and human milk composition remains limited.

Air pollution poses a significant threat to global human health and wellbeing. Components of air pollution have been associated with low birth weight, preterm birth, and gestational hypertension (Stieb et al., 2012; Guo et al., 2023; Liu et al., 2023). Current data indicates that both long- and short-term exposure to air pollution is linked to inflammation, respiratory infections, and neurological issues (Calderón-Garcidueñas et al., 2008; Yang et al., 2024). The World Health Organization classifies air pollution into two types: ambient (outdoor) and household (indoor) air pollution. Together, ambient and household air pollution exposure are estimated to cause approximately seven million premature deaths annually, of which it is estimated that household air pollution exposure results in approximately 3.1 million premature deaths, including 507,000 deaths of children under five years old (Brauer et al., 2024; World Health Organization, 2024). Importantly, in some settings, up to 50 % of ambient air pollution is attributable to household air pollution that has entered the ambient environment (World Health Organization, 2024). About 2–3 billion people use solid fuels such as wood, agricultural residue, coal, and charcoal for cooking, contributing to household air pollution (International Energy Agency International Renewable Energy Agency, 2024; Bennitt et al., 2025). The resulting smoke contains numerous pollutants, including fine particulate matter (PM_{2.5}), carbon monoxide (CO), black carbon (BC), and reactive metals (Naeher et al., 2007), which can enter the cardiorespiratory system. Therefore, reducing household air pollution through the promotion of cleaner cooking fuels is a key strategy for reducing air pollution levels overall.

Women are particularly at risk of exposure to household air pollution due to their role as primary cooks in many cultures. Pregnant and lactating women, as well as children, are especially vulnerable due to the potential adverse effects of pollutants on infant and child development. This exposure can lead to negative pregnancy outcomes, including low birth weight (Slama et al., 2008; Ghio et al., 2011; Rice et al., 2015; Ljungman et al., 2018). In one study, the association between PM_{2.5}

exposure and maternal circulatory homocysteine (Hcy) levels in 324 early pregnant women were analyzed; elevated Hcy is associated with adverse pregnancy outcomes such as hypertensive disorders of pregnancy. Results showed that Hcy increased as PM_{2.5} concentrations increased (along with constituents such as sulfate), increasing the risk of early pregnancy loss (Li et al., 2025). Although the exact mechanisms of how air pollution leads to adverse infant outcomes are not as clear, consistency in impact on inflammation and oxidative stress has been suggested.

Metabolomics, the study of the global detection and characterization of metabolites, is frequently used as a tool to understand biological changes in response to environmental exposures or health practices (Thornton and Nobeli, 2006; Liang and Walker, 2023). The metabolome provides a biological profile of chemicals, including those produced from pollutant exposure, and offers active chemical information about the organism's phenotype at a given time. This information allows for an understanding of the biological pathways of changes due to different exposure processes and their phenotypic outcomes (Pezzatti et al., 2020). Metabolomics approaches have been used in studies of air pollution, including a 12-week observational study that investigated the effects of indoor exposure to traffic-related air pollution using plasma and saliva samples collected from 54 students living in dormitories near a major highway (Liang et al., 2018). Similarly, another observational study used high-resolution metabolomics analysis of neonatal dried blood spots to examine associations between metabolites and mothers' prenatal (third trimester) exposure to PM_{2.5} from traffic-related air pollution (Ritz et al., 2022). Results from both studies indicated potential impacts of air pollution exposure on signaling pathways related to oxidative stress and inflammation. However, more research into the metabolic mechanisms associated with air pollution exposure is needed.

There are two main approaches in metabolomics analysis. The targeted approach is primarily hypothesis-driven and seeks to identify specific chemicals or compounds based on known metabolites (González-Riano et al., 2020). The untargeted approach is hypothesis-generating, allowing for the exploration of a broad range of unknown metabolites. For this reason, untargeted metabolomics approaches have been shown to have utility in exploratory studies of human milk composition. For example, one study among 30 healthy lactating women in China identified that distinct metabolites were present at three timepoints after delivery (Li et al., 2018). Another study in the US used untargeted metabolomics to investigate the impact of human milk on neonates at risk of diseases such as necrotizing enterocolitis (NEC). Although there was a small difference in the human milk metabolome between the mothers of infants with NEC and mothers of healthy control infants, more significant differences were found in the metabolome related to fatty acid metabolism, oligosaccharides, amino sugars, amino acids, vitamins and oxidative stress-related metabolites between mothers based on infant birth weight (Nolan et al., 2021). To our knowledge, no studies have examined the human milk metabolome in relation to exposure to air pollution.

Given the potential linkages between a mother's exposures, human milk, and infant health, it is crucial to understand the biological effects of exposure to harmful pollutants, specifically air pollution, on human milk composition (Holzhausen et al., 2023). In the Maternal and Developmental Risks from Environmental and Social Stressors (MADRES) study, researchers analyzed 382 third trimester maternal serum samples using an untargeted metabolomics approach to better understand the impact of exposure of ambient concentrations of PM_{2.5}, NO₂, and O₃. Fourteen metabolic pathways were significantly associated with PM_{2.5} exposure (with C21-steroid hormone biosynthesis and metabolism showing the most significant) while 16 pathways were significantly associated with birth weight (with vitamin A (retinol) metabolism being the most significant) (Chen et al., 2025). However, there is still limited information utilizing the human milk metabolome to understand biological mechanisms associated with air pollution and infant health.

Due to the significant evidence gap on the linkages between household air pollution exposure and the human milk metabolome, we employed an exploratory untargeted metabolomics approach to capture all possible results. Specifically, this study utilized untargeted metabolomics analysis of 75 human milk samples from mothers in Jalapa, Guatemala, aiming to understand the associations between household air pollution exposure during pregnancy and subsequent biological perturbations in the human milk metabolome.

2. Methods

2.1. Study design and population

We used data from a sub-study of 75 Guatemalan mothers who participated in the Household Air Pollution Intervention Network (HAPIN) trial. HAPIN was a multi-country randomized control trial of liquified petroleum gas (LPG) cookstoves and free fuel distribution compared to traditional biomass burning stoves. Primary outcomes of HAPIN included birth weight, incidence of pneumonia in infants, and stunting when infants were 12 months of age. Results related to these primary outcomes have been published elsewhere (Checkley et al., 2024; Clasen et al., 2022; McCollum et al., 2024). Eight hundred pregnant women were recruited through antenatal clinics in each of the following four sites: Jalapa, Guatemala; Tamil, Nadu, India; Puno, Peru; and Kayonza, Rwanda (Clasen et al., 2020). Details of the trial design have been published elsewhere (Barr et al., 2020; Clasen et al., 2020; Johnson et al., 2020). The present study focuses only on women from the Jalapa, Guatemala site, which was selected from among the four HAPIN trial sites due to interest among the Guatemala team.

Pregnant women of rural communities in Jalapa, Guatemala between 18 and 35 years of age who primarily used biomass stoves were eligible to participate in the HAPIN trial. These women had to have a single viable pregnancy, confirmed via ultrasound, of between 9 and 19 weeks' gestation. After enrollment and consent, the women were randomly assigned to either the intervention or control arm. Women in the intervention arm received an LPG stove with continuous free fuel supply for about 18 months. This also included educational materials and guidance on use of the LPG stoves. Women assigned to the control group kept using their biomass stoves but were compensated for their participation (Quinn et al., 2019). In Guatemala, this compensation took the form of periodic disbursement of cash-equivalent coupons that could be redeemed for items from a catalog (Quinn et al., 2019).

2.2. HAPIN data collection

Study participants were followed throughout their pregnancy and the first year of the infant's life. Prior to randomization (9–20 weeks gestation), baseline data were collected on socio-demographic and environmental characteristics such as age, highest level of education, lifestyle behaviors, and housing materials and other assets. After randomization, data were collected during household visits at two additional timepoints during pregnancy (24–28 weeks and 34–36 weeks gestation), within 24 h of birth, and when the infant was 3, 6, 9, and 12 months of age.

2.3. Air pollution exposure measurement

At each household visit, 24-h personal monitoring of exposure to PM_{2.5} was conducted using both gravimetric filter-based and concurrent nephelometric samples for each mother using the RTI Enhanced Children's MicroPEM (ECM, RTI International, Research Triangle Park, NC, USA) device. This device weighs about 150 g and measures 6x2x12 cm, has a 2.5-μm size-cut impactor, and operates with a flow rate of 0.3 L/min to collect PM_{2.5} in a 2 μm pore size polytetrafluoroethylene (PTFE) filter. The ECM collects PM_{2.5} concentrations continuously with an internal nephelometer sensor (Johnson et al., 2022). The Lascar

EL-USB-300 (Lascar Electronics) was used to collect CO concentrations at 1-min intervals simultaneously with the ECM. Both the ECM and Lascar were placed in front pockets on a specially designed apron worn by enrolled women, close to the breathing zone. BC concentrations were estimated from the PM_{2.5} PTFE filters using a SootScan transmissometer (Model OT-21, Magee Scientific). The depositions were measured using BC attenuation cross-section values from similar materials (Garland et al., 2017). Additional details on the exposure assessment methods and specific measurements calibration are described elsewhere (Johnson et al., 2020; Johnson et al., 2022). Due to data limitations, the current study uses exposure data only from prenatal household visits.

2.4. Human milk sample collection

All mothers enrolled in the HAPIN trial in Guatemala and who gave birth between February and August 2019 were recruited for this sub-study during the birth visit at the hospital. Exclusion criteria at the birth visit were if the newborn was currently sick or if the newborn was currently taking antibiotics. All other mothers were considered eligible and were invited to enroll. Recruitment ended when 75 women had been consented and enrolled. The sample size of 75 was based on budgetary constraints for the exploratory untargeted metabolomics analysis. Mothers were subsequently excluded if they delivered via cesarean section or did not intend to breastfeed their infant. Mothers were further excluded from human milk sampling at the six-month postpartum household visit if they had mastitis, had used antibiotics in the past 48 h, or if they were no longer nursing. Mastitis was determined by self-report and visual observation by a female surveyor at time of human milk collection.

Milk samples were collected during the household visit at six months postpartum following a standard protocol. A trained Guatemalan HAPIN staff member collected the milk samples using aseptic techniques. All milk samples were collected at the same time in the morning (±2 h) to reduce variation in human milk composition due to circadian variation (Hampel et al., 2017). Prior to collection, mothers were shown how to properly clean the nipple and surrounding area. Mothers were requested to fully empty one breast, via pump (Haakaa manual pump) or manual expression, into a 150 mL sterile collection bottle. The resulting sample was immediately placed into two sterile tubes, which were then placed in a cooler for transport to the field laboratory. Samples were aliquoted and stored at −20 °C in the field site. After all samples had been collected, they were transferred to a −80 °C freezer at the Universidad del Valle de Guatemala. Later, samples were shipped on dry ice to Emory University's School of Nursing Research Lab and then transferred to the Emory University Clinical Biomarker Laboratory for metabolomics profiling.

Fig. 1 shows the timeline for collection of baseline data, air pollution exposure measurement, and human milk sample collection.

2.5. High-resolution metabolomics profiling

Triplicate 10 μL human milk samples were analyzed using reverse-phase (C18) and hydrophilic interaction liquid chromatography (HILIC) coupled with high-resolution tandem mass spectroscopy (LC-HRMS) following an established protocol (Go et al., 2015; Liu et al., 2020). To provide a more complete and specific study, samples were analyzed using either negative or positive electrospray ionization (ESI) mode. A milk sample that was referenced against the National Institute of Standards and Technology (NIST) was added at the beginning and end of the study. Pooled control reference samples were analyzed at the beginning, middle and end of sample analyzed batch to account for normalization, background noise control, and quantification. Internal standards were also injected along with each sample (Table S1). The resulting metabolic profiles were extracted using apLCMS with any modifications by the R package xMSanalyzer version 2.0.6.1 (Yu and Jones, 2014; Uppal et al., 2013) to enhance data quality control and

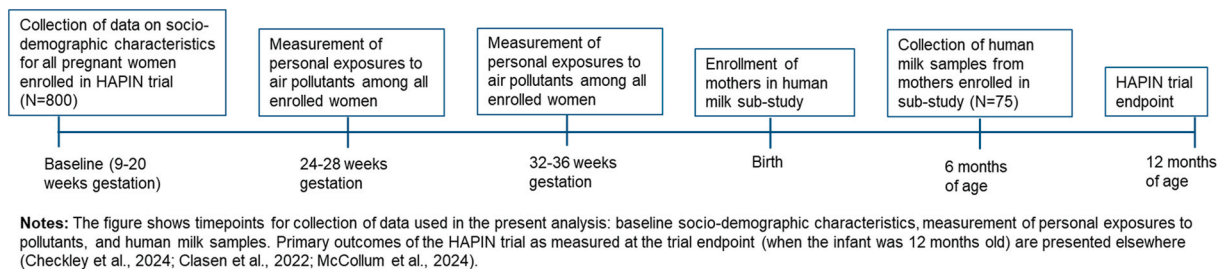


Fig. 1. Study timeline.

correct for batch effects using the ComBat method. The combination of the unique m/z , retention time, and ion intensity lead to the resulting metabolic features. Only features presented in $>15\%$ of samples, with a median coefficient of variation $<30\%$ and a Pearson correlation >0.7 among technical replicates were retained. As a result, we detected 19,199 and 11,594 metabolic features in HILIC and C18 columns, respectively. Metabolic features with a detection rate in less than 5 % of the study samples were excluded from subsequent analysis. Missing values in the remaining data were retained without imputation. As a result, we ended up with 19,199 (retention rate: 100 %) and 11,594 (retention rate: 100 %) metabolic features in HILIC and C18 columns, respectively, for the subsequent analysis. The relative concentrations of metabolic features were log2-transformed in the subsequent analyses.

2.6. Metabolome-wide association study (MWAS)

The continuous measured air pollutant concentration data collected during the two post-randomization prenatal household visits (24–28 weeks and 34–36 weeks gestation) were averaged and any outliers removed. Given the potential variability in exposure measurements for the same individual over time, these measurements were averaged to represent a proxy estimate of typical longer-term exposures over time during pregnancy. Perturbations in the human milk metabolome associated with exposure to averaged $PM_{2.5}$, BC, and CO concentrations were identified using an established untargeted metabolome-wide association study (MWAS) workflow (Liang et al., 2018; Li et al., 2021). This approach includes using data-driven significant feature detection using regression models, knowledge-driven methods for pathway analysis, and feature annotation/identification (Uppal et al., 2016). Linear regression models were used to examine the associations between each pollutant and each metabolic feature. Due to the relatively small sample size and the complexity of our models, we could only include one covariate in the models. We made a priori decision to control for housing materials, which we hypothesized could be associated with both pollutant exposure level and resulting metabolites. Specifically, we hypothesized that materials that allow for greater ventilation and air flow may lead to reduced exposure to household air pollution but may also lead to increased exposure to environmental factors, which could be associated with metabolomic features. We prioritized housing materials over other potential confounders (e.g., household wealth or maternal education) as we hypothesized that housing materials would be more directly related to our exposure and outcome. For the present analysis, housing materials were categorized into a categorical variable representing traditional materials (thatch, woven reed, mud, wattle, stone, fired brick, concrete/cement), modern materials (metal, fiberglass, fired tile, vinyl, ceramic), or a mix of traditional and modern materials. Two MWAS analyses were run for HILIC and C18 chromatography columns separately. The general equation for this assessment is as follows:

$$\log_2(Y_i^m)^* = \beta_0^m + \beta_1^m x_{\text{pollutant}_i} + \beta_2^m x_{\text{housing type}} + \epsilon_i^m$$

With Y_i^m = observed intensity of metabolic feature
 i = individual

m = metabolic feature

$\log(Y_i^m)$ = log-transformed feature intensity

β_1^m = coefficient of metabolic feature m for the air pollutant

Associations between each pollutant and the metabolic feature were displayed using Manhattan plots.

2.7. Pathway enrichment analysis

Pathway enrichment analysis was used to predict biological pathways in which the air pollution-associated metabolic features were enriched. To further pinpoint the metabolites, Metapone version 1.15.0, a comprehensive R/Bioconductor package metabolic pathway enrichment statistical matching program, evaluated the data (Tian et al., 2022). When analyzing untargeted metabolomics data, it can be difficult to identify found metabolites that are not already in a known network. Metapone tackles this by: 1) using a multi-system matching process incorporating weighted features and a permutation-based weighted hypergeometric test; 2) utilizing three reputable database sources; and 3) analyzing both positive and negative-ion data (Tian et al., 2022). Due to the small sample size, FDR correction was not used. Eligible metabolic features were chosen for pathway analysis based on identifying raw p -values <0.05 . All metabolic features measured in our samples were used as background metabolic features.

2.8. Chemical annotation

By matching with an in-house analytical standards library, metabolic features were confirmed with level 1 confidence using Metabolomics Standard Initiative (MSI) criteria of features whose m/z (± 10 ppm difference) and retention time (± 10 s) (Sumner et al., 2007). Using a level 1 criteria indicates that the resulting metabolites are accurate with high confidence by using the same reference standards as the human milk samples. We also used the MetaboAnalyst database to annotate the metabolic features based solely on m/z with Level 4 confidence in the significant pathways that might not have been identified using the Level 1 criteria.

2.9. Ethics

The study protocol was reviewed and approved by institutional review boards or ethics committees at Emory University (00089799), Universidad del Valle de Guatemala (146-08-2016/11–2016) and the Guatemalan Ministry of Health National Ethics Committee (11–2016). All participants provided written informed consent. The HAPIN trial is registered on [ClinicalTrials.gov](https://clinicaltrials.gov) (NCT02944682).

3. Results

3.1. Population characteristics

Participants ($n = 75$) comprised 38 mothers assigned to the HAPIN intervention group and 37 assigned to the control group. The average age of participants was 23.6 ± 3.8 years old with an average gestational

age of 14.6 weeks $\pm$ 2.9 weeks at baseline (Table 1). For housing material categorization, one participant had missing information. Four participants were removed from the analysis due to having air pollutant levels that were determined to be potential outliers based on a threshold of mean $\pm$ 3 standard deviation. Additional socio-demographic characteristics of the participants are shown in Table 1.

3.2. Metabolome association with air pollution

For the C18 negative column, 571, 492, and 690 metabolic features were associated with PM_{2.5}, BC, and CO respectively, while for the HILIC column, 931, 1381, and 909 features were associated with PM_{2.5}, BC, and CO respectively ($p < 0.05$). To visualize the extent of the relationship between each pollutant and metabolic feature, volcano plots were created (Fig. 2). Overall, a larger number of positive significant associations were observed across all three pollutants in the HILIC column compared to C18. Specifically, for HILIC, CO presented the most statistically significant associations by being associated with the smallest p -value. For C18, BC presented the most significant association.

3.3. Pathway enrichment analysis

We identified 17 major metabolic pathways associated with PM_{2.5}, BC, and CO (Fig. 3) at the $p < 0.05$ level. For PM_{2.5} we identified five pathways: alanine and aspartate metabolism; glycine and serine metabolism; methionine metabolism; sphingolipid metabolism; and urea cycle/amino group metabolism. For BC, we identified seven pathways: ABC transporters; amino sugar and nucleotide sugar metabolism; androgen and estrogen metabolism; caffeine metabolism; porphyrin metabolism; retinol metabolism; and starch and sucrose metabolism. For CO, we identified five pathways: alanine, aspartate and glutamate metabolism; degradation of aromatic compounds; glycosphingolipid metabolism; sphingolipid metabolism; and urea cycle/amino group metabolism. Both sphingolipid metabolism and urea cycle/amino group metabolism were associated with both PM_{2.5} and CO.

Additional detailed results are shown in Supplementary Tables 2–7.

3.4. Chemical annotation

By comparing our data to the in-house analytical standards library using level 1 confidence, we annotated a total of 204 metabolites. Of these, 84 were associated with BC, 57 associated with CO, and 63 associated with PM_{2.5}. MetaboAnalyst was used to annotate the significant metabolic features enriched in the significant pathways. After, these features were cross-referenced with confirmed metabolites using our in-house library. In total, we confirmed the chemical identity of 14

metabolic features (Table 2). Of these, eight were associated with BC, four were associated with PM_{2.5}, and two with CO. Most of these metabolites were found from analyzing the HILIC column (57.1 %). It is important to note that we observed variations in the levels of specific metabolites that are linked to each pollutant listed.

4. Discussion

In this study, we utilized untargeted metabolomics to explore the associations between exposure to household air pollution during pregnancy and biological perturbations in human milk at six months postpartum. Specifically, we applied cutting-edge high-resolution metabolomics to identify perturbations in the human milk metabolome associated with CO, PM_{2.5}, and BC among Guatemalan mothers participating in the HAPIN trial. By evaluating metabolomic profiles in human milk without predefined targets, we were able to identify a wide range of metabolites and metabolic pathways that may be influenced by air pollution exposure. While several prior studies have investigated air pollution metabolomics, they focused on human blood or urine (Liang et al., 2023). Our study therefore makes an important contribution by generating new evidence on air pollution and the human milk metabolome.

The metabolic features significantly associated with BC in our results included several metabolites that are critical to the synthesis of human milk oligosaccharides (HMOs). Among the metabolites identified in our results, alpha-D-Glucose and D-Galactose are monosaccharides that are building blocks of HMOs, while Galactose 1-phosphate (Gal1P) plays a role in the biosynthesis of HMOs (Kitaoka, 2012; Garwolinska et al., 2018). HMOs are complex carbohydrates that make up about 20 % of all human milk carbohydrates but cannot be digested by the infant's small intestine (Brand-Miller et al., 1998; Gnath et al., 2000; German et al., 2008). Instead, they play a key prebiotic role in the development of the gut microbiome and immune system by providing nutrients for beneficial bacteria such as bifidobacteria (Zhang et al., 2024). These same gut microbes can contribute to brain and neural development; protect the infant against diarrhea or serve as protection against bacteria and viruses including Streptococcus pneumonia; and, through their role in the immune system, may prevent food allergies or autoimmune conditions (Garwolinska et al., 2018; Walsh et al., 2020; Zhang et al., 2024). Therefore, alterations in metabolites related to HMOs may have implications for a range of infant biological systems and processes.

Other metabolic features significantly associated with pollutant exposure included non-protein molecules containing nitrogen such as free amino acids and nucleotides. Although the contribution of non-protein nitrogen to an infant's health is minimal, some reports show that microbial communities are heavily impacted by nitrogen availability and this can contribute to the wellness of the infant's gut microbiota (Holmes et al., 2017; Reese et al., 2018). In addition, nucleotides such as Uridine 5'-monophosphate (which we observed to be associated with BC) and guanine (associated with both CO and PM_{2.5}) play an important role in cell function and metabolism (Cosgrove, 1998). Nucleotides can be found in oligonucleotides in cells in human milk and as free nucleotides. Dietary nucleotides can play a variety of roles in infant development, including related to immune function, iron absorption, and even sleeping patterns (Faelli and Esposito, 1970; McMillan et al., 1977; Carver et al., 1991; Cosgrove, 1998; Pickering et al., 1998; Cubero et al., 2007; Cubero et al., 2009; Sánchez et al., 2009). Recent studies show that human milk nucleotide levels change throughout the day and that three specific nucleotides (adenosine-5'-monophosphate, uridine-5'-monophosphate and guanosine-5'-monophosphate) display increased concentrations at night (Sánchez et al., 2009; Garwolinska et al., 2018). Thus, it has been hypothesized that human milk nucleotides may be involved in inducing sleep in infants (Sánchez et al., 2009).

Among the 17 metabolic pathways that were associated with air pollution exposure, we identified pathways related to regulation of

Table 1
Sociodemographic characteristics of study population (N = 75).

Characteristic	Number (%) or Mean (SD)
Maternal age (years)	
<20	15 (20.0 %)
20 - 24	33 (44.0 %)
25-29	20 (26.7 %)
30-35	7 (9.3 %)
Maternal highest level of education completed, n (%)	
No formal education/some primary school	29 (38.7 %)
Primary school/some secondary school	33 (44.0 %)
Secondary/Vocational/Some University	13 (17.3 %)
Number of people sleeping in house, mean (SD)	5.29 ($\pm$ 2.3)
Main housing material	
Traditional (Thatch, Woven Reed, Mud, Wattle, Stone, Fired Brick, Concrete/Cement)	4 (5.7 %)
Modern (Metal, Fiberglass, Fired Tile, Vinyl, Ceramic)	3 (4.3 %)
Both Traditional and Modern (Overlays)	63 (90 %)
Gestational age at enrollment (weeks), mean (SD)	14.64 ($\pm$ 2.9)

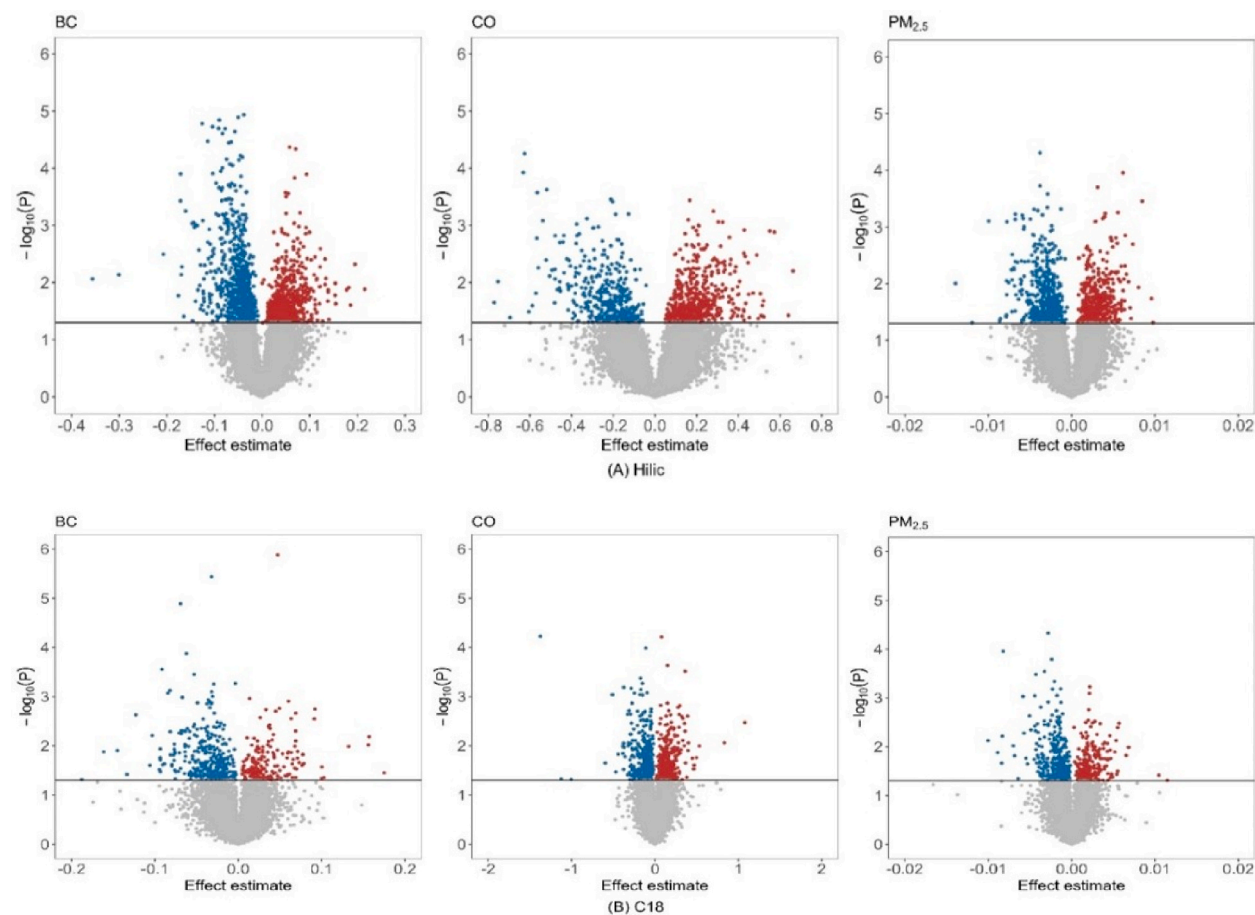


Fig. 2. Volcano plots of associations between each air pollutant and each metabolic feature from HILIC (A) and C18 (B) columns. The x-axis indicates the effect estimates from the air pollutant-metabolic feature models. The y-axis indicates the negative log10-transformed p-values of the effect estimates from air pollutant-metabolic feature models. The black solid line represents the threshold of p-values of 0.05. The dots above the black solid line were those with p-values <0.05. The red dots indicate positive associations between air pollutants and metabolic features, while the blue dots indicate negative associations between air pollutants and metabolic features. Note: BC, black carbon; CO, carbon monoxide; PM2.5, fine particulate matter.

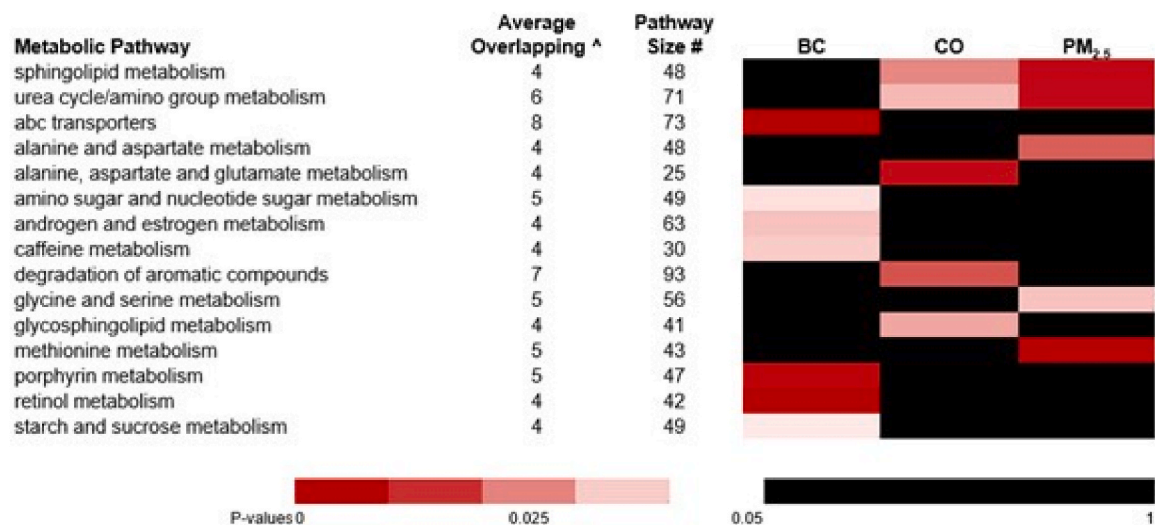


Fig. 3. Heatmap of metabolic pathways associated with air pollution exposure. It shows the average number of metabolic features matched in the specific metabolic pathway across the significant air pollutant-metabolic feature associations. The colors were shaded according to the P-value of associations. The pathways were ordered according to the total number of significant associations (p-value <0.05). BC, black carbon; CO, carbon monoxide; PM2.5, fine particulate matter.

cellular processes and function as well as detoxification. Sphingolipid and glycosphingolipid metabolism, associated with both CO and PM_{2.5},

Table 2Chemical identity of metabolic features significantly associated with BC, PM_{2.5}, and CO.

m/z	RT	Chemical Identity	Associated Column	Coefficient (95 % CI)	Pollutant	Pathway
203.0529	60.1	alpha-D-Glucose	HILIC	-0.019 (-0.037, -0.001)	BC	amino sugar and nucleotide sugar metabolism
203.0529	60.1	D-Galactose	HILIC	-0.019 (-0.037, -0.001)	BC	amino sugar and nucleotide sugar metabolism
261.037	264	Galactose 1-phosphate	HILIC	0.027 (-0.117, -0.012)	BC	starch and sucrose metabolism
261.037	278	Glucose 6-phosphate	HILIC	-0.064 (-0.117, -0.012)	BC	starch and sucrose metabolism
154.0622	23	Histidine	C18	-0.027 (-0.049, -0.004)	BC	abc transporters
175.1195	118	L-Arginine	HILIC	-0.054 (-0.093, -0.014)	BC	abc transporters
377.0856	20.4	Sucrose	C18	0.006 (0.001, 0.011)	BC	abc transporters
325.0431	149	Uridine 5'-monophosphate	HILIC	-0.048 (-0.092, -0.003)	BC	starch and sucrose metabolism
115.0037	21	Fumaric acid	C18	-0.001 (-0.002, -0.0003)	PM _{2.5}	alanine and aspartate metabolism
150.0416	21	Guanine	C18	0.002 (0.0001, 0.004)	PM _{2.5}	urea cycle/amino group metabolism
152.0567	50	Guanine	HILIC	0.002 (0.0004, 0.003)	PM _{2.5}	urea cycle/amino group metabolism
152.0572	49.8	Guanine	HILIC	0.002 (0.0004, 0.003)	PM _{2.5}	urea cycle/amino group metabolism

are processes that are linked to the synthesis and regulation of sphingolipids, which are needed for cellular functions such as cell membrane structure and signaling (Lahiri and Futerman, 2007). Urea cycle/amino group metabolism, similarly associated with both CO and PM_{2.5}, aids in the detoxification of ammonia, a byproduct of protein and amino acid metabolism. However, some data suggest that urea cycle alterations can lead to a higher risk of coronary artery disease (Precht et al., 2024). Glycine and serine metabolism, which was associated with PM_{2.5}, is critical for cellular growth as well as detoxification of harmful metabolites. It has also been seen to be involved as precursors for cancer cell growth (Amelio et al., 2014). Degradation of aromatic compounds, which was associated with CO, breaks down aromatic compounds into simpler products. It is also heavily involved in environmental degradation, an important process when harmful chemicals accumulate in the body (Cao et al., 2009). ABC transporters, which were associated with BC, are a family of membrane bound proteins that assist in transportation of substrates across the cellular membrane. In addition to this, ABC transporters also play a role in cellular detoxification (Rees et al., 2009).

Our MWAS results also revealed a closely connected network of metabolic pathways involved in systemic inflammation (salicylate, elaidic acid, anthranilate), protein synthesis (proline, guanosine), and cellular function and repair (nicotinamide). These results follow other studies that, for example, considered how oxidative stress and inflammation are induced by PM_{2.5} exposure (Barbier et al., 2023). Our results also align with the results in Ritz et al. (2022), which found that prenatal exposure to traffic-related PM_{2.5} altered serum metabolites in pathways related to oxidative stress and inflammation. This demonstrates that shared metabolic responses can be observed in both mother and infant. Oxidative stress and inflammation are associated with impaired child linear growth (Lui et al., 2024). Other long-term health implications surrounding such indications include a potential risk of increase of cardiovascular diseases, respiratory conditions, and indirect immune system dysregulation (Knip et al., 2000; Gong Jr et al., 2003; Oshita et al., 2019).

Our findings suggest that CO, PM_{2.5}, and BC could play a potentially important role, directly or indirectly, in affecting crucial health processes including cellular function while increasing the body's need for removal of harmful chemicals through detoxification methods. Similar results have been seen in previous findings from Ladd-Acosta et al., who observed associations between prenatal exposure to air pollution (in the form of nitrogen dioxide and ozone) and DNA methylation in cord blood and placenta samples collected in a multi-site study in the United States (Ladd-Acosta et al., 2019). Modeling studies have also observed associations between prenatal exposure to ambient air pollution (specifically, nitrogen dioxide) and infant anthropometric measures among Hispanic populations living in Southern California (USA) (Patterson et al., 2021). However, to our knowledge, no previous studies have examined relationships between air pollution and the human milk metabolome. Therefore, our results add new evidence and additional insight on associations between exposure to CO, PM_{2.5}, BC and human

milk perturbations.

A strength of our study is the use of high-resolution metabolomics techniques for detailed analysis of human milk samples to help identify the impacts of more than one air pollutant. Given that globally 48 % of infants are breastfed to 6 months, and globally, around 2.8 billion people are exposed to solid cooking fuels, further research into the effects of exposure to household air pollution on the composition of human milk remains necessary to understand environmental influences on early childhood nutrition (Bonjour et al., 2013; UNICEF, 2023; World Health Organization, 2018). At the same time, our study has several limitations that should be acknowledged, including the relatively small sample size, which limited the statistical power of our analysis and, hence, our ability to include multiple covariates in our models. Potential unmeasured confounders that might be associated with both exposure to household air pollution and the human milk metabolome could include household wealth and maternal education; omitting these variables may have affected the direction and magnitude of estimated associations in an unpredictable way. Other limitations include the single population of women from Jalapa, Guatemala, which limits generalizability, and the use of data on prenatal exposures to pollutants, which may not represent long-term exposures. Nevertheless, this study provides novel evidence of the potential influence of PM_{2.5}, CO, and BC on human milk composition and implications for future health outcomes resulting from metabolome disruption.

4.1. Conclusion

In this exploratory metabolomics study, we identified associations between pollutants (PM_{2.5}, CO, and BC) and the human milk metabolome, with implications for child growth, development of the immune system and gut microbiome, and other essential biological processes. Given the importance of human milk during the critical period of infancy for child growth and development, our results lay a foundation for future research into the effects of environmental exposures on the composition of human milk. By prioritizing the need to understand the connection between household air pollution and biological transformations, these studies can inform public health efforts aimed at reducing environmental exposures and help limit the harmful health effects of air pollution on women and young children.

CRedit authorship contribution statement

Jada Hoyle-Gardner: Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **Ziyin Tang:** Writing – review & editing, Visualization, Validation, Methodology, Formal analysis. **Donghai Liang:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Anaite Diaz-Artiga:** Writing – review & editing, Supervision, Project administration, Investigation. **Ajay Pillarisetti:** Writing – review & editing, Data curation. **Luke Naeher:** Writing – review & editing, Supervision, Project administration. **Kasthuri Sivalogan:** Writing – review & editing, Writing – original draft. **Erick**

Mollinedo: Writing – review & editing, Investigation. **Jiantong Wang:** Writing – review & editing, Data curation. **Thomas F. Clasen:** Writing – review & editing, Project administration, Funding acquisition. **Lisa M. Thompson:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Sheela S. Sinharoy:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

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A multidisciplinary, independent Data and Safety Monitoring Board (DSMB) appointed by the National Heart, Lung, and Blood Institute (NHLBI) monitored the quality of the data and protected the safety of patients enrolled in the HAPIN trial. The DSMB consisted of: Catherine Karr (Chair), Nancy R. Cook, Stephen Hecht, Joseph Mllum, Nalini Sathiakumar (deceased), Paul K. Whelton, and Gail Weinmann and Thomas Croxton (Executive Secretaries). Program Coordination: Gail Rodgers, Bill & Melinda Gates Foundation; Claudia L. Thompson, National Institute of Environmental Health Sciences; Mark J. Parascandola, National Cancer Institute; Marion Koso-Thomas, Eunice Kennedy Shriver National Institute of Child Health and Human Development; Joshua P. Rosenthal, Fogarty International Center; Concepcion R. Nierras, NIH Office of Strategic Coordination – The Common Fund; Katherine Kavounis, Dong-Yun Kim, Barry S. Schmetter (deceased), and Antonello Punturieri, NHLBI.

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the US National Institutes of Health or Department of Health and Human Services.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Donghai Liang, Lisa M. Thompson, and Thomas F. Clasen report financial support was provided by National Institutes of Health. Thomas F. Clasen reports financial support was provided by Bill and Melinda Gates Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2026.123687>.

Data availability

Data will be made available on request.

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