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Length of $PM_{2.5}$ exposure and alterations in the serum metabolome among women undergoing infertility treatment

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Background: Both acute and chronic exposure to fine particulate matter ($PM_{2.5}$) have been linked to negative health outcomes. Studies have used metabolomics to describe the biological pathways linking $PM_{2.5}$ with disease but have focused on a single exposure window. We compared alterations in the serum metabolome following various short- and long-term $PM_{2.5}$ exposures.

Methods: Participants were women undergoing *in vitro* fertilization at a New England fertility clinic (n = 200). Women provided their residential address and provided a blood sample during controlled ovarian stimulation. $PM_{2.5}$ exposure was estimated in the 1, 2, and 3 days, 2 weeks, and 3 months prior to blood collection using a validated spatiotemporal model. We utilized liquid chromatography with high-resolution mass spectrometry. We used generalized linear models to test for associations between metabolomic features and $PM_{2.5}$ exposures after adjusting for potential confounders. Significant features ($P < 0.005$) were used for pathway analysis and metabolite identification.

Results: We identified 17 pathways related to amino acid, lipid, energy, and nutrient metabolism that were solely associated with acute $PM_{2.5}$ exposure. Fifteen pathways, mostly, pro-inflammatory, anti-inflammatory, amino acid, and energy metabolism, were solely associated with long-term $PM_{2.5}$ exposure. Seven pathways were associated with the majority of exposure windows and were mostly related to anti-inflammatory and lipid metabolism. Among the significant features, we confirmed seven metabolites with level-1 evidence.

Conclusions: We identified serum metabolites and metabolic pathways uniquely associated with acute versus chronic $PM_{2.5}$ exposure. These different biologic pathways may help explain differences in disease states when investigating different lengths of $PM_{2.5}$ exposure.

Key Words: amino acid metabolism, anti-inflammatory, energy metabolism, lipid metabolism, $PM_{2.5}$, pro-inflammatory, untargeted metabolomics

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Introduction

Fine particulate matter ($PM_{2.5}$) air pollution is a complex mixture of liquid and solid particulates with a diameter of 2.5 micrometers or less and can penetrate deeply into the respiratory tract. $PM_{2.5}$ has been linked to a myriad of negative health outcomes in humans including but not limited to respiratory diseases, cardiovascular diseases, neurological and mental health issues, adverse reproductive and pregnancy outcomes, and mortality.[1–6] To better understand the underlying pathways between $PM_{2.5}$ and these health outcomes, several studies have used both targeted and untargeted methods to investigate potential alterations in the human metabolome.[7–17] Metabolomics is a relatively new field that focuses on global detection and relative quantification of small molecules, both endogenous and exogenous, in human tissues and fluids and evaluates how changes in these molecules are related to changes in exposures and disease states. Using metabolomics, several studies have observed alterations in pro-inflammatory and oxidative stress pathways when people are exposed to $PM_{2.5}$.[7,9,10,12–14,17,18] While they have yielded important findings,

What this study adds

Both short- and long-term exposure to $PM_{2.5}$ has been related to adverse health outcomes. However, the biological pathways underlying these health effects are largely unknown. We identified several unique serum metabolomic pathways associated with acute and chronic $PM_{2.5}$ exposure. Major pathways associated with acute $PM_{2.5}$ exposure included amino acid, energy, and lipid metabolism. Major pathways associated with chronic $PM_{2.5}$ exposure included pro-inflammatory and anti-inflammatory pathways. Seven unique metabolites were identified with level-1 evidence.

these studies are limited by the use of a singular time window for exposure to $PM_{2.5}$.

There are several downsides to focusing solely on one time window of $PM_{2.5}$ exposure. Chiefly, several studies examining the health effects of $PM_{2.5}$ exposure have demonstrated that both short- and long-term exposure to $PM_{2.5}$ are important but long-term exposure may elicit a greater or different response than short-term exposure. In studies of Medicare patients in New England, both acute (1–2 days) and chronic (1–7 years) measures of $PM_{2.5}$ exposure were associated with increased hospital admissions and mortality but the effect estimates for chronic $PM_{2.5}$ exposure were of greater magnitude than the acute exposures.[19,20] Recently, the same logic has also held true in studies examining adverse pregnancy outcomes, specifically preterm birth. Among a cohort of pregnancies in China, daily exposure to $PM_{2.5}$ in the one to six days prior to delivery and chronic exposure to $PM_{2.5}$ throughout pregnancy were both associated with an increased risk of preterm birth; however, the magnitude of association gradually increased as the moving average of $PM_{2.5}$ exposure expanded from one to 37 weeks prior to birth and the greatest effect estimate was observed for chronic exposure during the entire pregnancy.[21] While both acute and chronic exposures may influence risk of preterm birth, the underlying biological mechanisms may differ. Several pathways have been proposed to underlie the association between both acute and chronic $PM_{2.5}$ exposure and preterm birth including heightened oxidative stress, inflammation, and endocrine disruption.[22]

To our knowledge, there has only been one previous study that examined differences in the human metabolome during different windows of $PM_{2.5}$ exposure. Among 197 Belgian mother-infant pairs, Martens et al. used targeted metabolomics to measure 37 oxylipins in neonatal cord blood plasma samples and related these to *in utero* $PM_{2.5}$ exposures. Alterations in metabolites derived from the lipoxygenase pathway were only observed when examining total $PM_{2.5}$ exposure during pregnancy or second-trimester $PM_{2.5}$ exposure (but not first or third-trimester exposures).[23] Martens et al., hypothesized that this difference may be due in part to the thinning barrier between the maternal and fetal blood supplies with increasing gestational age and with the increasing fetal capillaries size until week ten of gestation.[23] These results support the hypothesis that the timing and duration of $PM_{2.5}$ exposures are important to consider, particularly for outcomes that may have critical windows of susceptibility and both short- and long-term exposure-response relationships.

To expand on the limited literature, our study sought to investigate the similarities and differences in how varying durations of $PM_{2.5}$ exposure may alter the serum metabolome. Specifically, we explored three acute, one intermediate, and one longer-term time window of $PM_{2.5}$ exposure and their association with metabolic features and metabolomic pathways identified using untargeted metabolomics. Untargeted metabolomics allows for a greater examination of the metabolome rather than targeting a single pathway or class of metabolites. Understanding the changes in the metabolome across exposure windows may offer novel insight into how acute and chronic exposure to $PM_{2.5}$ may lead to different disease states in humans and could lead to biomarkers for specific durations of exposure.

Materials and methods

Study population

The women included in our analysis were participants in the Environment and Reproductive Health (EARTH) study.[24] Briefly, the EARTH study was a prospective cohort that enrolled couples seeking infertility evaluation and treatment at the Massachusetts General Hospital (MGH) Fertility Center. The goal of the study was to evaluate how environmental and dietary factors influence fertility. Upon enrollment, women completed questionnaires on demographics, medical history, environmental exposures, diet, lifestyle, and reproductive health. Participants' height and weight were also measured via study staff to calculate body mass index (BMI; kg/m^2). Women provided their residential address, initially for reimbursement purposes, but later these were used for geocoding and linking to environmental exposure data. The EARTH study was approved by the Human Studies Institutional Review Boards of MGH and the Harvard T.H. Chan School of Public Health (IRB No. 1999P008167). All study participants signed an informed consent after the study procedures were explained by research study staff.

In 2019, we randomly selected 200 women using a random number generator (from the 345 women with complete air pollution data who underwent a fresh, autologous assisted reproductive technology (ART) cycle between 2005 and 2015)[25] for inclusion in a metabolomics sub-study. All of these women provided a non-fasting blood sample during controlled ovulation stimulation, between 2005 and 2015, which was used for metabolomic profiling. The blood samples were collected via venipuncture during a routine morning appointment (between 7 am and 10 am). Approximately 6-ml of blood was collected from each participant. Serum was centrifuged, aliquoted, and stored at −20°C initially before being transferred to Harvard for storage at −80°C.

Air pollution measures

We estimated individual ambient $PM_{2.5}$ exposure by linking women's geocoded residential address at enrollment to a spatiotemporal model of $PM_{2.5}$ exposure at a 1 km^2 spatial resolution.[26] The validated hybrid model of ground-level $PM_{2.5}$ concentrations used satellite-derived aerosol optical depth measurements, land use (e.g., measures of population density, elevation, traffic, percentages of land use, normalized difference vegetation index (NDVI), and point and source pollutant emissions), meteorological (e.g., air temperature, wind speed, daily visibility, sea-land pressure, and relative humidity) variables, and temporally resolved data on planetary boundary layer to estimate exposure.[26] All data used for the $PM_{2.5}$ model were publicly available and obtained from a variety of sources including satellites (aerosol optical depth data), the US Environmental Protection Agency (EPA) (monitoring data), the US Geological Survey National land Cover dataset (spatial data), the National Climatic Data Center (meteorological data), Moderate Resolution Imaging Spectroradiometer (MODIS) satellite NDVI (NDVI data), and the National Oceanic and Atmospheric Administration (planetary boundary layer). We derived daily estimated ambient $PM_{2.5}$ concentrations starting three months prior to the date of blood collection. Air pollution exposures per day were averaged across the following windows: one day, two days, three days, two weeks, and three months prior to blood collection to examine short-term (one-three days), intermediate (two weeks), and longer-term (three months) exposures to $PM_{2.5}$.

High-resolution metabolomics

Using established standard protocols,[11–13] samples were treated with two volumes of acetonitrile and were centrifuged. Samples were analyzed in triplicate. Prepared samples were analyzed via liquid chromatography with high-resolution mass spectrometry (LC-HRMS) (Dionex Ultimate 3000 RSLCnano; Thermo Orbitrap Fusion; Thermo Fisher Scientific, Waltham, MA). We utilized two column types, C18 hydrophobic reversed-phase chromatography (C18 Neg) with negative electrospray ionization (ESI) and hydrophilic interaction liquid chromatography (HILIC) with positive ESI. In the C18 column, analyte separation was achieved using water, acetonitrile, and 10 mM ammonium acetate during the mobile phase with the following gradient elution: initial one minute period, 60% water, 35% acetonitrile, and 5% ammonium acetate, followed by a linear increase to

0% water, 95% acetonitrile, and 5% ammonium acetate at three minutes and held for the remaining two minutes. In the HILIC column, analyte separation was achieved using water, acetonitrile, and 2% formic acid during the mobile phase with the following gradient elution: initial one-and-a-half-minute period, 22.5% water, 75% acetonitrile, and 2.5% formic acid, followed by a linear increase to 75% water, 22.5% acetonitrile and 2.5% formic acid at 4 minutes and a final hold of 1 minute. The mobile phase flow rate was 0.35 mL/min for the first minute and was increased to 0.4 mL/min for the final four minutes for both columns. In the C18 column, the gradient elution started at 60% aqueous condition could miss metabolites separated between 100% and 60% aqueous. However, the HILIC column is generally better for the detection of these metabolites. We applied to columns to maximize metabolomic coverage.[27–29] The sheath gas and auxiliary gas were set at 30 (arbitrary units) and 5 (arbitrary units) for the negative ESI, respectively. For the positive ESI, the sheath gas and the auxiliary gas were set at 45 (arbitrary units) and 25 (arbitrary units), respectively. The spray voltage was −3.0 kV for the negative ESI and 3.5 kV for the positive ESI. To ensure quality control and standardization, two controlled pooled reference plasma samples, NIST 1950[30] and pooled human plasma (Equitech Bio, Kerrville, TX), were included at the beginning and end of each batch. Using ProteoWizard, raw data were converted to.mzML files.[31] Files were further abstracted using R package apLCMS modified by xMSanalyzer.[32,33] We defined unique features (detected signals) using mass-to-charge ratio (*m/z*), retention time, and ion intensity. Features are unique metabolomic signals that have been detected but have not been identified by their chemical name. Features detected in less than 10% of samples were removed. Additionally, serum samples with a median coefficient of variation (CV) >30% and a Pearson correlation <0.7 among the technical replicates were not included in the analysis. We excluded these features because they had a low reproducibility across the replicates. Average intensity of the remaining features was log-transformed to allow for further analysis.

Statistical analysis

We followed the standard workflow for an untargeted metabolomics study (Supplemental Figure 1; http://links.lww.com/EE/A174). We used generalized linear models to evaluate the association between each metabolomic feature and $PM_{2.5}$ exposure window. Models were fit using the following equation:

$$Y_{ji} = \alpha + \beta_{1j} PM2.5_{ik} + \beta_{2j} Temp_{ik} + \beta_{3j} Age_i + \beta_{4j} BMI_i + \beta_{5j} Education_i + \beta_{6j} Smoking_i + e_{ij}$$

In these models, Y_{ji} was the natural log of the intensity for feature j and participant i. $PM2.5_{ik}$ was woman i's exposure to $PM_{2.5}$ averaged over exposure window k. Similarly, $Temp_{ik}$ was woman i's exposure to ambient temperature over exposure window k. Daily ambient temperatures were derived from the Parameter-elevation Regressions on Independent Slopes Model (PRISM)[34] and were averaged over the same windows as $PM_{2.5}$ exposure. Finally, these models also included the woman's age (Age_i), body mass index (BMI_i), education ($Education_i$), and smoking status ($Smoking_i$). The (summand) E_{ij} denotes the residual normal error. Covariates were selected based on a priori knowledge and biological relevance. We included ambient temperature and not season because these two variables were correlated and given changes in climate and weather, ambient temperature may be a better measure of time spent indoors and is more directly linked to fuel usage (for heating and cooling). Separate models were used for the HILIC [positive] and C18 [negative] columns. We identified significant features at increasingly stringent levels of statistical significance (*P* value: <0.05, <0.005, and <0.0005) which allowed us to select the most stringent significance level with interpretable results. Given the high number of statistical tests, we also corrected these raw p-values for multiple comparisons using the Benjamini–Hochberg false discovery rate (FDR) procedure at two thresholds (*q*-value: <0.20 and <0.05). In most cases, features with $P < 0.005$ were used. Analyses were conducted in R (v. 4.0.3, R Foundation for Statistical Computing, Vienna, Austria).

Metabolic pathway enrichment analysis and metabolite annotation

Pathway analysis was completed using Mummichog (v. 1.0.10) in Python (Python Software Foundation, Wilmington, DE) which has been described and validated elsewhere.[35] Briefly Mummichog is an innovative bioinformatic tool that computes biological pathways from a feature list using *m/z* and retention time without prior metabolite identification. Mummichog computes an adjusted *P* value for each pathway by resampling the reference input file using a gamma distribution.[35] We utilized a reference file for each technical column (C18 [negative] and HILIC [positive]) with the file consisting of features with a raw *P* value <0.005. Features with a raw *P* value <0.0005 and corrected *q*-values could not be used due to the lack of significant metabolites in the shorter-term exposure windows. We examined pathways with *P* values <0.05 in any of the five exposure windows and compared the significance and number of matched metabolites in each pathway. In this analysis, pathways could have the same number of overlapping features but different pathway *P* value because of the different number of significant underlying features in the reference files (e.g., C18 [Negative] 1-day: six significant features versus 3-months: 36 significant features). Heat maps were used to visually compare the pathways across each time window. A *P* value <0.05 was utilized for the pathway analysis since Mummichog computes an adjusted p-value and we utilized a stringent criterion for significant features ($P < 0.005$) so there was limited need to be more conservative than traditional statistical norms.

For metabolite confirmation, we selected the features that were significantly associated with any of the $PM_{2.5}$ exposure windows ($P < 0.005$). We examined extracted ion chromatography for retention time, isotope patterns, and peak quality. Significant features with high-quality peaks were then compared to authentic standards from our laboratory that were analyzed with the same methods (level-1 evidence).[36] Significant features were matched to authentic standards by comparing the *m/z*, retention time, and ion dissociation. For each identified metabolite, we used the Human Metabolome Database to determine their chemical superclass and class.

Results

Sample characteristics

The average age of women in our study was 34.8 years (standard deviation [SD]: 3.9) and the majority were white (86%; n = 171) (Supplemental Table 1; http://links.lww.com/EE/A174). Ninety-two percent had at least a college degree (n = 183) and 40% of the participants had an unexplained initial infertility diagnosis (n = 79). Demographic and clinical characteristics were similar between all eligible participants and those included in the metabolomics sub-study (Supplemental Table 1; http://links.lww.com/EE/A174).

The average 1-day $PM_{2.5}$ exposure was 8.7 μg/m^3 (SD: 4.0) while the average 3-month $PM_{2.5}$ exposure was 9.0 μg/m^3 (SD: 1.9) (Supplemental Table 2; http://links.lww.com/EE/A174). The correlation between 1-day and 2-day $PM_{2.5}$ exposures was 0.89 while the correlation between 1-day and 3-month $PM_{2.5}$ exposures was 0.26. Similar trends were observed across the

exposure windows with windows closer together in time having higher correlations compared to windows further apart.

Significant features (P < 0.005)

We detected 10,803 and 12,968 unique features using the C18 [negative] and HILIC [positive] columns respectively (Table 1). Using the 1-day exposure window, 28 and 68 features were significantly associated (*P* value <0.005) with the 1-day exposure window using the C18 [negative] and HILIC [positive] columns, respectively. In contrast, 136 and 267 features were significantly associated (*P* value <0.005) with the 3-month exposure window using the C18 [negative] and HILIC [positive] columns, respectively. Additionally, when using the corrected *q*-values (<0.05), no features were significantly associated with the 1-day exposure window but 21 and 83 features were significantly associated with the 3-month exposure window in the C18 [negative] and HILIC [positive] columns, respectively. In general, as the exposure window lengthened the number of significant features increased and this trend held across the various levels of statistical significance.

In total 267 and 484 unique features were significantly associated with at least one of the exposure windows in the C18 [negative] and HILIC [positive] columns, respectively (Figure 1). In the C18 column, the largest overlap of significant features occurred between the 2-week and 3-month exposure windows (n = 31) and the 2-day and 3-day exposure windows (n = 24). In the HILIC column, the largest overlaps again occurred between the 2-week and 3-month exposure windows (n = 46) and the 2-day and 3-day exposure windows (n = 26). Only four significant features were associated with all five exposure windows and all of these were detected in the HILIC [positive] column.

Metabolic pathways

Using the C18 [negative] significant features (*P* < 0.005), 26 significant pathways were identified that were associated with one or more $PM_{2.5}$ exposure windows. On the metabolite level, amino acids and inflammatory pathways had the most features identified using Mummichog across the exposure windows (Figure 2). In some instances, features identified by Mummichog were found in several pathways and this occurred in the 2-day, 3-day, 2-week, and 3-month exposure windows. Nine of the 26 pathways - D4&E4-neuroprostanes formation, hexose phosphorylation, nitrogen metabolism, parathio degradation, phosphatidylinositol phosphate metabolism, putative anti-inflammatory metabolites formed from eicosapentaenoic acid, tryptophan metabolism, valine, leucine, and isoleucine degradation, and xenobiotics metabolism were only associated with an acute exposure window (1–3 days prior to blood sample) but not the intermediate- or long-term exposure window (Table 2). An additional nine pathways including amino sugars metabolism, ascorbate and aldarate metabolism, beta-alanine metabolism, CoA catabolism, electron transport chain, glutamate metabolism glutathione metabolism, glycine, serine, alanine, and threonine metabolism, and histidine metabolism were only associated with the intermediate or long-term exposure windows but not the acute exposure windows. Four pathways were commonly altered across all or most (four out of five) exposure windows including arachidonic acid metabolism, arginine and proline metabolism, aspartate and asparagine metabolism, and leukotriene metabolism.

Using the HILIC [positive] significant features (*P* value <0.005), 20 pathways were significantly associated with one or more $PM_{2.5}$ exposure windows. In contrast to the findings from the C18 [negative] column, the categories of the metabolomic pathways related to the acute and long-term exposure to $PM_{2.5}$ in the HILIC [positive] column were strikingly different (Figure 3). Across the five exposure windows, lipid metabolism pathways generally had the highest number of features identified using Mummichog. In contrast, features involved in inflammatory pathways were most prominent in the 2-week and 3-month exposure windows. Features involved with amino acid metabolism pathways were uniquely associated with acute exposures. Generally, fewer features identified by Mummichog occurred across pathways. Nine pathways—carnitine shuttle, de novo fatty acid biosynthesis, di-unsaturated fatty acid beta-oxidation, fatty acid activation, fatty acid metabolism, histidine metabolism, mono-unsaturated fatty acid beta-oxidation, tryptophan metabolism, and vitamin E metabolism—were associated with at least one of the acute exposure windows but not the intermediate or long-term exposure windows (Table 3). Six pathways—arachidonic acid metabolism, leukotriene metabolism, nucleotide sugar metabolism, prostaglandin formation from arachidonate, putative anti-inflammatory metabolites—formed from eicosapentaenoic acid, and vitamin A (retinol) metabolism were associated with intermediate and long-term exposure windows but not the acute exposure windows. Three pathways, D4&E4-neuroprostanes formation, linoleate metabolism, and omega-3 fatty acid metabolism, were associated across all or most (four out of five) exposure windows in the HILIC [positive] column. Across both technical columns, tryptophan metabolism pathways were consistently associated with acute exposure to $PM_{2.5}$.

Metabolite identification

Using level-1 evidence, we identified seven unique metabolites that were significantly (*P* value <0.005) associated with various exposure windows. In the C18 [negative] column, one metabolite was associated with only the short-term exposure windows (glutamic acid) (Table 4). Glutamic acid was only associated with the 3-day exposure window. One metabolite, in the C18 [negative] column, was only associated with the intermediate- and long-term exposure (hypoxanthine). Three metabolites in the C18 [negative] column were associated with both the short- (e.g., 3-day) and intermediate- (e.g., 2-week) -term

Table 1.

Number of significant metabolomic features associated with different $PM_{2.5}$ exposure windows among 200 women in the EARTH study from 2005 to 2015 in the Northeast United States.

	C18 [Negative] (n = 10,803)			HILIC [Positive] (n = 12,968)		
	Raw *P* values	Corrected *Q* values		Raw *P* values	Corrected *Q* values	
Exposure Window	<0.005	<0.20	<0.05	<0.005	<0.20	<0.05
1 day	28	0	0	68	0	0
2 days	56	0	0	74	2	0
3 days	74	0	0	100	2	1
2 weeks	85	14	5	163	36	12
3 months	136	37	21	267	209	83

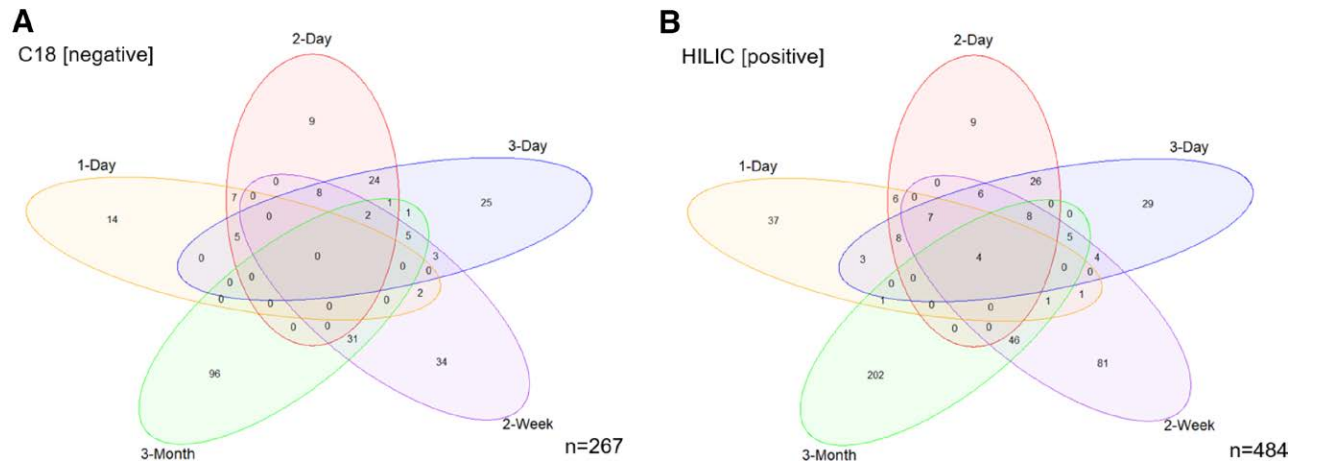


Figure 1. Number of significant features associated with each $PM_{2.5}$ exposure window among 200 women in the EARTH study from 2005 to 2015 in the Northeast United States using the C18 [Negative] and HILIC [Positive] technical columns.

exposure windows (N-Acetyl-serine, N-Methyl-aspartic acid, and O-Acetyl-serine).

In the HILIC [positive] column, two identified metabolites, Bis(2-Ethylhexyl)Phthalate (DEHP) and Retinoic acid, were associated with both the intermediate- and long-term exposure window. Across the seven unique metabolites we identified, the most common superclass was organic acids and derivates (n = 4; 57.1%) (Supplemental Table 3; http://links.lww.com/EE/A174).

Discussion

Key findings

In this metabolomics study among women undergoing infertility treatment, shorter- versus longer-term $PM_{2.5}$ exposure windows were largely associated with unique alterations in specific metabolites and metabolomic pathways while fewer pathways were common across all exposure windows. We identified 17 pathways solely associated with acute exposure to $PM_{2.5}$ and 15 pathways solely associated with the chronic exposure to $PM_{2.5}$. Only seven pathways were found to be commonly altered across the majority (four out of five) of exposure time windows. Furthermore, we were able to identify seven unique metabolites associated with $PM_{2.5}$ exposures of varying duration, using level-1 evidence, several of which were involved in the observed pathways.

We identified 17 pathways (eight in C18 [negative], eight in HILIC [positive], and one overlapping pathway) associated with acute (e.g., 1-3 day) exposure to $PM_{2.5}$, including many metabolites involved in amino acid metabolism, lipid metabolism, energy and nutrient metabolism, and free radical formation. Several studies have also identified many of these same pathways when studying acute exposure to air pollution.[9,11,13,15] For amino acid metabolism, several studies have observed an association between acute $PM_{2.5}$ exposure and tryptophan metabolism[9,15] which we also observed. In addition to tryptophan, we also found alterations with histidine metabolism, and valine, leucine, and isoleucine degradation. Under normal circumstances, these amino acids and their metabolism are involved in numerous responses including immune response, cell signaling, and hormone formation.[37–40] However, some of these amino acids have both antioxidant and pro-inflammatory metabolites and depending on which metabolites are upregulated, there could be serious consequences for the human body.[41,42] Thus, acute $PM_{2.5}$ exposure is concerning due to the potential damage from oxidative stress through these pathways. In addition to amino acid metabolism, six lipid metabolism pathways were associated with acute exposure to $PM_{2.5}$. Of these six lipid metabolism pathways, only three, carnitine shuttle, de novo fatty acid biosynthesis, and fatty acid activation, have previously been linked to acute exposure to $PM_{2.5}$.[11] These pathways may be activated after acute exposure to $PM_{2.5}$ as a means for the body to expend energy to repair itself from oxidative stress induced by short-term $PM_{2.5}$exposure. Finally, several anti-inflammatory pathways were associated with acute exposure to $PM_{2.5}$ including vitamin E metabolism and putative anti-inflammatory metabolites formed from eicosapentaenoic acid. Activation of these pathways is likely the body's immediate defensive response to short-term $PM_{2.5}$ exposure-creating antioxidants that will help the body combat an increase in oxidative stress.

We also observed 15 pathways (nine in C18 [negative] and six in HILIC [positive]) associated with intermediate- or longer-term exposure to $PM_{2.5}$, which in our study was defined as average exposure over the past 2-weeks or 3-months. These 15 pathways included several pro-inflammatory pathways, energy pathways, and anti-inflammatory pathways. Among the pro-inflammatory pathways, both leukotriene metabolism and prostaglandin formation from arachidonic acid have been previously observed in relation to long-term exposure to air pollution.[16,17] Interestingly, we also observed a relationship between long-term exposure to $PM_{2.5}$ and arachidonic acid metabolism. Previously, this pathway has only been associated with short-term exposures to air pollution.[9] These three pathways together indicate a large and likely sustained pro-inflammatory response with chronic $PM_{2.5}$ exposure. Unlike previous long-term exposure window studies, we observed a relationship between vitamin A metabolism and putative anti-inflammatory metabolites from eicosapentaenoic acid. Interestingly, the putative anti-inflammatory pathway was associated with the acute exposure window in the C18 [negative] column whereas it was associated with the long-term exposure in the HILIC [positive] column. We also observed an association between long-term $PM_{2.5}$ exposure and ascorbate and aldarate metabolism, another anti-inflammatory pathway, that has been previously identified by others.[16] These three anti-inflammatory pathways taken together may be the body's attempt to compensate for the sustained inflammatory response from the upregulated pro-inflammatory pathways in an attempt to maintain homeostasis. We again observed a relationship between $PM_{2.5}$ exposure and energy metabolism pathways as well as amino acid metabolism pathways. Both the electron transport chain and the nucleotide sugar metabolism pathways were associated with long-term exposure to $PM_{2.5}$; however, neither of these pathways have previously been associated with long-term $PM_{2.5}$ exposure. The increased need for energy may be due in part to fuel cellular efforts to repair damages from

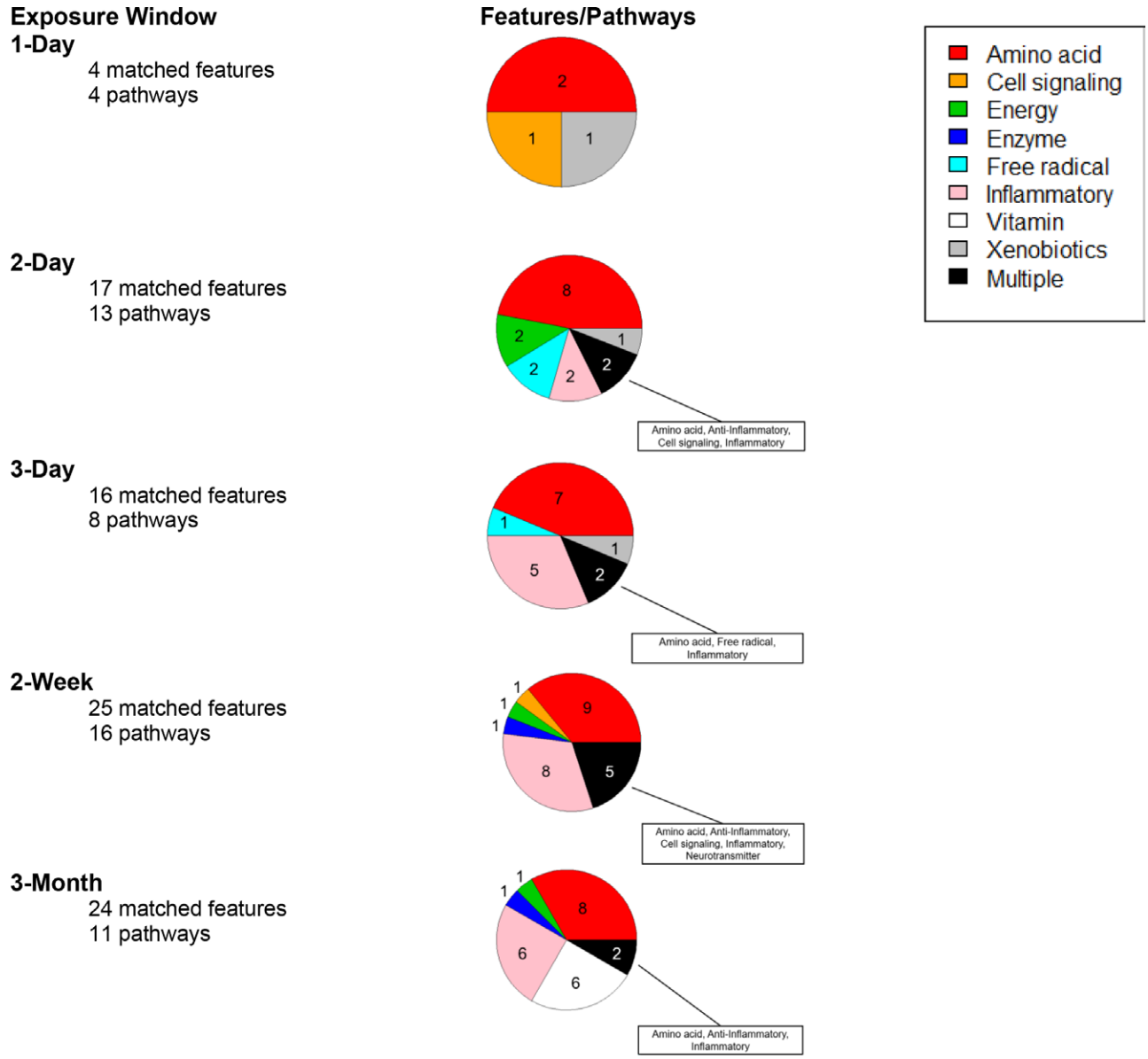


Figure 2. Number of features linked to pathways using Mummichog and classification of pathways modified by $PM_{2.5}$ Exposure in the C18 [Negative] Column among 200 women in the EARTH study from 2005 to 2015 in the Northeast United States. Each pie chart represents a single exposure window with the total number of features that matched to known metabolites in pathways identified using Mummichog. The colors for the pie charts represent the type of pathway a feature was found to be a part of, with some features being identified as a metabolite present in several pathways (denoted by the black color). Because of this, the total number of features will not add to the total number of matched features in Table 2. The numbers in the pie chart denote the number of features found in each type of pathway.

oxidative stress. With regards to amino acid metabolism, we observed three pathways associated with long-term exposure, beta-alanine metabolism, glycine, serine, alanine, and threonine- and histidine metabolism. All three of these amino acid pathways have previously been associated with long-term exposure to $PM_{2.5}$.[16,17] Additionally, histidine metabolism was associated with long-term exposure to $PM_{2.5}$ in the C18 [negative] column but was associated with acute exposure to $PM_{2.5}$ in the HILIC [positive] column.

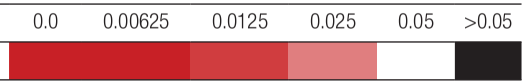
Across the five $PM_{2.5}$ exposure windows, we observed seven pathways (four in C18 [negative] and three in HILIC [positive]) that were associated with at least four of the five exposure windows. Similar to the pathways associated with only the acute or long-term exposure, we again observed associations with anti-inflammatory pathways and lipid metabolism. Interestingly, studies of both acute-[12,15] and long-term[16] exposure to $PM_{2.5}$ have observed alterations in the arginine and proline metabolism and aspartate and asparagine metabolism which adds credence to our finding of these pathways being associated with $PM_{2.5}$ exposures of varying duration. We observed two lipid metabolism pathways, omega-3 fatty acid metabolism and linoleate metabolism associated across several windows of $PM_{2.5}$ exposure. Thus far omega-3 fatty acid metabolism has only been associated with acute exposure to $PM_{2.5}$[12] while linoleate metabolism has only been associated with long-term exposure to $PM_{2.5}$.[16,17,43] Lastly, we observed several inflammatory pathways that were associated with at least four of the five exposure windows including arachidonic acid metabolism, D4&E4-neuroprostanes formation, leukotriene metabolism, and prostaglandin formation from arachidonate. Three of these pathways, arachidonic acid, leukotriene, and prostaglandin formation, were only associated with the long-term exposure window in the HILIC [positive] column but in the C18 [negative] column these three pathways were commonly dysregulated

Table 2.

Pathways (Features with *P* values <0.005) associated with different exposure windows of $PM_{2.5}$ among 200 women in the EARTH study from 2005 to 2015 in the Northeast United States using the C18 [Negative] technical column.

				Overlap					P-Value				
	Pathway			**1D**	**2D**	**3D**	**2W**	**3M**	**1D**	**2D**	**3D**	**2W**	**3M**
	Number of Significant Features	**Classification**	**Size**	**28**	**56**	**74**	**85**	**136**					
Acute Only (n=9)	Phosphatidylinositol phosphate metabolism	Cell signaling	19	**1**	1	1	1	1					
	Xenobiotics metabolism	Xenobiotic	41	**1**	1	1	2	0					
	Tryptophan metabolism	Amino acid	86	**2**	**4**	4	1	2					
	Hexose phosphorylation	Energy	20	0	**2**	2	2	1					
	Valine, leucine, and isoleucine degradation	Amino acid	25	0	**2**	2	1	1					
	Nitrogen metabolism	Cell signaling	6	0	**1**	1	1	1					
	Putative anti-Inflammatory metabolites formation from eicosapentaenoic acid	Anti-inflammatory	7	0	**1**	1	1	1					
	D4&E4-neuroprostanes formation	Free radical	14	0	**2**	**2**	1	1					
	Parathio degradation	Xenobiotic	2	**1**	**1**	**1**	0	0					
Long-term Only (n=9)	Glutamate metabolism	Neurotransmitter	14	0	1	1	**3**	1					
	Aminosugars metabolism	Cell signaling	20	0	1	1	**3**	1					
	Glycine, serine, alanine, and threonine metabolism	Amino acid	43	0	1	3	**4**	1					
	Glutathione Metabolism	Anti-inflammatory	10	0	1	1	**2**	**2**					
	Electron transport chain	Energy	2	0	0	0	**1**	**1**					
	Beta-Alanine metabolism	Amino acid	13	0	1	1	**2**	**2**					
	Histidine metabolism	Amino acid	31	0	1	1	**3**	**3**					
	CoA Catabolism	Enzyme	3	0	0	0	**1**	**1**					
	Ascorbate (Vitamin C) and Aldarate Metabolism	Vitamin	35	0	1	1	3	**6**					
Acute & Long-term (n=8)	Lysine metabolism	Amino acid	24	0	**2**	2	**3**	1					
	Methionine and cysteine metabolism	Amino acid	30	0	**2**	2	**3**	2					
	Prostaglandin formation from arachidonate	Inflammatory	39	0	2	**3**	**5**	3					
	Urea cycle/amino group metabolism	Amino acid	62	0	1	**4**	**5**	**5**					
	Arachidonic acid metabolism	Inflammatory	29	0	**3**	**4**	**5**	**4**					
	Arginine and Proline Metabolism	Amino acid	36	0	**3**	**5**	**9**	**5**					
	Aspartate and asparagine metabolism	Amino acid	63	0	**4**	**6**	**8**	**6**					
	Leukotriene metabolism	Inflammatory	31	0	**2**	**5**	**6**	**4**					

***P* value**



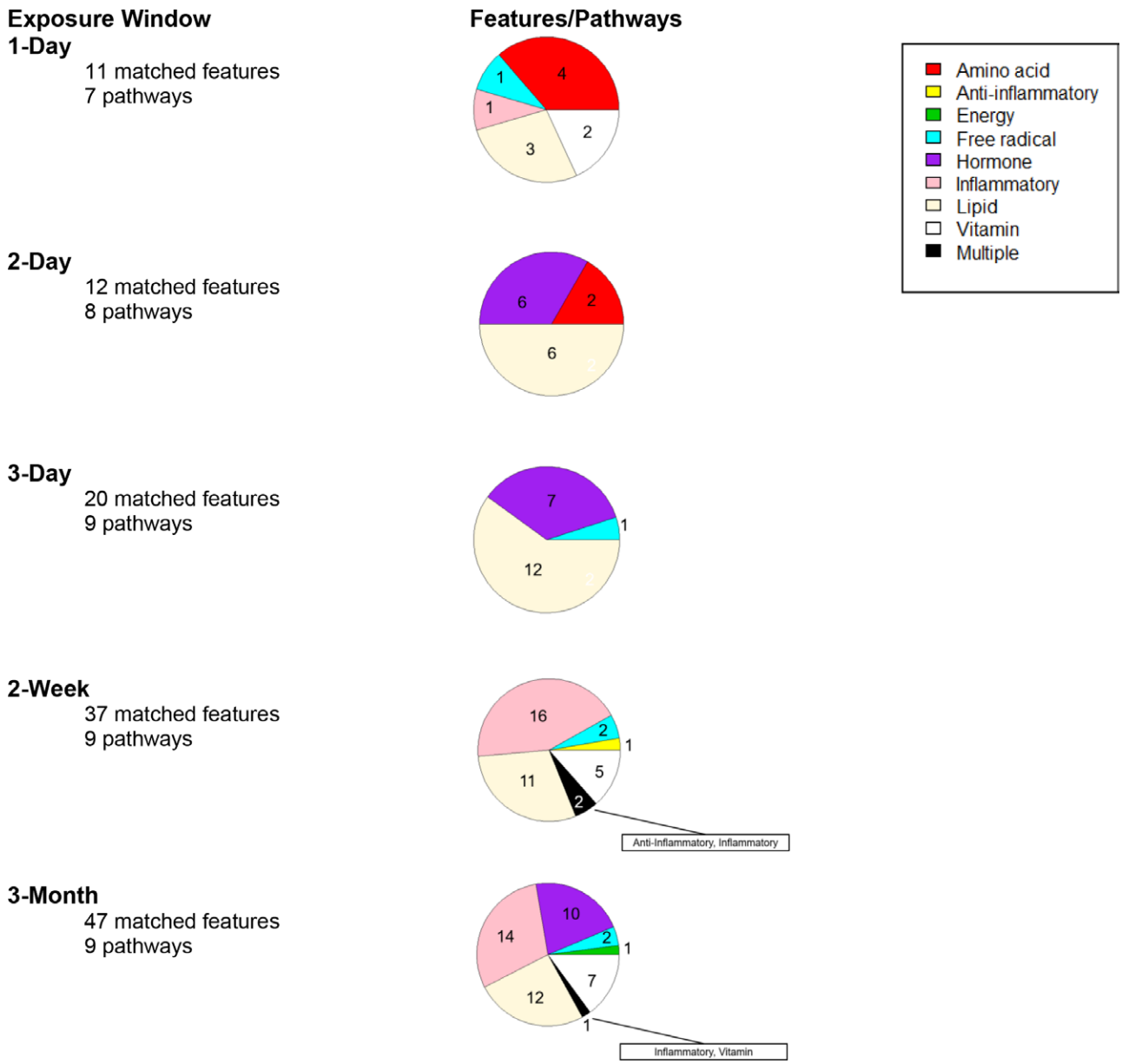


Figure 3. Number of features linked to pathways using Mummichog and classification of pathways modified by $PM_{2.5}$ Exposure in the HILIC [Positive] Column among 200 women in the EARTH study from 2005 to 2015 in the Northeast United States. Each pie chart represents a single exposure window with the total number of features that matched to known metabolites in pathways identified using Mummichog. The colors for the pie charts represent the type of pathway a feature was found to be a part of, with some features being identified as a metabolite present in several pathways (denoted by the black color). Because of this, the total number of features will not add to the total number of matched features in Table 3. The numbers in the pie chart denote the number of features found in each type of pathway.

across several $PM_{2.5}$ exposure windows. In contrast, the D4&E4 pathway was associated with acute $PM_{2.5}$ exposure in C18 [negative] column but was associated with several $PM_{2.5}$ windows in the HILIC [positive] column.

Overall, we were able to identify seven unique metabolites using level-1 evidence. Similar to the pathway analysis, we observed differences in metabolites by $PM_{2.5}$ duration of exposure. In the short-term windows, we identified one metabolite, glutamic acid. Glutamic acid is involved in several metabolomic pathways that were commonly altered with short-term $PM_{2.5}$ exposure including arachidonic acid metabolism, arginine and proline metabolism, aspartate metabolism, and the urea cycle. We identified three metabolites, DEHP, retinoic acid, and hypoxanthine associated with both the intermediate- and long-term $PM_{2.5}$ exposure windows. Retinoic acid is a part of vitamin A metabolism which is a pathway we observed being associated with long-term exposure to $PM_{2.5}$. There were three metabolites commonly associated with 3-day and 2-week average exposure to $PM_{2.5}$, N-Acetyl-serine, N-Methyl-aspartic acid, and O-Acetyl-Serine. N-Acetyl-serine, N-Methyl-aspartic acid, and O-Acetyl-serine are all types of amino acids derivatives and offer credence to our finding of amino acid pathways related to $PM_{2.5}$ exposure. N-Methyl-aspartic acid is needed for normal synaptic transmission and plasticity but when overstimulated can be excitotoxic.[44] The degree to which these metabolites can be used as biomarkers of short and long-term exposure to $PM_{2.5}$ warrants further study.

Clinical and policy implications

We observed that exposure to $PM_{2.5}$ of varying durations (from days to several months) led to different alterations in the serum

Table 3.

Pathways (features with *P* values <0.005) associated with different exposure windows of $PM_{2.5}$ among 200 women in the EARTH study from 2005 to 2015 in the Northeast United States using the HILIC [Positive] technical column.

				Overlap					*P* value				
	Pathway			**1D**	**2D**	**3D**	**2W**	**3M**	**1D**	**2D**	**3D**	**2W**	**3M**
	Number of Significant Features	**Classification**	**Size**	**68**	**74**	**100**	**163**	**267**					
Acute Only (n=9)	Mono-unsaturated fatty acid beta-oxidation	Lipid	2	**2**	0	0	0	0					
	Vitamin E metabolism	Vitamin	22	**2**	0	0	2	3					
	Tryptophan metabolism	Amino acid	107	**4**	2	2	1	5					
	Histidine metabolism	Amino acid	34	0	**2**	2	1	0					
	De novo fatty acid biosynthesis	Lipid	34	0	**4**	**4**	2	2					
	Fatty acid activation	Lipid	34	1	**4**	**4**	1	2					
	Fatty Acid Metabolism	Lipid	23	0	**2**	**2**	2	2					
	Carnitine shuttle	Lipid	33	1	1	**4**	0	1					
	Di-unsaturated fatty acid beta-oxidation	Lipid	5	**3**	**1**	**1**	0	0					
Long-term Only (n=6)	Putative anti-Inflammatory metabolites formation from eicosapentaenoic acid	Anti-Inflammatory	7	0	0	0	**3**	2					
	Arachidonic acid metabolism	Inflammatory	36	1	1	2	**13**	**11**					
	Prostaglandin formation from arachidonate	Inflammatory	27	0	1	1	**13**	**9**					
	Leukotriene metabolism	Inflammatory	30	0	0	0	**9**	**7**					
	Vitamin A (retinol) metabolism	Vitamin	26	0	1	1	**5**	**8**					
	Nucleotide Sugar Metabolism	Energy	1	0	0	0	0	**1**					
Acute & Long-term (n=5)	Prostaglandin formation from dihomo gama-linoleic acid	Inflammatory	3	**1**	0	0	**2**	**2**					
	C21-steroid hormone biosynthesis and metabolism	Hormone	78	1	**4**	**7**	5	**10**					
	Omega-3 fatty acid metabolism	Lipid	13	**2**	**2**	**2**	**3**	2					
	D4&E4-neuroprostanes formation	Free radical	4	**1**	0	**1**	**2**	**2**					
	Linoleate metabolism	Lipid	46	1	**3**	**5**	**9**	**12**					

P value

0.0	0.00625	0.0125	0.025	0.05	>0.05

Table 4.

Significant ($P < 0.005$) metabolites identified using level 1 evidence and associated with different exposure windows of $PM_{2.5}$ among 200 women in the EARTH study from 2005 to 2015 in the Northeast United States using the C18 [Negative] and HILIC [Positive] technical columns.

	Metabolites	ESI	1D	2D	3D	2W	3M
Acute	Glutamic acid	-					
Long	Bis(2-ethylhexyl)phthalate	+					
	Hypoxanthine	-					
	Retinoic acid	+					
Acute & long	N-acetyl-serine	-					
	N-methyl-aspartic acid	-					
	O-acetyl-serine	-					

metabolome of reproductive-aged women. The different alterations in the serum metabolome may explain the different health effects that have been observed when comparing acute versus long-term exposure to $PM_{2.5}$. Our results may be particularly relevant for perinatal studies focused on pregnancy loss or pre-term birth where air pollution has been shown to have both acute and long-term adverse impacts and the biological mechanisms are largely unknown.[6,21,45–47] Until further evidence is available, our results support the hypothesis that air pollution largely acts on different biological pathways when encountered acutely versus chronically and this may have important implications for future studies when determining the most biologically relevant time window to focus on. Additionally, to identify sensitive biomarkers of air pollution exposure, metabolomics can be a powerful hypothesis-generating tool. In this analysis, we highlight the specific metabolic features and pathways that are linked to short-term or long-term, or both, exposure windows, which can contribute to follow-up biomarker development studies.

Strengths and Limitations

Our study has several important limitations. First, this study utilized data collected from women residing in the Northeastern US who were undergoing infertility treatment which potentially limits the generalizability of our findings. The majority of our women were also white and of high socioeconomic status, which is typical of studies focusing on infertility clinic populations, but may limit the applicability of our findings to other race/ethnicities and socioeconomic status. Nevertheless, the results were consistent with many existing air pollution and metabolomic applications conducted in population-based and highly selected populations. Second, our exposure measure only captured ambient exposure to $PM_{2.5}$ and did not capture indoor air pollution and occupational exposure to $PM_{2.5}$. Because we were unable to include these exposures in our measure, women's personal exposure to $PM_{2.5}$ is likely misclassified. However, we have no reason to believe that this misclassification would be differential, thus the likely consequence is that our results are biased towards the null. In addition, $PM_{2.5}$ exposure in this population is generally low in comparison to other regions of the world and therefore may not be generalizable to reproductive-aged women who live in areas with high exposure to $PM_{2.5}$. Third, we were unable to separate the effect of the length of the time window and the effect of acute versus chronic $PM_{2.5}$ exposure on the serum metabolome. The shorter time windows (1-, 2- and 3-day) could be subject to greater noise and variation when compared to the longer time windows (2-week and 3-month). In our results, we observed that as the length of time window declined, the number of significant features also decreased. Future studies using personal monitors would be the ideal way to address this limitation; however, conducting a study like this in a large representative sample remains expensive and challenging. Fourth, we utilized average $PM_{2.5}$ exposure windows which could mask potentially important temporal variations. For example, in a 3-month exposure window, $PM_{2.5}$ could rapidly rise and fall and this would be recorded as the same average as a 3-month exposure window that had a steady amount of $PM_{2.5}$. By not accounting for these temporal variations, we could have missed out on identifying important effects on the serum metabolome. While we examined a range of acute and chronic exposure windows that were defined a priori, there could be other critical time windows of exposure that were not investigated in our study. Future studies should consider the advantages and disadvantages of using averaged air pollution exposure windows versus other methods that may account for temporal variation in air pollution exposure and select critical time windows using a more data-driven approach. Fifth, because Mummichog relies on the number of significant features to determine P values for each pathway, it is possible that some pathways with the same number of overlapping features were significant in one time window and not in another (for example, phosphatidylinositol phosphate metabolism in the C18 [neg] column). Additionally, because we are testing multiple pathways, it is possible that some of the metabolomic pathways were associated with time windows by chance. Because of these concerns, our pathway analysis results should be interpreted with caution and will need to be confirmed by additional studies. Sixth, because we utilized non-fasting blood samples, dietary factors could have influenced our results. However, it is unlikely that diet and $PM_{2.5}$ exposure were related, which means that diet is unlikely to be a confounder. Additionally, we utilized a comprehensive metabolomic workflow that has been successful in analyzing non-fasting samples. In addition, we observed similar metabolomic alterations to other air pollution studies using fasting blood samples[12] which may indicate that diet had a minimal impact on our results. Future studies should consider the potential difference in results that non-fasting and fasting blood samples could provide with regard to metabolomic analyses. Next, we attempted to adjust for the false positive rate. However, due to a lack of interpretable data for the short-term exposures, we were unable to use the most stringent, FDR corrected q-values. Therefore, our results should be carefully interpreted and will need to be confirmed by additional studies with larger samples sizes. Finally, the samples used in this analysis were stored for a long period of time at –80°C prior to analysis, which could have negatively impacted the quality of the sample. However, a review of studies investigating pre-analytic factors, found samples under this condition did not have any significant negative impacts on quality after 30 months of storage[48]; longer storage times have not been investigated so the impact on sample quality remains a question and should be investigated in future studies. Our study does have several strengths. We utilized a validated measure of ambient exposure to $PM_{2.5}$ and

used a standard protocol for metabolomics analysis including laboratory standards to confirm metabolite identification with level-1 evidence using these same protocols. Lastly, due to the prospective nature of the EARTH Study, we were able to adjust for several potential confounders including age, smoking status, education, and BMI.

Conclusion

In our study of reproductive-aged women, we found that short versus long-term exposure to ambient $PM_{2.5}$ had differential impacts on the serum metabolome as many specific metabolites and metabolic pathways were only associated in the acute window or the long-term window, with very few being commonly altered across all time windows examined. Differences in pathways activated by $PM_{2.5}$ exposure windows may explain how differences in health outcomes arise depending on the exposure windows utilized. Researchers should be aware that $PM_{2.5}$ exposures of differing duration may lead to different biological responses in the human metabolome and should take this into consideration when planning and studying the health effects of $PM_{2.5}$.

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