Integrated molecular response of exposure to traffic-related pollutants in the US trucking industry

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abstract

Exposure to traffic-related pollutants, including diesel exhaust, is associated with increased risk of cardiopulmonary disease and mortality; however, the precise biochemical pathways underlying these effects are not known. To investigate biological response mechanisms underlying exposure to traffic related pollutants, we used an integrated molecular response approach that included high-resolution metabolomic profiling and peripheral blood gene expression to identify biological responses to diesel exhaust exposure. Plasma samples were collected from 73 non-smoking males employed in the US trucking industry between February 2009 and October 2010, and analyzed using untargeted high-resolution metabolomics to characterize association with shift- and week-averaged levels of elemental carbon (EC), organic carbon (OC) and particulate matter with diameter 2.5 μm ($PM_{2.5}$). Metabolic associations with EC, OC and $PM_{2.5}$ were evaluated for biochemical processes known to be associated with disease risk. Annotated metabolites associated with exposure were then tested for relationships with the peripheral blood transcriptome using multivariate selection and network correlation. Week-averaged EC and OC levels, which were averaged across multiple shifts during the workweek, resulted in the greatest exposure-associated metabolic alterations compared to shift-averaged exposure levels. Metabolic changes associated with EC exposure suggest increased lipid peroxidation products, biomarkers of oxidative stress, thrombotic signaling lipids, and metabolites associated with endothelial dysfunction from altered nitric oxide metabolism, while OC exposures were associated with antioxidants, oxidative stress biomarkers and critical intermediates in nitric oxide production. Correlation with whole blood RNA gene expression provided additional evidence of changes in processes related to endothelial function, immune response, inflammation, and oxidative stress. We did not detect metabolic associations with $PM_{2.5}$. This study provides an integrated molecular assessment of human exposure to traffic-related air pollutants that includes diesel exhaust. Metabolite and transcriptomic changes associated with exposure to EC and OC are consistent with increased risk of cardiovascular diseases and the adverse health effects of traffic-related air pollution.

Graphical Abstract
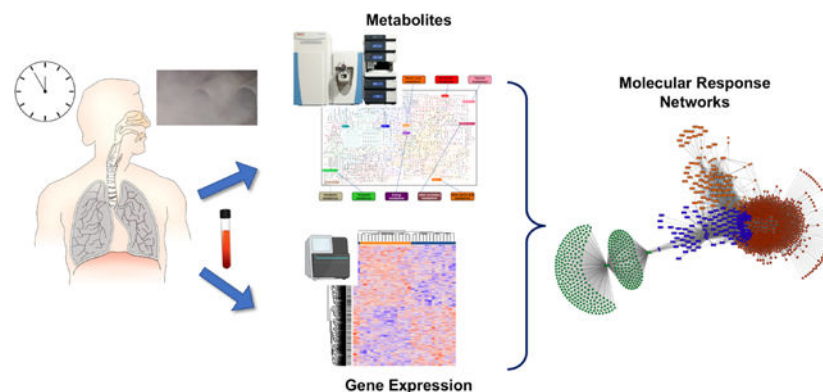
Keywords

Metabolomics; Exposomics; High-resolution mass spectrometry; Multi-omics; Traffic-related pollutants; Diesel exhaust

INTRODUCTION

Exposure to traffic-related pollution has been linked to increased risk of all-cause mortality, cardiovascular disease (CVD), cardiopulmonary outcomes and lung cancer mortality, (Hart 2016; Pope et al. 2002; Pope et al. 2009) with 2010 estimates suggesting 3.1 million deaths were attributable to ambient particulate exposure alone. (Lim et al. 2012) Suspected mechanisms that contribute to disease risk include increased oxidative stress, endothelial dysfunction and inflammation; however, study in human populations has shown conflicting results. (Bates et al. 2015b; Brugge et al. 2013; Chuang et al. 2007; Donaldson et al. 2001; Sorensen et al. 2003)

Exhaust emissions consist of a complex mixture of particle-bound and free chemicals. Source, fuel type, combustion efficiency and distance from the source contribute to both the individual constituents and composition of particulate matter present in traffic-related air pollution. However, in general, combustion related particulate matter includes ultrafine particles with an elemental carbon core (EC) and a particulate organic carbon (OC) fraction that originates both from chemicals produced during incomplete combustion as well as condensation of organic volatile gases on the particle surface during atmospheric transport. Evidence suggests the composition of particulate matter, specifically EC, are a robust indicator of combustion-based exposures from traffic. (Janssen et al. 2012) EC is a major component of carbonaceous particulates arising from diesel exhaust lacking modern emission controls and is also present to a lesser extent in exhaust from other mobile sources, including spark-ignition vehicles. (Costantini et al. 2016; Grahame et al. 2014; Schauer 2003; Sheesley et al. 2009) Several studies have shown using EC as a measure of traffic-related exposures results in higher effect estimates associated with cardiopulmonary disease when compared to $PM_{2.5}$ (particulate matter with diameter 2.5 μm); however, these findings have not been consistent across different populations and it is not clear if EC is independently associated with outcomes or an indirect measure of particle mass. (Beelen et al. 2008; Lipfert et al. 2006; Luben et al. 2017; Ostro et al. 2007; Sarnat et al. 2015) Cumulative EC exposure based upon 5- and 1-year lag periods has been associated with lung cancer mortality in trucking industry workers in the US, (Garshick et al. 2008) while the International Agency for Research on Cancer has classified diesel exhaust as a Group 1 carcinogen. (Benbrahim-Tallaa et al. 2012) Although health risks of EC have been documented, the contribution of EC and other traffic-related pollutants such as particle associated organic chemicals (assessed by measuring OC) to metabolic responses has not been well characterized.

The human metabolome is a functional measure of the interaction between the genome, diet, environment, and metabolism. (Wishart et al. 2013) Identifying metabolic changes associated with environmental exposures has the potential to improve understanding of response to exposures, providing new insight into the biological changes underlying exposure-related diseases. Untargeted chemical profiling techniques based upon high resolution mass spectrometry now make possible measurement of up to 20,000 chemical features, providing sufficient metabolic characterization for precision medicine and exposome research. (Liu et al. 2016) When combined with complementary response measures, such as gene expression, protein levels or epigenetic changes, it is possible to

develop an *in vivo*, systems biology approach to investigate how environmental exposures contribute to disease risk in humans. Development of aggregated biological response patterns across multiple 'omic layers represents a new paradigm for combining toxicology with molecular and environmental epidemiology.

In the present study, we used untargeted high-resolution metabolomics (HRM) and whole blood RNA gene expression to characterize the integrated molecular response of US trucking industry workers following occupational exposure to diesel exhaust. Using exposure levels measured at the workplace and blood samples collected at multiple time points throughout the workweek, we evaluated metabolic alterations associated with shift- and week-averaged EC and organic carbon (OC) in particles <1 μm in diameter ($PM_{1.0}$), and fine particulate matter ($PM_{2.5}$). Using metabolic changes associated with week-averaged exposures, we applied a data-driven network approach to identify correlating gene expression pathways. Our objective was to provide an integrated assessment of human molecular response to traffic-related pollutants and identify biological pathways that may underlie the observed effects of these exposures.

METHODS

Study population

Details on the study population, recruitment and sample collection have been reported previously. (Neophytou et al. 2013; Neophytou et al. 2014) Briefly, 95 participants were recruited from 10 unionized trucking terminals located throughout the northeastern US (Connecticut, Massachusetts, Maryland, New Jersey, New York and Pennsylvania), and classified based upon job duties with different patterns of roadway traffic and freight terminal exposures from working in proximity to diesel powered trucks. Job classifications include pickup and delivery drivers, freight dock workers, and office clerks. Participants were selected for enrollment in the study if they had at least two days off prior to the start of the study. Measurements and sample collection were completed between February 2009 and October 2010, with terminals sampled one at a time for up to 8 continuous days. To reduce confounding due to smoking and sex differences, the present analysis was restricted to 73 non-smoking, male workers. All participants provided informed consent, and the study protocol was approved by the Institutional Review Board of the Brigham and Women's Hospital and the Human Subject Committee of the Harvard T.H. Chan School of Public Health.

Exposure assessment

Stationary samplers for exposure assessment were placed at indoor office spaces, terminal docks and within truck cabs for all 10 terminal locations during the full workweek (6–9 days). Exposure measures of traffic-related pollutants included $PM_{2.5}$, and EC and OC in $PM_{1.0}$. Collection protocols and detailed methodology on assigning exposures are described elsewhere. (Neophytou et al. 2013) $PM_{2.5}$ was collected on a 37-mm Teflon filter (Gelman/Pall, Port Washington, NY, USA) after passing through a precision-machined cyclone pre-selector (GK2–5-SH, BGI, Inc., Waltham, MA USA) to remove particles greater than 2.5 μm in aerodynamic diameter. The method was consistent with the EPA PQ200

Federal Reference Method. (Tainio et al. 2005; Yanosky and MacIntosh 2001) EC and OC were measured by collecting $PM_{1.0}$ on a 22-mm quartz tissue filter preceded by precision machined cyclone separator (SCC1.062 Triplex, BGI Inc., Waltham, MA USA), which was then analyzed by thermal-optical carbon analyzer using the NIOSH 5040 method. (NIOSH 2003) Individualized, personal exposures were then estimated for each participant using a weighted average of the reported time spent at each work location on each workday.

Biomarker sampling

A total of four blood samples was collected from each participant over the course of the workweek. The first blood sample was collected from each participant prior to the day's work shift on their first day back after at least two days off, followed by a second blood sample at the end of the first work shift. Pre- and post-shift samples were collected again on the last day of the same workweek. Following each blood draw, blood tubes were stored at 4°C until processing. EDTA plasma samples for metabolomic analysis were centrifuged, aliquoted, and stored in the vapor phase of liquid nitrogen freezers at < −130°C. RNA was extracted from PaxGene tubes using the Qiagen RNAEasy kit and stored at −80°C.

High-resolution metabolomics

Untargeted HRM profiling was completed using verified protocols. (Accardi et al. 2016a; Go et al. 2015) Plasma aliquots were removed from storage and thawed on ice. A 65 μL aliquot of plasma was then added to 130 μL of acetonitrile containing a mixture of stable isotopic standards that included $[^{13}C_6]$-D-glucose, $[^{15}N]$-indole, $[2-^{15}N]$-L-lysine dihydrochloride, $[^{13}C_5]$-L-glutamic acid, $[^{13}C_7]$-benzoic acid, $[3,4-^{13}C_2]$-cholesterol, $[^{15}N]$-L-tyrosine, $[trimethyl-^{13}C_3]$-caffeine, $[^{15}N_2]$-uracil, $[3,3-^{13}C_2]$-cystine, $[1,2-^{13}C_2]$-palmitic acid, $[^{15}N,^{13}C_5]$-L-methionine, $[^{15}N]$-choline chloride, and 2'-deoxyguanosine-$^{15}N_2,^{13}C_{10}$-5'-monophosphate, vortexed, and allowed to equilibrate for 30 minutes. (Soltow et al. 2013) Triplicate 10 μL aliquots were analyzed by reverse-phase C_{18} liquid chromatography (Targa 100 mm x 2.1mm x 2.6 μm, Higgins Analytical Inc) with detection by a Thermo Scientific Q-Exactive high-resolution mass spectrometer. Analyte separation was accomplished using water, acetonitrile and 2% [v/v] formic acid in water (solution A) mobile phases operating under the following gradient: initial 2 min period of 80% A, 5% water, 15% acetonitrile, followed by linear increase to 0% A, 5% water, 95% acetonitrile at 6 min and then held for an additional 4 min. Mobile phase flow rate was held at 0.35 mL/min for 6 min, and then increased to 0.5 mL/min. The high-resolution mass spectrometer was equipped with an electrospray ionization source operated in positive ion mode with spray voltage of 4.5 kV, probe, capillary temperature 275°C, sheath gas flow 45 (arbitrary units), auxiliary gas flow 5 (arbitrary units) and S-lens RF level of 69. Resolution was set at 70,000 (FWHM) and mass-to-charge (*m/z*) scan range 85–1275. Spectra were collected in full scan only without MSMS. Samples were analyzed in batches of 20, in addition to a quality control (QC) pooled reference sample included at the beginning and end of each batch of samples to evaluate batch effects and reproducibility of the detected metabolite features.

Upon injection of all study and quality control samples, mass spectral features with replicate coefficient of variation (CV) 100% were extracted and aligned using *apLCMS* (Yu et al.

2013) with modifications by *xMSanalyzer* (Uppal et al. 2013) and batch effect correction by *ComBat* (Johnson et al. 2007). Detected chemical signals were defined by accurate mass-to-charge ratio (*m/z*), retention time and intensity, referred to as metabolite features throughout. Prior to statistical analysis, replicate injections were averaged, and metabolite features not detected in >50% of the participants were removed, resulting in 7,042 and 7,035 metabolite features for the shift- and week-averaged analyses, respectively. All participant covariates and untargeted metabolomics data underlying this article are available under Study ID ST001930 in the Metabolomics Workbench at http://dx.doi.org/10.21228/M84Q3H.

Whole blood RNA gene expression

Gene expression associations with $PM_{2.5}$, EC and OC in this cohort have been described previously. (Chu et al. 2016) Analysis was conducted using the Illumina HumanHT-12 v4 Expression BeadChip, with RNA labeling and array hybridization performed according to protocol. Image capture was performed using the Illumina BeadArray Reader. Standard QC and pre-processing procedures were applied, with background correction and quantile normalization procedures completed using the R package *lumi*. (Du et al. 2008) The final data set was $\log_2$ transformed prior to network integration and included information for 47,295 probes. Previous analysis of gene expression profiles from this cohort show no significant differences were present when comparing pre- and post-shift gene expression on day 1, or across post-shift samples on days 1 and 4. (Chu et al. 2016) Thus, to reduce adding any additional variability due to diurnal effects and simplify interpretation of correlation results only post-shift measurements from both days were considered.

Metabolome wide-association study of traffic-related pollutants

Due to the availability of repeat HRM and exposure measures in this study, we completed metabolome-wide association study (MWAS) of $PM_{2.5}$, EC and OC separately for shift- and week-averaged workplace exposures. The association of each metabolite feature with $PM_{2.5}$, EC and OC were determined using linear mixed effects regression models, which included a random intercept for each subject to account for baseline inter-individual differences. Fixed effects for both models included the interquartile range (IQR) normalized exposure measure, age, day (factor for first and last day of the workweek), and body mass index (BMI, calculated from measured height and weight). The effect of day as a continuous variable and race was also evaluated; however, accounting for time differences between blood sample collection or race as a fixed factor in the regression models did not impact MWAS results and were excluded as covariates. We first tested for daily associations with $PM_{2.5}$, EC and OC using metabolite feature measured in post-shift blood samples collected on both days and the corresponding exposures measured over the course of that workday. Week-averaged effects were evaluated using the average exposure calculated using the first and last shift for each participant and metabolomic results measured in blood samples collected at all time points. To account for diurnal effects, we included sampling time (pre- or post-shift) as an additional covariate to allow metabolite peak intensity to vary with time irrespective of exposure measures. Regression analysis was performed using the R package *lme4* and completed separately for each exposure and time combination. (Bates et al. 2015a) The likelihood ratio test of the complete model against the null model, which excluded the corresponding exposure measures as the independent variable, was used to

obtain p for each metabolite feature-exposure pair. Model fits were evaluated by calculating the conditional and marginal r^2. To account for multiple hypothesis testing and estimate the expected proportion of incorrectly rejected null hypotheses (i.e. false positives), a Benjamini-Hochberg false discovery rate (FDR) was applied to identify metabolite features associated with each pollutant. (Benjamini and Hochberg 1995) Associated metabolite features were identified using an FDR threshold of 20% to balance the rate of type I and II errors, which when combined with dose-response linear regression and metabolic pathway enrichment has been shown to improve detection of biological activity and reduce false positives. (Li et al. 2013; Walker et al. 2016)

Metabolite annotation and pathway enrichment

The metabolite features associated with each exposure were first matched using accurate m/z detected using MS1 and retention time to a database of 120 metabolites previously confirmed with MS^2 and co-elution studies (Level 1 identification). (Accardi et al. 2016b; Liu et al. 2020; Schymanski et al. 2014) To generate additional biological insight into effects of exposure EC, OC and $PM_{2.5}$, metabolite features not matching the database of 120 metabolites were annotated using MS1 detected accurate m/z based upon positive electrospray ionization adducts and the Human Metabolome Database (HMDB) release 3.0. (Wishart et al. 2013) Metabolite annotations were assigned using evidence scoring (Uppal et al. 2016a) and ± 10 parts-per-million (ppm) mass tolerance ($m_{error}/m_{theoretical} \times 10^6$). While these annotations provide low confidence matches (Level 4 annotation) that predict chemical formulas only and do not inform on compound structures, combining low confidence annotations with functional activity patterns within the untargeted data improves the ability to uncover novel insight into metabolite response profiles to exposures. (Uppal et al. 2016b) Thus, we combined annotation of features with pathway-based functional enrichment analysis using *Mummichog* to characterize metabolic activity patterns associated with each exposure. (Li et al. 2013) Enriched metabolite pathways were selected using a *Mummichog* scoring threshold 0.05.

Metabolome x transcriptome exposure response pathways

To evaluate integrated metabolic and gene expression patterns associated with exposure to traffic-related pollutants, we performed a network-based correlation analysis using xMWAS to characterize relationships between annotated metabolites associated with week-averaged exposures and peripheral blood transcriptomic results. (Uppal et al. 2018) Correlations with $|r|$ 0.4 and $p < 0.05$ were then selected for visualization in Cytoscape, with gene and metabolite pathway enrichment determined using MetaCore (Thomson Reuters) and *Mummichog*, respectively. An enrichment score of 0.1 was used to identify metabolite pathways and $p < 0.05$ for gene expression pathways. Previously reported relationships between gene pathways present in the integrated network and exposure to traffic related pollutants were evaluated using the Comparative Toxicogenomics Database (CTD). (Davis et al. 2013) To identify previous gene-exposure relationships, we used the top two most connected gene nodes from each cluster for searching reported gene-chemical interactions within CTD.

RESULTS

Study population

A summary of participant characteristics is provided in Table 1. HRM profiling was limited to 73 individuals who were otherwise healthy, non-smoking males from all three-job categories with blood samples available on both sampling days. Comparison of exposure levels at the beginning and end of the workweek showed no differences between the two workdays ($p > 0.7$). Week-averaged exposures showed a weak correlation between $PM_{2.5}$ and EC (Pearson $r = 0.39$, $p = 0.0007$) and between $PM_{2.5}$ and OC (Pearson $r = 0.27$, $p = 0.021$), while no correlation was present between EC and OC (Pearson $r = 0.03$, $p = 0.78$). Dockworkers and pickup and delivery drivers had higher $PM_{2.5}$ and EC exposures compared to office clerks, while OC was lowest among dockworkers. (Neophytou et al. 2013; Neophytou et al. 2014) Participant shift-and week-averaged exposure levels for EC, OC and $PM_{2.5}$ are provided in Supplementary Figure 1.

Shift-averaged exposure MWAS

To evaluate acute effects of exposure to EC, OC and $PM_{2.5}$, we used an MWAS framework to test the relationship between shift-averaged exposures and metabolite features measured in post-shift samples obtained on both workdays (Figure 1A). Using an FDR threshold of 20%, 68 and 48 unique m/z features were associated with EC and OC, respectively (Supplementary Tables 1 and 2). Only 3 metabolites were identified at level 1, the remaining were annotations based upon accurate mass matching only. Annotated metabolites associated with EC included metabolic intermediates related to inflammation, endothelial function, lipid peroxidation and co-factors (Table 2); enriched pathways included butanoate metabolism ($p = 0.01$) and urea cycle/amino group metabolism ($p = 0.04$). For OC, metabolite annotations were consistent with nucleotide metabolism, porphyrin metabolites and lipid peroxidation products (Table 2), and enriched pathways included changes in selenoamino acid metabolism ($p = 0.003$), glycerophospholipids ($p = 0.003$), pyruvate metabolism ($p = 0.009$), serine metabolism ($p = 0.01$), glycolysis/gluconeogenesis ($p = 0.03$) and arginine/proline metabolism ($p = 0.03$). None of the metabolite feature intensities were associated with shift $PM_{2.5}$ at FDR<20%.

Week-averaged exposure MWAS

Previous results in this cohort have shown changes in gene expression, inflammatory markers, oxidative stress and urinary exposure biomarkers are largely dependent on week-averaged, rather than shift-averaged exposures. (Chu et al. 2016; Neophytou et al. 2013; Neophytou et al. 2014) Therefore, we next performed MWAS for $PM_{2.5}$, EC and OC using plasma samples collected at all timepoints and averaged exposures for the workweek (Figure 1B). Using FDR <20%, 845 and 650 metabolite features were associated with week-averaged EC and OC levels, respectively; none met the FDR criteria for $PM_{2.5}$ (Supplementary Tables 3 and 4). Comparison of the results for week-averaged MWAS showed 168 metabolite features were associated with both EC and OC. For EC 49 metabolite features were associated with both shift- and week-averaged exposure levels, while OC included 28 metabolite features associated with both exposure time periods. (Figure 2). The majority of the metabolite features showed a positive association with

exposure at FDR<20%, including 634 for EC and 384 for OC that increased with increasing exposure levels.

Metabolite identification for both exposures was first completed by comparison to a reference database of confirmed metabolites (Level 1 identification), which was then complemented with annotation by accurate mass matching and confidence scoring, which allows prediction of chemical formulas and potential metabolite structures (Level 4 annotation). Annotated endogenous metabolites associated with week-averaged EC included lipid peroxidation products, oxidative stress biomarkers, endothelial function-related metabolites and co-factors (Table 3). Lipid peroxidation products and oxidative stress metabolites included 4-oxo-2-nonenal, glutathione and 12-oxo-10E-octadecenoic acid. Pathway enrichment identified 13 metabolic pathways, including changes to fatty acid metabolism, pro-inflammatory lipid signaling, co-factor metabolism and nitrogen catabolism (Figure 3). Only urea cycle/amino group metabolism was also associated with shift-averaged EC.

Annotation of metabolite features associated with week-averaged OC show changes to key metabolic intermediates, including co-factor metabolism, increased oxidative stress, and disruption to nitric oxide (NO) production and endothelial function (Table 4). Pathway enrichment identified 23 pathways associated with week-averaged OC, which were related to endothelial function, co-factor metabolism, inflammatory signaling, nitrogen catabolism, mitochondrial bioenergetics and amino acid metabolism (Figure 3). Pathways including selenoamino acid metabolism, serine metabolism, arginine/proline metabolism, pyruvate metabolism and glycerphospholipid were associated with both shift- and week-averaged OC exposure.

Network correlation of molecular response

We used an integrative network approach developed for combining multi-omic datasets to identify correlated gene and metabolite pathways associated with week-averaged EC and OC exposure (Figure 4). A high degree of connectivity was observed for the two datasets, resulting in 1,234 molecular probes and 146 metabolites with $|r|$ 0.4 and p 0.05. Multi-level community detection identified four clusters in the molecular response network. Excluding cluster 2, the remaining were largely gene expression dominated, with clusters 1, 3 and 4 including 120, 370 and 699 genes, respectively, associated with 65, 2 and 22 metabolites. Cluster 2 contained 57 metabolites and 45 genes. To assess the biological functions associated with each of the clusters, we tested for the presence of metabolite and gene expression pathways enriched in each cluster. The complete results are provided in Supplementary Table 5. Cluster 1 showed association with 15 metabolic and 23 gene expression pathways, which included pathways related to inflammation and immune response, DNA damage, cell adhesion and vascular function. Metabolites present in cluster 2 included 26 metabolic and 13 gene expression pathways. These were consistent with developmental processes, endothelial NO synthases, nucleotide metabolism and oxidative stress processes. Oxidative stress pathways included changes to ascorbate/aldarate metabolism, sulfur amino acid metabolism and TNF-alpha-induced ROS-dependent Caspase-3 signaling, with the metabolites ascorbic acid, glutathione and cysteamine also

present in the correlation cluster. Cluster 3 consists almost entirely of gene expression nodes, with the vitamin E metabolite 7'-carboxy-alpha-chromanol and pyrroline the only two identified metabolites present. Due to this, 60 gene expression pathways were enriched from a diverse range of processes, including adipocyte differentiation, apoptosis, immune response, oxidative and reticulum stress response, inflammation and red blood cell adhesion. Similarly, cluster 4 only showed enrichment in Vitamin E metabolism. The gene expression results showed 47 pathways, including NO synthase signaling in muscle tissue, platelet aggregation, TNF signal transduction, mitochondrial dysfunction, immune response and regulation of lung epithelial progenitor cell differentiation.

Integration of gene expression and metabolites associated with EC and OC identified four main clusters of gene-metabolite correlations. We next evaluated the top two most connected genes present in each cluster to see if these genes had previously been linked to traffic related pollutants by searching CTD for literature reported gene-chemical interactions. For cluster 1, MATR3 and PPM1A showed interaction with benzo[a]pyrene, and exposures listed as volatile organic chemicals and vehicle emissions. In addition, BPDE, dinitrotoluene and nanoparticle exposures had reported effects on expression of MATR3. Cluster 2 nodes included one gene providing no matches in CTD (LOC100131165). The second gene, SNORA14A, only showed interactions with 1-(4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)ethane, a methylated analog of resveratrol that has been shown to reduce levels of induced NO synthase. Chemical interactions listed in CTD for the topmost connected gene expression nodes in cluster 3 included environmental pollutants originating from exhaust emissions. For KIDINS220, traffic-related pollutants included dinitrobenzene, benzene and PAH dipole epoxides, while dinitrotoluenes, PAHs and dipole epoxide metabolites, carbon nanotubes, tobacco smoke and vehicle emissions have documented interactions with expression of IVNS1ABP listed in CTD. Cluster 4 resulted in similar interactions, including dinitrotoluenes, dioxin and PAH-related chemicals for the topmost connected gene in that cluster (UBE2D2), while interactions with dinitrotoluene, dinitrobenzene, a PAH dipole epoxide, dioxins and nitroarenes were present for OTUB1.

DISCUSSION

The present study provides an integrated molecular characterization of the biological response to $PM_{2.5}$, EC and OC based upon trucking industry workers whose jobs included operating and working in proximity to diesel powered trucks in trucking terminals and on roadways. Untargeted metabolomics was used to characterize metabolic changes associated with EC and OC from shift- and week-averaged exposures. The results showed week-averaged EC and OC were associated with the greatest number of metabolic alterations, and included metabolite changes consistent with oxidative stress, endothelial function and inflammation. Changes in these pathways were also present in gene expression profiles correlated with EC and OC-associated metabolites, providing new insight into biological effects underlying exposure to traffic-related pollutants and cardiovascular disease risk.

Metabolomic profiling of exposure to traffic-related pollutants

Due to the ability of metabolomics to characterize multiple key biological processes related to human health and disease, metabolomics approaches studying the effects of traffic related pollution include a wide range of exposure scenarios and timescales, which were recently detailed extensively by Jin et al. (2021). These studies include controlled exposure to ambient air pollution and specific constituents, (Cheng et al. 2018) observational studies that provide comprehensive assessment to traffic related pollutants, (Liang et al. 2018; Vlaanderen et al. 2017) natural and non-natural intervention studies, (Li et al. 2017; Mu et al. 2019) and long-term exposure assessment using location-based exposure estimates. (Menni et al. 2015; Walker et al. 2018; Yan et al. 2019) In several of these studies, how the metabolome mediates relationships between air pollution and other health phenotypes, including pregnancy, asthma and cardiovascular disease, has provided new insight into potential pathways underlying adverse effects of these exposures. (Inoue et al. 2020; Jeong et al. 2018; Liang et al. 2019) While reported metabolites associated with traffic-related pollution have varied depending on the study design and metabolomic platforms used, most studies have consistently shown exposure is associated with endothelial pathways that include critical intermediates for NO, alterations in vitamin E metabolites, antioxidants, sulfur-containing amino acids, fatty acids and lipid peroxidation products consistent with oxidative stress, and changes in inflammatory signaling pathways that include prostaglandins and leukotrienes. Many of these metabolites have also been identified in metabolomic studies of diseases linked to air pollution exposure, including lung cancer, chronic obstructive pulmonary disease (COPD) and cardiovascular disease. (Cruickshank-Quinn et al. 2018; Fitzpatrick et al. 2014; Seow et al. 2019) To date, these studies have largely focused on ambient exposures, which can include emissions from many sources. In the present study, we provide metabolomic characterization specific to workers with occupational exposures primarily from diesel exhaust. Metabolomic and gene expression changes in our study were consistent with many of the pathways described by others, suggesting diesel exhaust exposure results in similar biological response pathways. However, these associations were largely driven by exposure composition, measured as EC and OC, rather than $PM_{2.5}$. These results were unexpected, since $PM_{2.5}$ and other particulate exposures have been linked to metabolic changes and all-cause mortality. (Cohen et al. 2017; Walker et al. 2018) Lack of association with $PM_{2.5}$ could be due to the focus on monitoring exposures during working hours, which would be expected to catch the majority of diesel-related EC and OC exposure, while $PM_{2.5}$ levels could vary considerable throughout the day based upon other factors, such as residence location, home ventilation, commute, and cooking. These results highlight the importance of considering air pollution composition and levels outside of monitoring periods when evaluating metabolic effects of exposure.

Both EC and OC were associated with dose-associated alterations to central metabolic pathways, including amino acids, co-factors, lipids and fatty acids and consistent with changes in oxidative stress, endothelial dysfunction and inflammation. Due to the complexity and varied composition of EC and OC and associated exhaust constituents in particulate matter, findings on the health effects of these two pollutants have largely been inconsistent across different population studies. (Schauer 2003) However, both toxicological and epidemiological studies suggest EC as a marker of traffic exposure and important

contributor to adverse health effects linked to traffic-related exposures. (Janssen et al. 2011) Only a small number of studies have evaluated metabolic effects associated with EC and diesel exhaust. (Liang et al. 2019; Liang et al. 2018) To measure acute response to biodiesel fuel exhaust exposure, Surowiec et al. (2016) evaluated human lung lavage fluids from different lung regions following a 1-hr controlled exposure to biodiesel exhaust emissions, and similar to the associations with EC and OC in the present study identified changes in amino acids, including aspartic acid, ornithine, fatty acids, benzoic acid metabolites and co-factor metabolism following exposure. Additional studies in humans show diesel exhaust results in altered DNA methylation profiles, (Jiang et al. 2014) gene expression, (Peretz et al. 2007) and changes to bioactive lipids in lung lavage fluids. (Gouveia-Figueira et al. 2017) Model systems provide additional evidence of metabolic changes associated with exposure to diesel emissions. Oeder et al. (2015) applied a multi-omic approach that included proteomic, transcriptomic and metabolomic assessment of exposure to emissions from diesel and heavy fuel oil using human epithelial lung cells. The results showed diesel fuel resulted in pathway alterations across multiple omic-layers that were not observed for the heavy fuel oil particulates. Metabolomic profiling of mouse models using mixed vehicle emissions that included both gasoline and diesel exhaust have identified similar metabolic changes in oxidative stress-related metabolites, inflammation and lipid peroxidation products, in addition to alterations in intermediates of NO production critical for proper vascular and endothelial function. (Brower et al. 2016)

Metabolomic response profiles of occupational EC exposure

Week-averaged EC levels were associated with antioxidant metabolites and biomarkers of increased oxidative stress, which have been linked to risk of CVD. Increased oxidative stress and formation of *in vivo* ROS has been well documented in exposure to particulate matter. (Bates et al. 2015b; Lodovici and Bigagli 2011; Neophytou et al. 2014) Previous HRM studies have also identified similar changes in oxidative stress-related metabolites associated with ultrafine particulates from near highway exposures, (Walker et al. 2018) including increased levels of lipid peroxidation products, which have been implicated in the pathogenesis of multiple chronic diseases. (Akude et al. 2010; Selley 1998) In addition, the two antioxidants GSH and ascorbic acid were identified. GSH and its corresponding oxidized form, glutathione disulfide (GSSG) are one of the primary thiol/disulfide redox couples and provide multiple critical functions, which include protecting against oxidative stress, chemical detoxification and arginine turnover during NO synthase. (Hofmann and Schmidt 1995) Ascorbic acid, which is a protective antioxidant with functions similar to GSH, contributes in multiple ways to endothelial functions, including chemical stabilization of tetrahydrobiopterin during NO synthesis, vascular endothelial function, and has beneficial effects on vascular dilation. (Brown and Hu 2001; Heller et al. 2001; Jackson et al. 1998; Padayatty et al. 2003) Increased oxidation, particularly of thiol couples, and subsequent shift towards a more positive steady state redox potential has been shown to regulate early events in atherosclerosis and is associated with CVD and endothelial dysfunction. (Ashfaq et al. 2008; Go and Jones 2005) A high burden of oxidative stress has also been associated with mortality in patients with coronary heart disease. (Patel et al. 2016)

Additional associations with EC exposure consistent with CVD risk include inflammatory and endothelial-related metabolites. Thromboxane A2 (TxA$_2$) provides multiple physiological functions, which include signaling for activation of new platelets and increased aggregation, vasoconstriction, endothelial adhesion molecule expression, cell proliferation and acts as a hypertensive agent. (Ding and Murray 2005; Ishizuka et al. 1998; Smyth 2010) Elevated biosynthesis of TxA$_2$ and increased receptor expression are elevated in CVD. (Smyth 2010) Additional inducers of TxA$_2$ production and receptor expression include inflammation, oxidative stress and NO, with TxA$_2$ and other prostaglandin formation almost entirely driven by NO following inflammatory events. (Mollace et al. 2005) This is consistent with the EC-associated increase in hydroxy-L-homoarginine, which is a minor metabolic byproduct of NO production by oxidation of the arginine (Arg) analogue, homoarginine. (Moali et al. 1998) Increased prostaglandin I2 (PGI$_2$) and hepoxilin A3-C (HxA$_3$-C) provides additional evidence of disruption to this pathway. The physiological functions of PGI$_2$ include mediation of vasodilation and platelet inhibition, (Cheng et al. 2002; Whittle et al. 1978) and homeostatic control for the vasoactive effects of TxA$_2$. HxA$_3$-C is the glutathione adduct of hepoxilin A3, which is also an arachidonic acid metabolite with pro-inflammatory and vasodilation signaling properties. Taken together, these results provide strong evidence that exposure to EC results in changes to physiologically active signaling pathways that are implicated in a wide range of processes related to CVD and endothelial dysfunction.

Metabolomic response profiles of occupational OC exposure

Both shift- and week-averaged OC exposures resulted in changes to metabolites providing antioxidant effects and markers of oxidative stress. Metabolite changes suggesting increased oxidative stress include methionine sulfoxide, (Moskovitz et al. 1997) and LGSH, which is a byproduct of GSH dependent degradation of methylglyoxal (MG) by glyoxalate I. (Chang and Wu 2006) MG has been implicated in a number of disease processes, including hypertension, diabetes and formation of atherosclerotic lesions. (Chang and Wu 2006) Pathways consistent with antioxidant processes were also detected, including vitamin E metabolism and ascorbate/aldarate metabolism. Alpha-tocopherol was positively associated with OC, which has previously been observed to protect against decreasing lung function following PM$_{2.5}$ exposure, (Menni et al. 2015) and has been associated with exposure to other traffic-related pollutants. (Liang et al. 2018)

OC exposure was associated with the greatest number of metabolites related to endothelial function. This included alterations to arginine and proline metabolism, and changes to metabolites with functions that include NO production from arginine, required co-factors, decreased thrombotic signaling molecules and endogenous inhibitors of NO. Many of these changes indicate OC results in disruption to NO production itself, while EC associations suggest increased platelet formation and aggregation. Changes consistent with possible disruption to NO included agmatine, acetylagmatine and n-(omega)-hydroxyarginine. Agmatine, which is an endogenously produced decarboxylation product of Arg, inhibits inducible NO synthase and acts as a modulator of NO production. (Raghavan and Dikshit 2004) Decreased levels suggest elevated rates of NO production, which is consistent with the changes in n-(omega)-hydroxyarginine but not citrulline. N-(omega)-hydroxyarginine is

the first oxidative product of arginine by NO synthase and can as both an inhibitor of Mn dependent arginase enzymes and catalytic intermediate in NO production. (Cox et al. 2001) Taken together, these results suggest OC exposure contributes to metabolic alterations that have important implications for endothelial function.

Differential metabolomic response to EC and OC occupational exposures

While many of the underlying biological processes associated with OC were similar to those observed for EC, specific metabolite changes differed and overlapping metabolites showed opposing direction of change following exposure to the two pollutants. A possible explanation for this trend is different patterns of exposure and particulate origin, which is consistent with the lack of correlation between EC and OC. (Zhang et al. 2011) Prior characterization of this cohort has shown exposure patterns differ based upon job title and pollutant, with pickup/delivery drivers and dockworkers exhibiting highest exposure levels of EC, while office workers and drivers showed the highest OC levels. (Neophytou et al. 2013) The OC fraction of particulate matter consists of a complex mixture of polar and non-polar chemicals that can be formed after initial combustion, including organic matter condensation, binding of semi-volatile organics on the particulate surface and interaction with aerosols, in addition to atmospheric changes occurring during transport. Thus, while EC represents exposures from diesel fuel exhaust, the measured OC could arise from a variety of sources and may not be due to diesel emissions alone.

Gene expression by metabolome molecular correlation networks

Following characterization of the metabolome, we tested for additional associations with gene expression levels to characterize potential biological activity networks linking metabolite and gene expression pathways. Exposure to environmental chemicals can influence local and global changes in gene transcription and enzyme activity, resulting in varied biological changes that can influence metabolic changes and contribute to underlying toxicological mechanisms. Integrating gene expression results with MWAS has the potential to enhance understanding of how functional molecular mechanisms interact due to environmental exposures, providing a systems biology framework for studying toxicological effects of air pollution *in vivo*. Enriched gene pathways were consistent with the identified metabolic changes associated with EC and OC exposure, providing additional evidence that exposure to traffic-related pollution leads to changes in endothelial function, inflammation and oxidative stress. Most importantly, enriched metabolite and gene expression pathways within each cluster consisted of different biochemical processes, suggesting that the biological response to traffic-related air pollution is systemic and has important implications for homeostatic control and health. Comparison to curated data in CTD showed the most-connected genes for each cluster have previously been demonstrated to interact with pollutants present in exhaust emissions, including PAHs and diol epoxide metabolites, VOCs and nitroaromatics. Thus, not only were the *in vivo* identified biological changes from gene expression and the metabolome consistent, using CTD we show the most connected transcript in each cluster have been associated with gene changes due to traffic-related pollutants *in vitro*.

Limitations

We acknowledge several limitations of this work. First, this study was limited to an all-male, non-smoking, predominantly Caucasian cohort employed in the US unionized trucking industry. Therefore, we were unable to examine differences due to sex or other unaccounted for confounders. Although samples were collected over the course of a workweek, only exposures occurring at the workplace were considered, and we could not account for those that occurred during commuting or at home. Second, results showed week-averaged exposures resulted in the greatest molecular changes; however, these were estimated based upon one week only, and due to the length of the study we could not evaluate the effects of longer exposure periods or how levels varied over time. Third, the results from this study are correlative in nature. While we accounted for age, BMI and measurement day in our model, we could not account for unknown and uncharacterized confounders. In addition, we evaluated correlation between gene expression levels and the metabolome. Previous results in humans have shown signals in whole blood generally reflect organism wide processes; (Bartel et al. 2015) however, although the metabolome represents an integrated profile of multiple body compartments and processes, it is not possible to assess cell specific metabolite or gene changes. Fourth, only a small number of metabolites were identified by comparison to standards, and the majority of metabolite features were annotated using MS1 accurate mass only. Limited reference standards, low abundance of many of the metabolite features and lack of fragmentation spectra from MSMS render characterization and identification challenging. Finally, exposure was determined using aggregate measures that included $PM_{2.5}$, EC and OC. We were unable to characterize other exposures associated with vehicle-exhaust exposures (e.g. nitrogen oxides, volatile or semi-volatile organics, or even individual PAHs or nitro-PAHs). Despite these limitations, we identified EC and OC exposure-associated changes consistent with increased oxidative stress, endothelial dysfunction and inflammation, which are common risk factors for diseases related to air pollution and diesel exhaust exposure. Integrating gene expression and metabolome profiles showed these changes were present across both molecular layers, providing additional evidence of biological response pathways underlying environmental exposure to traffic related pollutants. Continued application of these approaches in humans will provide improved understanding of how environmental exposures contribute to adverse health outcomes.

CONCLUSIONS

To elucidate the molecular response of occupational exposure to traffic-related pollutants in the US trucking industry, we used an MWAS framework to identify plasma metabolic changes in workers following shift- and week-averaged exposure to EC, OC and $PM_{2.5}$. MWAS of EC and OC identified dose-associated metabolic alterations consistent with endothelial function, inflammation and oxidative stress. These include annotated metabolites that function as thrombotic signaling molecules, antioxidants, biomarkers of oxidative stress, lipid peroxidation products and intermediate metabolites in NO production. Week-averaged levels of both pollutants were associated with the greatest number of metabolite alterations observed in study participants, suggesting persistent workplace exposure influence biological changes that are not mediated by time off. Data-driven integration of

gene expression levels with exposure-associated metabolites identified additional functional changes related to endothelial function, NO synthase, inflammation and immune response, including changes that have been reported as associated with traffic-related pollutants in other studies. Taken together, the results show exposure to traffic-related pollutants that includes diesel exhaust emissions influences metabolite and gene expression pathways implicated in cardiopulmonary disease risk and provide insight into the molecular pathways underlying the adverse effects of traffic-related pollution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Diesel exhaust pollutants were evaluated for workers in the US trucking industry.

- Metabolomic alterations were associated with elemental carbon and organic carbon.

- No metabolites were associated with $PM_{2.5}$ exposure.

- Exposure-associated metabolites were related to oxidative stress and nitric oxide production

- Gene and metabolite networks support associations with cardiopulmonary disease
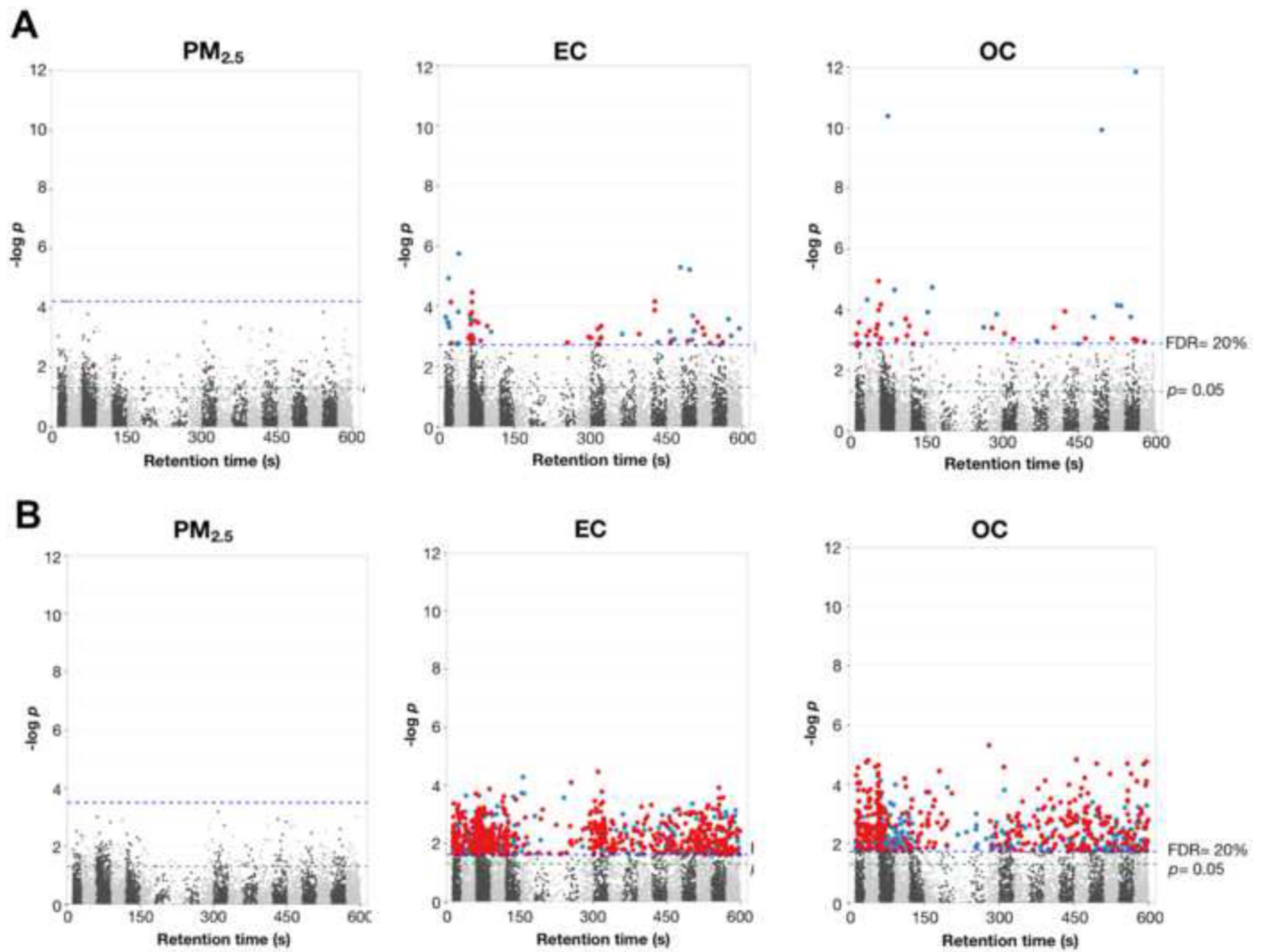
Figure 1:

Manhattan plot showing −log p as a function m/z feature retention time for A) daily and B) week-average exposure to $PM_{2.5}$, EC and OC. MWAS was completed using linear mixed effects models and post-shift metabolite intensity only. Fixed effects included daily shift length exposure, age, day and BMI. Red= positive association; Blue= negative association

Figure 2:
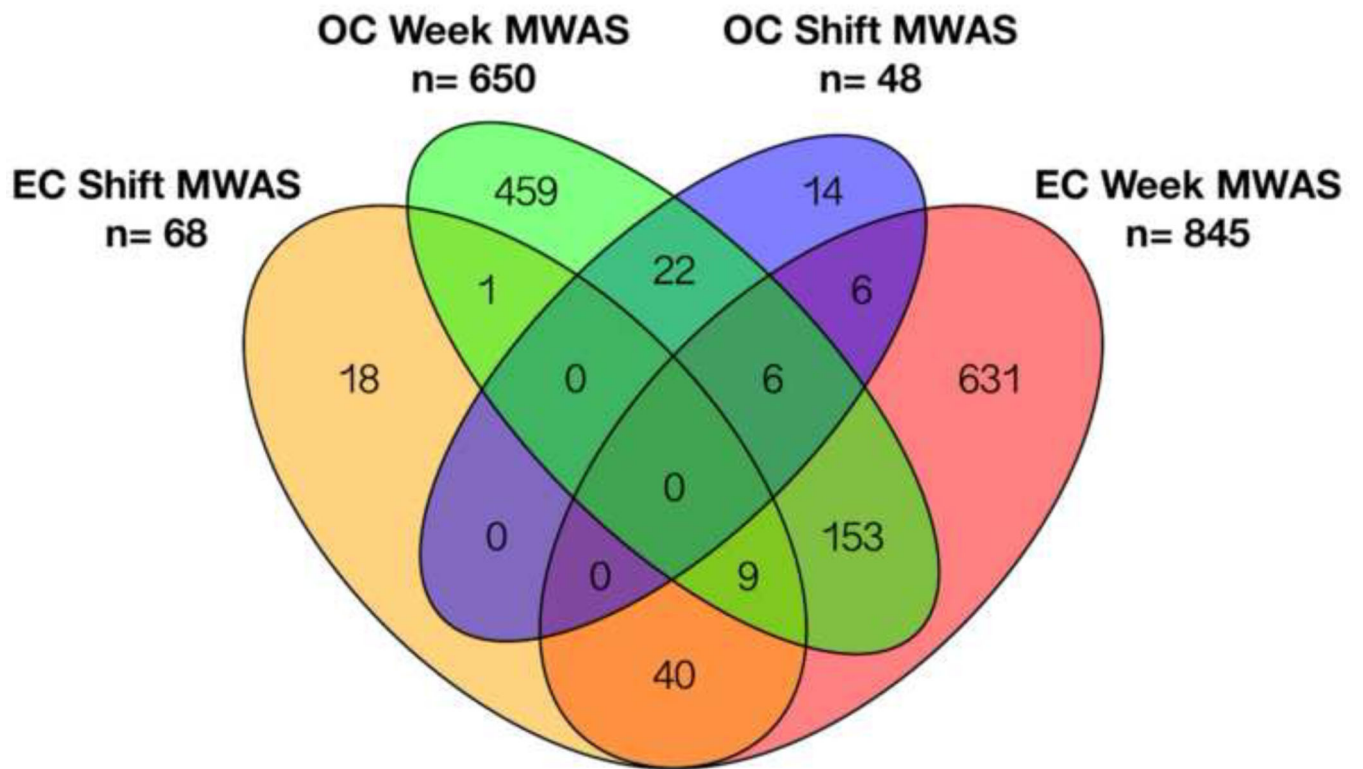Overlapping features associated with shift-length and week averaged exposure to the two pollutants at false discovery rate threshold 20%.
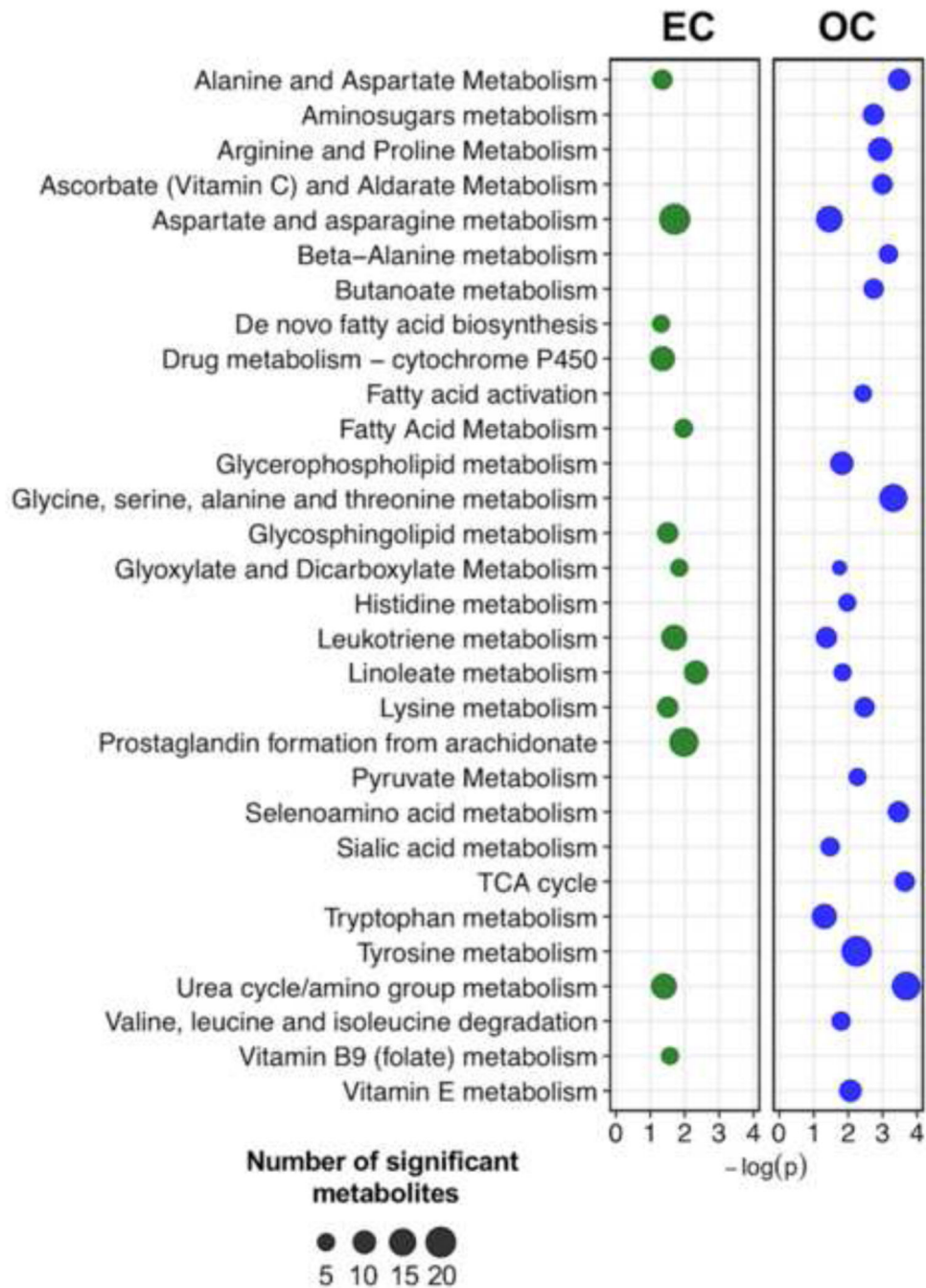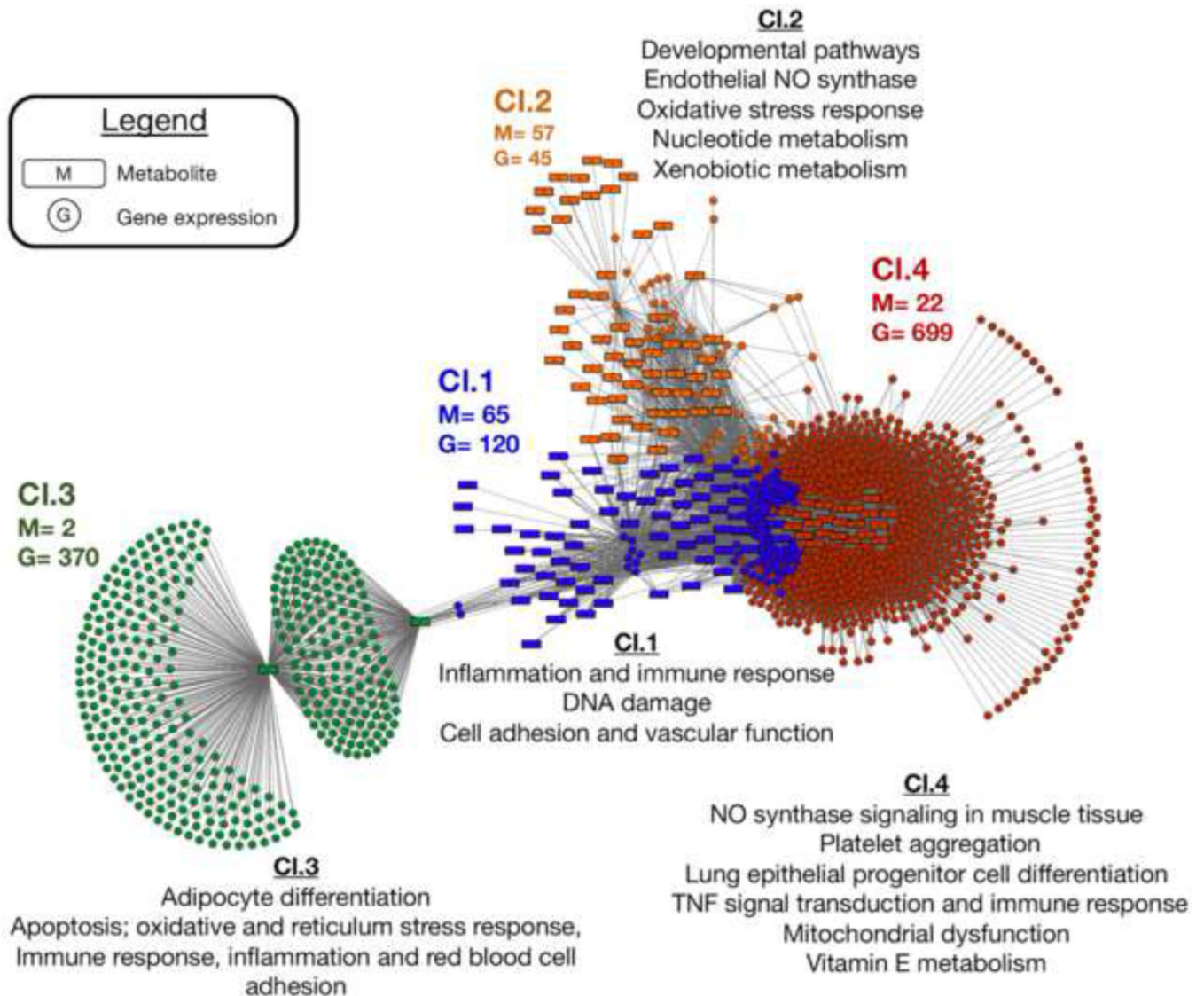
Figure 3:
Metabolic pathways associated with week-averaged exposure to elemental and organic carbon. Dot size is a function of the number of metabolites from that pathway associated with the corresponding exposure measure. Only pathways with *P*<0.05 were considered.

Figure 4:

Metabolite and gene expression integration network. Discriminatory features and network correlations were determined using the R package xMWAS. The top discriminatory genes and identified metabolites were first selected using multilevel sparse partial least squares regression analysis, which were then evaluated for correlation with $|r|$ 0.4 and $p< 0.05$. Clusters of gene expression and metabolite profiles were identified using a using multi-level community detection algorithm that groups nodes showing a high-degree of connection among each other and are less connected to other nodes in the network (cluster 1= blue nodes, cluster 2= orange nodes; cluster 3= green nodes; cluster 4= red nodes). Each cluster was then evaluated for biological activity and overlap between metabolite and gene expression pathways. Three of clusters that were identified included both metabolites and genes; cluster 3 was predominantly genes and only included two metabolites, suggesting a minimal degree of overlap between metabolic and gene expression related processes present in cluster 3. For each cluster, the main pathways linked to endothelial function,

inflammation, oxidative stress and related are provided; a full list of gene expression and metabolite pathways present in the correlation network are provided in Supplementary Table 5. Cl= Cluster; M= metabolite node, G= gene expression node.

Table 1.

Population characteristics of 73 male unionized trucking industry workers included in HRM profiling

Characteristic	Total
Number of individuals	73
Age (years, mean±SD)	49.8 ± 8.3
BMI (mean±SD)	29.8 ± 4.5
Race (*n*, (%))	
White	68 (93%)
Hispanic	5 (7%)
Primary job title (*n*, (%))	
Pick-up and delivery driver	38 (52%)
Dockworker	14 (19%)
Officeworker	21 (29%)
Average first day of work exposures (mean±SD)	
$PM_{2.5}$ (μg/m³)	10.0 ± 5.7
EC (μg/m³)	0.6 ± 0.5
OC (μg/m³)	8.8 ± 2.9
Average last day of work exposures (mean±SD)	
$PM_{2.5}$ (μg/m³)	9.7 ± 4.8
EC (μg/m³)	0.6 ± 0.4
OC (μg/m³)	8.6 ± 4.3
Average work-week exposures (mean±SD)	
$PM_{2.5}$ (μg/m³)	10.0 ± 4.5
EC (μg/m³)	0.6 ± 0.4
OC (μg/m³)	8.7 ± 3.1
Median work-week exposures (median, IQR)	
$PM_{2.5}$ (μg/m³)	9.7, 6.3
EC (μg/m³)	0.5, 0.6
OC (μg/m³)	8.9, 4.0

Table 2.

Annotated metabolites related to oxidative stress and endothelial function associated with shift-averaged exposure to elemental carbon (EC) and organic carbon (OC) detected in plasma from 73 male unionized trucking industry workers.

m/z	Associated exposure	Identity	Number of matches[1]	Annotation confidence[2]	Exposure β (95% CI)	p-value
155.1067	EC	4-oxo-2-Nonenal	13	4	−0.9 (−1.3, −0.5)	4.88E-06
389.1350	EC	16-Hydroxy-4-carboxyretinoic acid	15	4	−2.4 (−3.7, −1.0)	1.09E-03
105.0341	EC	Benzoic acid	1	1	−3.7 (−5.8, −1.5)	8.18E-04
167.9929	EC	2-Mercaptobenzothiazole	1	4	2.3 (0.9, 3.7)	1.61E-03
422.2357	EC	Leukotriene E4	16	4	3.2 (1.4, 5.1)	8.66E-04
223.1054	EC	Dihydrobiopterin	2	4	3.4 (1.5, 5.5)	1.15E-03
854.373	EC	Heme A	1	4	3.9 (2.0,5.8)	7.20E-05
130.0337	OC	Methionine-sulfoxide	1	1	−1.8 (−2.2, −1.3)	1.40E-12
311.1286	OC	Porphyrin	25	4	−1.8 (−2.7, −1.0)	7.10E-05
136.0622	OC	Adenine	2	4	−2.4 (−3.8, −1.0)	1.05E-03
157.1224	OC	4-hydroxynonenal	28	4	−2.8 (−4.2, −1.4)	1.65E-04
589.2770	OC	Mesoporphyrin	2	4	2.7 (1.1, 4.3)	1.35E-03
402.0927	OC	Lactoylglutathione	11	4	3.3 (1.6, 5.1)	2.50E-04
107.0537	OC	Serine	1	1	3.5 (1.7, 5.3)	2.00E-04
132.0657	OC	L-Glutamate-5-semialdehyde	19	4	3.9 (1.8, 6.1)	3.70E-04

[1.]Total number of compounds matching accurate mass *m/z* using HMDB 3.0 at 10 ppm mass accuracy.

[2.]Identification confidence based upon Schymanski et al. (2014). Level 4 confidence levels are based upon accurate mass matching only, and do not inform on compound structures. The full list of metabolite matches from HMDB 3.0 are provided in Supplementary Table 1 (EC) and Supplementary Table 2 (OC).

Table 3.

Annotated metabolites related to oxidative stress, endothelial function and cofactor metabolism associated with week-averaged exposure to elemental carbon (EC) detected in plasma from 73 male unionized trucking industry workers.

m/z	Identity	Number of Matches[1]	Identification confidence[2]	Change per IQR (95% CI)	*p*
196.1335	4-oxo-2-nonenal	3	4	0.5 (0.1, 0.9)	1.5E-02
263.2366	Linoleic acid	1	1	1.6 (0.5, 2.8)	6.9E-03
297.2410	12-oxo-10E-octadecenoic acid	8	4	1.8 (0.6, 2.9)	3.2E-03
371.1007	Glutathione	11	4	2.4 (0.7, 4.1)	6.0E-03
177.0400	Ascorbic acid	1	1	3.9 (1.9, 5.9)	2.3E-04
351.2144	Prostaglandin I2	19	4	1.7 (0.3, 3.1)	1.7E-02
335.2214	Thromboxane B2	5	4	2.6 (.5, 4.8)	1.5E-01
375.2141	Thromboxane A2	6	4	2.7 (0.8, 4.5)	5.8E-03
624.2953	Hepoxilin A3-C	7	4	2.9 (1.0, 4.8)	3.6E-03
854.3730	Heme A	1	4	3.5 (1.7, 5.2)	2.2E-04
227.1097	Hydroxy-L-homoarginine	5	4	2.6 (0.8, 4.4)	6.3E-03
169.0592	Glutamine	1	1	−0.5 (−1.0, −0.1)	1.7E-02
156.0278	Aspartic acid	1	1	2.7 (0.6, 4.8)	1.1E-02
445.1649	Dihydrofolic acid	1	1	−2.2 (−3.7, −0.7)	5.4E-03
459.1986	Riboflavin	12	4	−3.8 (−6.0, −1.6)	8.2E-04

[1] Total number of compounds matching accurate mass *m/z* using HMDB 3.0 at 10 ppm mass accuracy.

[2] Identification confidence based upon Schymanski et al. (2014). Level 4 confidence levels are based upon accurate mass matching only, and do not inform on compound structures. The full list of metabolite matches from HMDB 3.0 are provided in Supplementary Table 3.

Table 4.

Annotated metabolites related to oxidative stress, endothelial function and cofactor metabolism associated with week-averaged exposure to organic carbon (OC) detected in plasma from 73 male unionized trucking industry workers.

m/z	Identity	Number of matches[1]	Identification confidence[2]	Change per IQR (95% CI)	p
221.0940	Hippuric acid		1	−2.5 (−4.2, − 0.8)	5.2E-03
204.0086	Methionine sulfoxide	1	1	0.8 (0.3, 1.4)	3.5E-03
402.0921	Lactoylglutathione	11	4	3.4 (1.0, 5.7)	5.1E-03
177.0691	Homocysteine	1	1	−2.4 (−4.3, −0.4)	1.7E-02
156.1021	4-hydroxyhexenal	16	4	−2.6 (−4.5, −0.6)	9.3E-03
214.0578	Citrulline	1	1	−0.7 (−1.2, −0.1)	1.6E-02
303.1179	Dihydrobiopterin	5	4	−3.8 (−5.6, −2)	9.8E-05
335.2214	Thromboxane B2	5	4	−3.8 (−6.2, −1.4)	2.4E-03
227.1097	Hydroxy-Lhomoarginine	5	4	−3.9 (−5.9, 1.8)	4.2E-04
169.0860	Agmatine	11	4	−4.2 (−6.4, −1.9)	3.8E-04
209.0081	Ornithine	4	4	1.3 (0.3, 2.2)	1.1E-02
217.1045	Acetylagmatine	1	4	1.9 (0.4, 3.3)	1.1E-02
229.0682	N-(omega)-Hydroxyarginine	8	4	2.7 (0.9, 4.4)	2.9E-03
132.0657	Lactate	19	4	3.1 (1.2, 4.9)	1.7E-03
195.9779	Threonine	1	1	−3.7 (−5.6, −1.9)	1.5E-04
156.0278	Aspartic acid	1	1	−3.6 (−6, −1.2)	4.2E-03
216.1960	alpha-Tocopherol	4	4	1.9 (0.6, 3.2)	6.1E-03
134.0635	Asparagine	1	1	2.6 (1, 4.2)	1.7E-03
107.0537	Serine	1	1	4.1 (2.2, 6)	6.1E-05
202.1074	Pantothenic acid	17	4	3.9 (1.6, 6.3)	1.4E-03

[1.] Total number of compounds matching accurate mass m/z using HMDB 3.0 at 10 ppm mass accuracy.

[2.] Identification confidence based upon Schymanski et al. (2014). Level 4 confidence levels are based upon accurate mass matching only, and do not inform on compound structures. The full list of metabolite matches from HMDB 3.0 are provided in Supplementary Table 4.