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Authors
Yan, Qi
Liew, Zeyan
Uppal, Karan
et al.

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Maternal Serum Metabolome and Traffic-Related Air Pollution Exposure in Pregnancy

Qi Yan[a], **Zeyan Liew**[b,c], **Karan Uppal**[d], **Xin Cui**[e,f], **Chenxiao Ling**[a], **Julia E. Heck**[a], **Ondine S. von Ehrenstein**[a], **Jun Wu**[g], **Douglas I. Walker**[h], **Dean P. Jones**[d,i], **Beate Ritz**[a,j,*]

[a]Department of Epidemiology, UCLA Fielding School of Public Health, Los Angeles, CA, USA

[b]Department of Environmental Health Sciences, Yale School of Public Health, New Haven, CT, USA

[c]Yale Center for Perinatal, Pediatric, and Environmental Epidemiology, Yale School of Public Health, New Haven, CT, USA

[d]Clinical Biomarkers Laboratory, Division of Pulmonary, Allergy, and Critical Care Medicine, School of Medicine, Emory University, Atlanta, GA, USA

[e]Perinatal Epidemiology and Health Outcomes Research Unit, Division of Neonatology, Department of Pediatrics, Stanford University School of Medicine and Lucile Packard Children's Hospital, Palo Alto, CA, USA

[f]California Perinatal Quality Care Collaborative, Palo Alto, CA, USA

[g]Program in Public Health, UCI Susan and Henry Samueli College of Health Sciences, Irvine, CA, USA

[h]Department of Environmental Medicine and Public Health, Icahn School of Medicine at Mount Sinai, New York, NY, USA

[i]Department of Medicine, Emory University, Atlanta, GA, USA

[j]Department of Neurology, UCLA School of Medicine, CA, USA

Abstract

Background: Maternal exposure to traffic-related air pollution during pregnancy has been shown to increase the risk of adverse birth outcomes and neurodevelopmental disorders. By utilizing high-resolution metabolomics (HRM), we investigated perturbations of the maternal serum metabolome in response to traffic-related air pollution to identify biological mechanisms.

Corresponding author: Beate Ritz, Mailing address: x650 Charles E Young Dr S, Los Angeles, CA 90095, Telephone number: +1-310-206-7458, britz@ucla.edu.

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Conflict of Interest
None.

Methods: We retrieved stored mid-pregnancy serum samples from 160 mothers who lived in the Central Valley of California known for high air particulate levels. We estimated prenatal traffic-related air pollution exposure (carbon monoxide, nitric oxides, and particulate matter less than 2.5 microns) during first-trimester using the California Line Source Dispersion Model, version 4 (CALINE4) based on residential addresses recorded at birth. We used liquid chromatography-high resolution mass spectrometry to obtain untargeted metabolic profiles and partial least squares discriminant analysis (PLS-DA) to select metabolic features associated with air pollution exposure. Pathway analyses were employed to identify biologic pathways related to air pollution exposure. As potential confounders we included maternal age, maternal race/ethnicity, and maternal education.

Results: In total we extracted 4,038 and 4,957 metabolic features from maternal serum samples in hydrophilic interaction (HILIC) chromatography (positive ion mode) and C18 (negative ion mode) columns, respectively. After controlling for confounding factors, PLS-DA (Variable Importance in Projection (VIP) >= 2) yielded 181 and 251 metabolic features (HILIC and C18, respectively) that discriminated between the high (n=98) and low exposed (n=62). Pathway enrichment analysis for discriminatory features associated with air pollution indicated that in maternal serum oxidative stress and inflammation related pathways were altered, including linoleate, leukotriene, and prostaglandin pathways.

Conclusion: The metabolomic features and pathways we found to be associated with air pollution exposure suggest that maternal exposure during pregnancy induces oxidative stress and inflammation pathways previously implicated in pregnancy complications and adverse outcomes.

Keywords

High resolution metabolomics; Air pollution; Oxidative stress; Inflammation; Exposome

1. Introduction

Maternal exposures to air pollutants including nitrogen oxides (NO2, NOx), particulate matter (PM10, PM2.5), carbon monoxide (CO) and ozone (O3) and their mixtures represented by various sources have been associated with pregnancy complications and adverse birth outcomes such as preeclampsia, gestational diabetes, preterm birth and low birth weight (Dadvand et al. 2013; Eze et al. 2015; Pedersen et al. 2013; Pedersen et al. 2014; Stieb et al. 2012; Wu et al. 2009). It has also been shown to affect fetal development especially neurodevelopment in offspring (Becerra et al. 2013; Glinianaia et al. 2004; Guxens et al. 2014; Suades-Gonzalez et al. 2015). However, there is still not much data available to elucidate relevant biologic pathways and mechanisms underlying air pollution related adverse pregnancy outcomes, even though it has been hypothesized that endocrine disruption, oxidative stress, inflammatory response, and DNA damage are major contributors (Hougaard et al. 2008; Kelly 2003; Risom et al. 2005).

High resolution metabolomics (HRM) is a powerful analytical approach to profile thousands of chemicals in biological specimens and to identify metabolic disturbances. Recently, researchers have used HRM to study the effects of environmental exposures such as metals,

tobacco smoking, and polycyclic aromatic hydrocarbons on human metabolism (Garcia-Sevillano et al. 2015; Gu et al. 2016; Jones et al. 2016; Wang et al. 2015).

There are currently no metabolomics studies that focus on air pollution exposure in pregnant women, however several previous studies have used HRM as a tool to study acute effects of air pollution on the human metabolome. The Cooperative Health Research in the Region of Augsburg (KORA) study in Germany conducted targeted metabolomics for 138 metabolites in older adults' serum samples and found lysophosphatidylcholines (LPCs) to be associated with short-term air pollution exposures, especially nitrogen dioxide (NO_2) (Ward-Caviness et al. 2016). A randomized, crossover trial that enrolled 55 healthy college students showed that short-term exposure to particulate matter less than 2.5 microns (PM2.5) induced metabolic changes associated with stress hormone levels, insulin resistance, and markers of oxidative stress and inflammation (H Li et al. 2017). Two other experimental studies using a crossover design to assess short-term ambient air pollution exposure identified a variety of metabolic features (van Veldhoven et al. 2018; Vlaanderen et al. 2017). One study utilized a multi-platform approach including gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) to identify metabolites in bronchial wash and bronchoalveolar lavage fluid samples from fifteen healthy subjects exposed to biodiesel exhaust (Surowiec et al. 2016). Recently, a panel study of healthy college students characterized the metabolic profile of plasma and saliva samples after traffic-related air pollution exposure and identified metabolites and pathways associated with oxidative stress, inflammation, and nucleic acid damage and repair (Liang et al. 2018). Most of the previous studies concentrated on short-term (hours or days) air pollution exposure, had relatively small sample sizes and assessed healthy adults.

Only two studies to date focused on long-term air pollution exposure. A cross-sectional study conducted within the TwinsUK cohort, found that oxidative stress and inflammation related metabolites such as α-tocopherol, benzoate, and glycine were associated with both long term air pollution (annual average PM2.5 concentration) and lung function (Menni et al. 2015). Another cross-sectional study with 59 healthy participants has linked year-long ultrafine particle exposure with metabolic variations related to antioxidant pathways and endothelial function (Walker et al. 2018).

We conducted an untargeted metabolomics study focusing on pregnant women since they and their offspring might be more susceptible to the adverse effects of air pollution. Specifically, in our study population, serum samples from 160 pregnant women were collected during the second trimesters of pregnancy, and average air pollution exposures (CO, nitric oxides (NOx), and PM2.5) during the first trimester of pregnancy were estimated based on residential addresses using an emissions based dispersion model– i.e. the California line source dispersion model, version 4 (CALINE4) (Benson 1984). We then utilized liquid chromatography with high-resolution mass spectrometry (LC-HRMS) to obtain maternal metabolic profiles. The HRM platform we adopted has also been used in three previous studies that examined ambient air pollution exposure (Ladva et al. 2018; Liang et al. 2018; Walker et al. 2018), and been shown to be sensitive enough to capture metabolic perturbations in blood samples induced by air pollution. The aims of this study were to identify changes in specific metabolic profiles associated with air pollution exposure

in pregnant women, and to conduct comprehensive pathway analysis to investigate potential biological mechanisms related to adverse health effect from air pollution exposure during pregnancy.

2. Method

2.1. Study population

We utilized the California birth records to randomly select mothers of children born between 2005 and 2010 from a previous case-control study of autism, all of whom were controls matched to children with autism in a 1:10 ratio by sex and birth year. All eligible mothers lived in the California Central Valley region according to the residential address recorded on the birth certificate (n = 1,466). In addition, mothers were eligible for the present study if their live born autism-free children did not have a birth defect recorded, were born at a gestational age between 21–46 weeks and had a recorded birth weight between 500g and 6800g (n = 1,433). The California central valley is known for its extremely high use of agricultural pesticides. Here, we restricted our analyses to mothers who according to our geographic information system (GIS) and state mandated pesticide use report based assessments had not been exposed to suspected neurotoxic pesticides (organophosphates, pyrethroids, glyphosates, fungicides, or neonicotinoids) (Rull and Ritz 2003). Considering the limited number of serum samples available for analysis via HRM and the generally high air pollution exposure levels in the study region, we further selected mothers based on the air pollution levels we modeled at residences to increase the statistical power of our study. Specifically, we used the population of 1,433 women originally randomly selected from birth certificates to estimate percentiles of air pollution exposure, and selected mothers from the extremes of the exposure distribution, considering women as “highly exposed” in the first trimester of pregnancy if three highly correlated CALINE4 modeled traffic related air pollutant levels (CO, NOx, and PM2.5) fell at or above the 75th percentile of the distribution, and as “low exposed” if their air pollutant levels fell below the 35th percentile. This was necessary because we did not have enough subjects available for a balanced selection of exposed and unexposed at the extremes of the air pollution distribution. Furthermore, mothers had to have utilized the California Prenatal Screening Program (CPSP) and their mid-pregnancy blood serum sample be stored and available (n = 160). About 74% of California women participate in the CPSP, which is a sequential serum screening program for birth defects during pregnancy that collects blood samples in mid-pregnancy (around the 16th week of gestation) (Cunningham and Tompkinison 1999). Maternal serum samples were collected by obstetrical care service providers and mailed to the state laboratory for analyses when subjects participated in the CPSP and specimens leftover after testing were stored at −20 °C at the California Biobank.

From birth records we obtained information on child sex, birth year, maternal age, race/ethnicity, education, and parity. Other potential confounders such as BMI or diet were not available in the birth records limiting this type of study.

2.2. Air pollution estimation

Residential addresses as recorded on birth certificates were geocoded using OpenSource geocoding software (Goldberg et al. 2008). We estimated each woman's average air pollutant exposure (i.e. CO, NOx, and PM2.5) during the first trimester, using a modified version of the CALINE4 that estimates pollution from sources within 1500 m of residential locations as described in detail elsewhere (Heck et al. 2013). In brief, input data for the prediction process included roadway geometry, traffic counts, emission factors, and meteorological parameters (wind speed, wind direction, temperature, stability class, and mixing heights). Year and season (winter and summer) and emission factors for CO, NOx and PM2.5 were obtained from the EMFAC2011 vehicle emissions model (California Air Resources Board 2013). CALINE4 predictions in this study do not incorporate background levels of pollutants, thus solely represent the contribution from local traffic emissions (Benson 1989; Broderick et al. 2005; Levitin et al. 2005; Marmur and Mamane 2003; Wu et al. 2016).

Subjects were classified based on their air pollution levels such that 98 pregnancies were considered "highly exposed" in the first trimester, while 62 were "low exposed" (Supplemental Table 1).

2.3. High-resolution metabolomics

HRM profiling was completed according to established methods (Walker et al. 2018; Walker et al. 2019). Serum samples were transported from California Biobank to Emory and stored at −80 °C. Batches of 40 serum samples were removed from storage and thawed on ice. Each sample was then thoroughly vortexed, and 65 μL of serum was treated with 130 μL of LC-MS grade acetonitrile. The extract was equilibrated for 30 min on ice and centrifuged at 16,100 × g for 10 min to remove precipitated proteins. The resulting supernatant was transferred to an autosampler containing a low volume insert and maintained at 4°C until analysis (<24 h). NIST 1950 (Simon-Manso et al. 2013) was analyzed at the beginning and end of the entire analytical run and for additional quality control (QC) two replicate pooled human plasma samples were analyzed at the beginning, middle, and end of each batch of 40 samples for normalization and batch effect evaluation.

Sample extracts were analyzed in triplicate using a dual column, dual polarity approach that includes hydrophilic interaction (HILIC) chromatography with positive ESI and C18 chromatography with negative ESI (Ultimate 3000, Q-Exactive HF, Thermo Fisher, *m/z* range 85–1275) (Walker et al. 2018). Following a 10 μL sample injection, HILIC separation was accomplished using a 2.1 cm × 5 cm × 2.5 μm HILIC column (Waters XBridge BEH Amide XP HILIC) and acetonitrile gradient (A= water, B= acetonitrile, C= 2% formic acid) consisting of an initial 1.5 minute period of 22.5% A, 75% B, 2.5% C, followed by linear increase to 77.5% A, 20% B, 2.5% C at 4 min and hold for 1 minute. Separation by C18 was with 2.1 cm × 5 cm × 3 μm column (Higgins endcapped C18) with C= 10mM ammonium and the following gradient: initial 0.5 minute period of 60% A, 35% B, 5% C, followed by linear increase to 0% A, 95% B, 5% C at 1.5 min and then held for an additional 3 minutes. Mobile phase flow rate was held at 0.4 mL/min for 1.5 minutes, and then increased to 0.5 mL/min. The mass spectrometer was operated using ESI mode at a resolution of 120,000

and mass-to-charge ratio (*m/z*) range 85–1275. Source tune settings included capillary temperature, sheath gas, auxiliary gas, sweep gas and spray voltage settings of 300°C, 45 (arbitrary units), 25 (arbitrary units), 1 (arbitrary units) and +3.5 kV, respectively for positive mode, and 200°C, 30 (arbitrary units), 5 (arbitrary units), 1 (arbitrary units) and +3.0 kV for negative mode. S-Lens RF level was maintained at 45. High-resolution detection of *m/z* features was accomplished by maximum injection time of 10 milliseconds and AGC target of 1×10^6. Raw data files were extracted and aligned using apLCMS (Yu et al. 2013) with modifications by xMSanalyzer (Uppal et al. 2013). Uniquely detected ions consisted of *m/z*, retention time and ion abundance, referred to as *m/z* features. Prior to data analysis, *m/z* features were batch corrected using ComBat (Johnson et al. 2007).

2.4. Statistical analyses

For three replicates of each feature, intensities were summarized using the median, except when more than 50% of the replicates were missing (in this instance the value was set to missing). Metabolomic data was then filtered to keep only features present in at least 80% of one comparison group and >50% of all samples together. After filtering, missing values were imputed by one-half of the lowest signal detected for that feature across all samples.

Feature intensities were log2 transformed before analyses. To control for potential confounding, we used residuals of intensities derived from linear regression against potential confounders including maternal age, maternal race/ethnicity, and maternal education.

We performed partial least squares discriminant analysis (PLS-DA) to identify features associated with air pollution exposure. PLS-DA is a supervised, multivariate analysis approach for dimensionality reduction that maximizes covariance between intensities of metabolic features and air pollution exposure (Wold et al. 2001). We selected features with a Variable Importance in Projection (VIP) scores >= 2 (Le Cao et al. 2009). Fold change was calculated as the ratio of raw intensities between high and low-exposed groups. To evaluate the performance of selected features, we conducted 10-fold cross-validation tests utilizing the support vector machine (SVM) and calculated the classification accuracy of the selected features. All feature selection approaches were implemented with the R package mixOmics v6.3.1.

2.5. Pathway analysis and annotation

Discriminative features selected by PLS-DA were annotated using xMSannotator (Uppal et al. 2017). Accurate mass *m/z* for adducts formed under positive/negative ESI mode was matched to the Human Metabolome Database (HMDB), Kyoto Encyclopedia of Genes and Genomes (KEGG), and LipidMaps with a mass error threshold of 10 ppm. xMSannotator also takes into consideration correlation of intensities and retention time, and assigns confidence scores based on a multilevel scoring algorithm which ensures the accuracy of annotation.

In order to identify enriched metabolic pathways comparing high and low exposed groups, we conducted pathway enrichment analysis utilizing Mummichog (v. 1.0.10) (Li et al. 2013). All discriminating features previously selected by PLS-DA with VIP >= 2 were included in this pathway enrichment analysis. Mummichog is a novel pathway and module

enrichment analysis algorithm designed specifically for high-resolution liquid chromatography-mass spectrometry. Although the tentative annotation results in mummichog may include false positives, the enriched pathways inferred by the algorithm have been proven to be valid and to reflect real biological activity (S. Li et al. 2016; Shuzhao Li et al. 2016; S Li et al. 2017; Uppal et al. 2016). All metabolites annotated by mummichog were required to present in at least their primary adduct (M+H or M-H for positive and negative mode respectively) to reduce the false positive match rate. The P-value threshold we relied on was 0.05. Only enriched pathways with at least 3 overlapping metabolites were kept for further evaluation and interpretation.

Some discriminating features were further confirmed by matching the accurate mass *m/z* and retention time to authentic chemical standards analyzed using LC-MS/MS. The error tolerance was 5 ppm for *m/z* and 15 seconds for retention time.

3. Results

Demographics of the 160 subjects are provided in Table 1. Mothers who were younger, less educated, or of Hispanic origin were more likely to be highly exposed to air pollution in this study population.

In total, we detected 14,555 features (6,139 in HILIC column and 8,416 in C18 column), but after filtering for missing values, 8,995 features remained (4,038 in HILIC column and 4,957 in C18 column). We identified 181 and 251 unique metabolic features that were associated with air pollution exposure during pregnancy from both HILIC and C18 column respectively (Figure 1) using PLS-DA and adjusting for maternal age, ethnicity and education while setting the VIP scores greater than 2. The balanced classification rate derived from 10-fold CV showed that both sets of discriminatory features effectively separate the classes (82.5% and 70.4% for HILIC and C18 respectively).

Annotation of PLS-DA discriminatory features included 187 *m/z* features matching to one or more unique metabolites with medium or high confidence scores. Because compounds were present in the databases used for annotation that share the same chemical formula but different structure, we confirmed the annotation results by matching the retention time and *m/z* to authenticated chemical standards verified by tandem mass spectrometry. In total, we confirmed 6 metabolites including serine, creatinine, L-histidine, myo-inositol, linoleic acid, and heptadecanoic acid (confidence level 1). Their chemical identities are shown in Table 2. Among them, creatinine was positively associated, while four other metabolites were negatively associated with air pollution.

Using mummichog, we examined whether the features that were selected by PLS-DA were enriched within specific metabolic pathways. The result indicated that 24 metabolic pathways were differentially enriched with a P-value < 0.05 (Table 3). Tentative annotation results of metabolites in each pathway are provided in Supplemental Table 2. Nine are lipid-related metabolic pathways including fatty acid activation, de novo fatty acid biosynthesis, and glycosphingolipid metabolism. Changes in these pathways may indicate associations between air pollution exposure and oxidative stress; features in the linoleate pathway were

annotated as oxidative stress biomarkers or antioxidants including linoleic acid, 13-HODE, 13-oxo-ODE, gamma-linolenic acid, and azelaic acid. Linoleic acid was further confirmed by an authentic standard (confidence level 1). Disruption of eicosanoid metabolism such as the leukotriene metabolism and prostaglandin formation from arachidonate indicates an inflammatory response. Putative annotations in the leukotriene metabolism pathway included 12-oxo-LTB4 and 20-OH-LTE4, all of which showed increased abundance in the high air pollution exposure group. Other pathways associated with oxidative stress included vitamin E metabolism, xenobiotic metabolism, urea cycle/amino group metabolism, and some amino acid metabolism pathways such as histidine metabolism and lysine metabolism. Perturbations were also observed in nucleotide metabolism and several carbohydrate metabolism related pathways.

4. Discussion

The untargeted HRM approach we employed provided us with an opportunity to explore the relationship between traffic related air pollution exposures and metabolic signatures in maternal blood in mid-pregnancy. Measuring more than 10,000 metabolic features in serum samples using HRM and comparing metabolic profiles according to exposure, we illustrated that high air pollution exposures during pregnancy are linked to widespread perturbations in the maternal serum metabolome. Sorting these features according to biologic pathways allowed us to identify potential mechanisms by which air pollution may affect pregnant women and fetuses. Of particular importance is that the enriched pathways identified were related to oxidative stress and inflammatory reactions, which have been implicated in many pathological conditions, including adverse birth outcomes such as preterm birth, intrauterine growth restriction, low birth weight, and preeclampsia (Sultana et al. 2017) as well as neurodevelopmental disorders (Patterson 2009).

We observed alterations in fatty acid metabolism, phospholipid metabolism, linoleate metabolism, and eicosanoids including leukotriene and prostaglandin metabolism (see Table 3 and Figure 2). Traffic-related air pollutants may act directly as free radicals or generate free radicals and cause oxidative stress (Kelly 2003). One of the primary targets of reactive oxygen species derived from air pollutants is the cell membrane. Oxidative stress can induce the activation of phospholipase A2 (PLA2) which then hydrolyze phospholipid (PL) from the cell membrane to generate polyunsaturated free fatty acid and lyso-PL (Anthonymuthu et al. 2018; Sato et al. 2016). Major polyunsaturated fatty acids (PUFA) released through this step include linoleic acid and arachidonate acid. Due to the existence of double carbon bonds, these fatty acids can be subsequently oxidized by oxygenase (Sato et al. 2016). As one of the omega-6 polyunsaturated fatty acids, linoleic acid generates 13-hydroperoxyoctadecadenoic acid (9-HPODE) and 13-HPODE through lipoxygenases. These products then convert to 9-hydroxyoctadecadienoic acid (9-HODE) and 13-HODE (Tam et al. 2013), and both are potential biomarkers for oxidative stress affecting lipids. In our study, we observed associations with the linoleate pathway, specifically, air pollution exposure increased metabolomic features we matched to 13-HODE; while linoleic acid and 13-keto-9Z,11E-octadecadienoic acid (13-oxo-ODE), a downstream product derived from 13-HODE were decreased in the highly exposed group. In particular, we were able to confirm the identification of linoleic acid and gamma-linolenic acid within the linoleate pathway

using authentic standards. Gamma-linolenic acid is an anti-inflammatory metabolite that can inhibit the biosynthesis of leukotriene B4 (Horrobin 1992; Liang et al. 2018). Although below the discriminative feature selection threshold (VIP=1.31) in our data, we found gamma-linolenic acid to be negatively associated with air pollution exposure, which is consistent with previous studies (Liang et al. 2018; Walker et al. 2018).

In addition to linoleic acid, arachidonic acid is another omega-6 PUFA affected by air pollution induced oxidative stress. As a key inflammatory intermediate, arachidonic acid is being released from cell membranes and converted to eicosanoids through several pathways including: the lipoxygenase (LOX) pathway, where arachidonic acids is dioxygenated to produce hydroperoxyeicosatetraenoic acid (HPETE) and then converted to leukotrienes and other lipoxins; and the cyclooxygenase (COX) pathway that produces prostaglandin (Tam et al. 2013). Both leukotrienes and prostaglandins are major proinflammatory mediators.

In our study, two of the features found to be enriched in the leukotriene pathways were annotated as 12-oxo-leukotriene B4 and 10,11-dihydro-12-oxo-leukotriene B4. 12-oxo-LTB4 was found to be lower in the exposed group, while 10,11-dihydro-12-oxo LTB4 was increased. Both are downstream metabolites of LTB4, but possibly due to the rapid metabolism of 12-oxo-LTB4 to 10,11-dihydro-12-oxo-LTB4 by reductase (Powell et al. 1996; Wainwright and Powell 1991) only the end product in this pathways is found to be increased. LTB4 is a potent lipid chemoattractant that can induce an inflammatory response. It is synthesized through the LOX pathway and recruits leukocytes leading to inflammatory reactions (Ford-Hutchinson et al. 1980). Due to their pro-inflammatory effect, altered levels of LTB4 and its metabolites during pregnancy have been linked to various adverse health outcomes (e.g. asthma) and birth complications such as preterm birth and preeclampsia (Biagi et al. 1990; Busse 1998; Romero et al. 2007). Recently, a lipidomics study conducted in 197 mother-newborn pairs measured the association between in-utero PM2.5 exposure and oxylipin profiles in newborns. Consistent with our study, they also found significant differences in cord blood levels for metabolites derived from the LOX pathway (Martens et al. 2017).

Prostaglandins are a type of eicosanoid derived from arachidonic acids through COX. Mummichog annotated 8 features to be enriched in prostaglandin pathways including multiple prostaglandins and related metabolites. Previously, animal and human studies have linked excessive prostaglandins with NO2 inhalation (Yan et al. 2016), ozone (Peden 1999; Peden 2001), PM2.5, and sulfate (W Li et al. 2016). Increased levels of 8-epi-prostaglandin F2a are widely used as indicators of lipid peroxidation (Tacconelli et al. 2010), and the pro-inflammatory effects of prostaglandins are well documented (Funk 2001). Prostaglandins play important roles during pregnancy including vasodilatation and uterine contraction. Perturbations of the prostaglandin pathway are most likely responsible for abnormal placental and uterine blood flow. Several in-vivo and in-vitro studies have associated prostaglandins with preeclampsia (Kaaja et al. 1995; Ogburn et al. 1984).

In addition to lipid-related pathways, vitamin E metabolism is also associated with oxidative stress. Vitamin E, including tocopherols and tocotrienols, are lipid-soluble antioxidants and lipid peroxy radical scavengers (Chow 1991; DellaPenna and Mène-Saffrané 2011; Noctor

et al. 2015). Vitamin E can protect membrane lipids from oxidation by physically or chemically quenching singlet oxygen or by donating a hydrogen atom to the PUFA peroxy radical thereby interrupting the peroxidative reaction (DellaPenna and Mène-Saffrané 2011). Two previous metabolomics studies found the activity of vitamin E metabolism to be decreasing with air pollution exposure (Liang et al. 2018; Menni et al. 2015). In our study, metabolites of tocotrienols showed alterations with a decrease in antioxidant and increase in oxidized metabolites among the exposed. Within the Vitamin E metabolism pathway, a feature tentatively annotated as 13'-carboxy-alpha-tocotrienol (mz:452.2952, M-H) was found to be higher while a feature tentatively annotated as alpha-tocotrienol (mz:423.3265, M-H) was lower in the exposed group.

Several amino acid metabolism pathways were also enriched including the methionine, cysteine, and histidine pathways which are sulfur-containing amino acids that are readily oxidized (Berlett and Stadtman 1997; Pisoschi and Pop 2015). Previous studies described oxidation of methionine to be associated with ambient PM exposure in mice (Lai et al. 2016; Lee et al. 2014). In our samples, histidine and its pathway were found to be reduced among the air pollution exposed. Notably, we were able to annotate histidine with confidence level 1 using the authentic standard. Histidine has been reported to have anti-inflammatory effects, and previous studies found that histidine was negatively associated with inflammation and oxidative stress (Liang et al. 2018; Niu et al. 2012; Uchida 2003; Watanabe et al. 2008).

Numerous in-vitro and in-vivo studies have shown that exposure to air pollution induces oxidative stress and inflammatory reactions (Daher et al. 2014; Dick et al. 2003; Ghio et al. 2012; Guerra et al. 2013; Happo et al. 2013). Moreover, the pregnancy period is especially vulnerable to oxidative stress due to the necessary increased energy expenditure (Nagiah et al. 2015). Two studies measuring specific biomarkers for oxidative stress indicated that air pollution exposure during pregnancy elevated oxidative stress responses (Anderson et al. 2018; Nagiah et al. 2015). Consistent with these results, our untargeted systematic approach also identified pathways predominantly enriched for oxidative stress and inflammatory responses. Maternal oxidative stress may cause damage to all major cellular elements and might especially affect the placenta and its function, therefore contributing to adverse birth outcomes such as spontaneous abortion, preeclampsia, intrauterine growth restriction, low birth weight, and preterm delivery (Al-Gubory et al. 2010; Duhig et al. 2016; Lavigne et al. 2018; Peter Stein et al. 2008).

Our study has some limitations. Although model-based air pollution estimation has a high spatial resolution, they do not necessarily translate into personal exposure because 1) there are inherent uncertainties in an emissions based dispersion model; 2) emission sources other than local traffic are not addressed by the model; 3) lack of information about work location and subjects' time-activity. Thus, we did not estimate 'personal' air pollution exposure but rather the contributions by local traffic sources to personal exposure. Local traffic noise might be a confounder, if it influences the same metabolic pathways as air pollution. However, we recently found that traffic noise estimates were only moderately correlated with CALINE4 estimates of traffic related air pollution (r=0.4) at the residences in Northern CA counties (unpublished data); most likely because air pollution and noise physically

behave differently and CALINE4 estimates take factors such as meteorology into consideration.

Besides air pollution exposure, active or passive smoking and diet may explain differences in metabolites. We did not have information on the subjects' smoking behavior or dietary intake during pregnancy, however in order for smoking or dietary differences to have confounded our analyses, they would have had to be related to air pollution exposures. By controlling for maternal age, maternal race/ethnicity, and maternal education, we hope to have at least partially addressed potential confounding. Additionally, while there is no active or second-hand smoking information on the birth records from these years, we were able to identify cotinine levels in the serum by matching the accurate mass m/z (177.1022) and retention time (32.8s) to authentic standards. Previous studies have shown that cotinine is a reliable marker of smoking [4–6]. Cotinine levels among women with high or low traffic-related air pollution exposure were generally extremely low, indicating that the percentage of active or passive smokers in our study is low - as expected in California. Also, the intensities of cotinine were not statistically significantly different (t-test p-value=0.34) for exposed versus unexposed women's samples. Nevertheless, residual uncontrolled confounding is possible. Also, women with less than a high school education are likely more recent immigrants from Mexico (Hoggatt et al. 2012). If these women are introducing a 'healthy migrant' bias this may bias our results.

To assess whether air pollution impacts on metabolomic profiles differed by ethnicity, we also conducted analyses in subgroups of Hispanic and Non-Hispanic women. We found that high level of traffic-related air pollution was associated with 9 metabolic pathways in both subgroups; importantly, we identified linoleate metabolism as well as methionine and cysteine metabolism pathways in both groups consistent with the induction of oxidative stress.

Another limitation is an inherent challenge in the untargeted metabolomics analysis. Without further metabolite identification using tandem MS, we could only tentatively annotate the extracted features using computational approaches. Adopting a pathway and network analysis approach, we were able to improve annotation results, but there may still be some false matches that could have influenced the interpretation. It is recommended to improve the identification of metabolites using either tandem MS or internal standards in future studies.

5. Conclusions

In summary, we applied HRM to identify perturbations in the serum metabolome associated with traffic-related air pollution exposure during pregnancy. We observed metabolic pathways consistent with oxidative stress and inflammatory reactions, which may contribute to adverse health outcomes in offspring and we corroborated previous results of metabolome studies conducted in air pollution exposed non-pregnant adults.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- High-resolution metabolomics detected metabolic signals associated with air pollution exposure in maternal mid-pregnancy serum.
- High levels of air pollution in early pregnancy affected oxidative stress and inflammation according to pathway enrichment analyses.
- Maternal metabolism derived markers pointed to mechanisms in which air pollution may cause adverse pregnancy and birth outcomes well documented in epidemiologic studies worldwide.

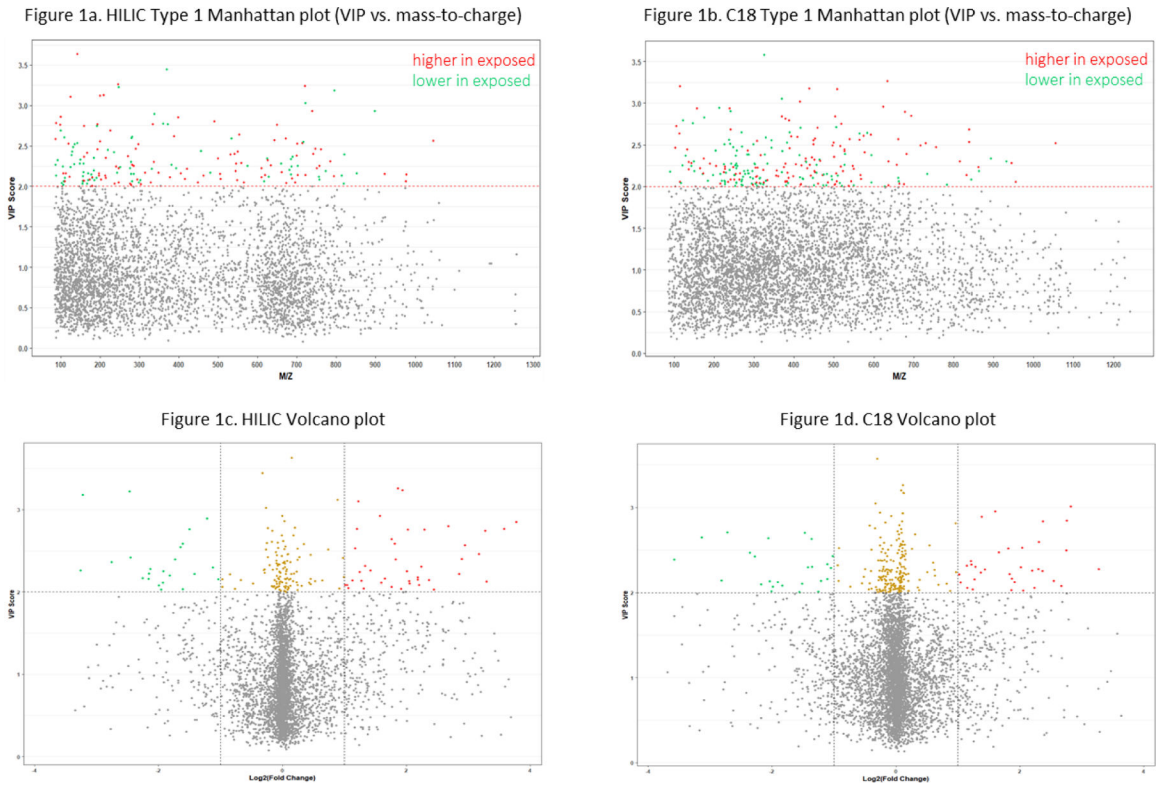

Figure 1. Identification of metabolic features associated with air pollution exposure during pregnancy.

A) Type 1 Manhattan plot for features in the HILIC column (positive ion mode), VIP score vs *m/z*. 181 *m/z* features were found above the VIP >= 2 threshold. Red dots represent the features that were higher in the high air pollution exposure group and the green dots represent the features that were lower in the high air pollution exposure group; B) Type 1 Manhattan plot for features in the C18 column (negative ion mode), VIP score vs mass-to-charge. 251 *m/z* features were found above the VIP >= 2 threshold; C) Volcano plot for features in the HILIC column; D) Volcano plot for features in the C18 column.

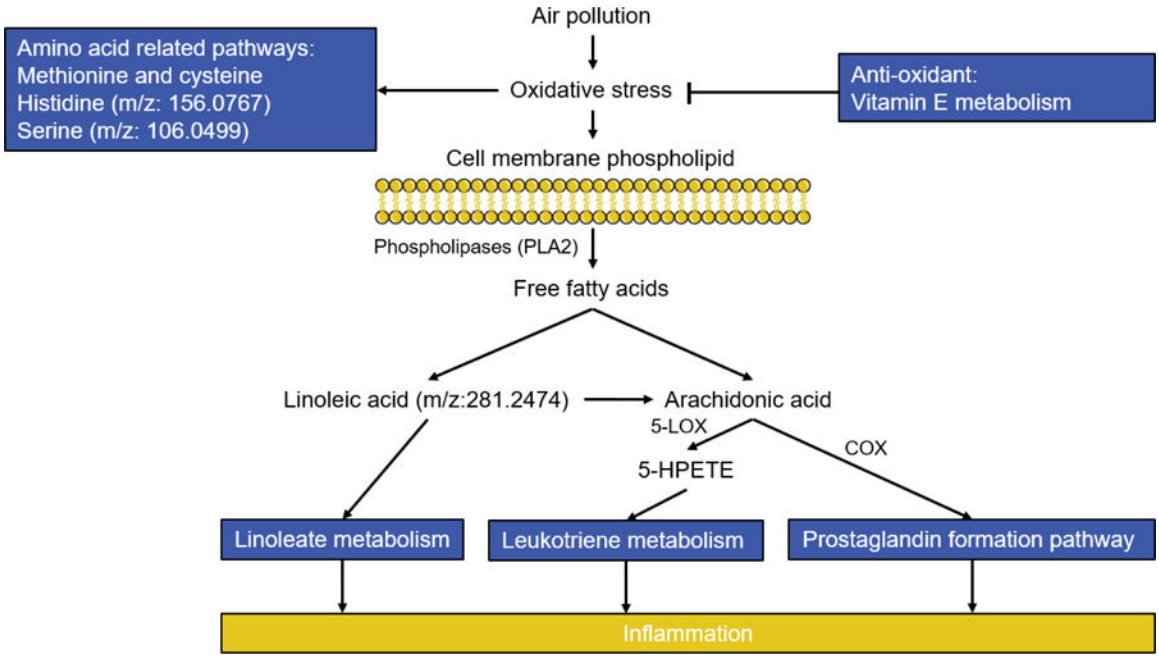

Figure 2. Enriched metabolic pathways in response to air pollution induced oxidative stress.
Pathways in blue boxes are significantly enriched pathways.

Table 1

Demographic characteristics of mothers and children

	High Exposure n=98		Low Exposure n=62	
	n	%	n	%
Maternal age at time of pregnancy (years)				
<= 18	18	18.4	5	8.1
19–25	42	42.9	17	27.4
26–30	24	24.5	20	32.3
> 30	14	14.3	20	32.3
Maternal race/ethnicity				
Non-Hispanic White	11	11.2	21	33.9
Hispanic	70	71.4	30	48.5
Others (African American/Black, Asian)	17	17.4	11	17.7
Maternal education				
Less than 12th grade	47	48.0	11	17.7
High school graduate or equivalent	24	24.5	22	35.5
Some college	20	20.4	12	19.4
College or more	7	7.1	17	27.4
Mother born in the US				
Yes	61	62.2	46	74.2
No	37	37.8	15	24.2
Missing	0	0.0	1	1.6
Preterm birth				
Yes	12	12.2	7	11.3
No	86	87.8	55	88.7
Child sex				
Male	79	80.8	46	74.2
Female	19	19.2	16	25.8

Table 2

Confirmed [a] chemical identity of the discriminatory metabolic features associated with high traffic-related air pollution

m/z	RT (s)	Adduct Form	Metabolite	Fold Change (Log2)	Column
106.0499	94.2	M+H[1+]	Serine	−0.05	HILIC
114.0662	47.0	M+H[1+]	Creatinine	0.45	HILIC
156.0767	112.7	M+H[1+]	L-Histidine	−0.05	HILIC
215.0328	28.6	M+Cl[1−]	Myo-Inositol	1.24	C18
269.2485	271.5	M-H[1−]	Heptadecanoic acid	−0.25	C18
281.2474	33.6	M+H[1+]	Linoleic acid	−0.15	HILIC

[a]Chemical identification was conducted by matching peaks by accurate mass and retention time to authentic reference standards in an in-house library run under identical conditions using tandem mass spectrometry.

Table 3

Enriched metabolic pathways associated with traffic-related air pollution

Column	Pathway	Overlap size	Pathway size	P-value [a]
HILIC	Urea cycle/amino group metabolism	7	40	0.0015
	Glycosphingolipid metabolism	5	22	0.0015
	Histidine metabolism	4	17	0.0020
	Glycerophospholipid metabolism	4	30	0.0081
	Linoleate metabolism	3	19	0.0100
	Glycine, serine, alanine and threonine metabolism	4	36	0.0162
	Pyrimidine metabolism	3	30	0.0435
C18	Fatty acid activation	10	16	0.0002
	De novo fatty acid biosynthesis	7	15	0.0002
	Glycosphingolipid metabolism	7	24	0.0003
	Keratan sulfate degradation	3	6	0.0006
	Fatty Acid Metabolism	4	13	0.0008
	TCA cycle	3	14	0.0049
	Prostaglandin formation from arachidonate	8	54	0.0061
	Lysine metabolism	4	24	0.0074
	Glycerophospholipid metabolism	6	40	0.0074
	Xenobiotics metabolism	8	59	0.0110
	Glycolysis and Gluconeogenesis	4	27	0.0123
	Methionine and cysteine metabolism	6	44	0.0127
	Fructose and mannose metabolism	3	19	0.0141
	Vitamin E metabolism	4	29	0.0167
	Butanoate metabolism	3	20	0.0168
	Linoleate metabolism	3	20	0.0168
	Phosphatidylinositol phosphate metabolism	3	22	0.0231
	Purine metabolism	5	42	0.0276
	Leukotriene metabolism	5	43	0.0307
	Sialic acid metabolism	3	28	0.0491

[a] P-value calculated by mummichog are gamma-adjusted p-values based on permutation tests by resampling from the reference list (Li et al. 2013).