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Journal Title: Environment International
Volume: Volume 127
Publisher: Elsevier: Creative Commons Licenses | 2019-06-01, Pages 503-513
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1016/j.envint.2019.04.003
Permanent URL: https://pid.emory.edu/ark:/25593/tqycc

Final published version: http://dx.doi.org/10.1016/j.envint.2019.04.003

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Accessed August 7, 2024 6:56 AM EDT
Perturbations of the arginine metabolome following exposures to traffic-related air pollution in a panel of commuters with and without asthma

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ARTICLE INFO
Handling Editor: Hanna Boogaard
Keywords:
Traffic-related air pollution
High-resolution metabolomics
Asthma
Metabolomics-wide association study
Environmentally mediated responses

ABSTRACT
Background: Mechanisms underlying the effects of traffic-related air pollution on people with asthma remain largely unknown, despite the abundance of observational and controlled studies reporting associations between traffic sources and asthma exacerbation and hospitalizations.
Objectives: To identify molecular pathways perturbed following traffic pollution exposures, we analyzed data as part of the Atlanta Commuters Exposure (ACE-2) study, a crossover panel of commuters with and without asthma.
Methods: We measured 27 air pollutants and conducted high-resolution metabolomics profiling on blood samples from 45 commuters before and after each exposure session. We evaluated metabolite and metabolic pathway perturbations using an untargeted metabolome-wide association study framework with pathway analyses and chemical annotation.
Results: Most of the measured pollutants were elevated in highway commutes (p < 0.05). From both negative and positive ionization modes, 17,586 and 9087 metabolic features were extracted from plasma, respectively. 494 and 220 unique features were associated with at least 3 of the 27 exposures, respectively (p < 0.05), after controlling confounders and false discovery rates. Pathway analysis indicated alteration of several inflammatory and oxidative stress related metabolic pathways, including leukotriene, vitamin E, cytochrome P450, and tryptophan metabolism. We identified and annotated 45 unique metabolites enriched in these pathways, including arginine, histidine, and methionine. Most of these metabolites were not only associated with multiple pollutants, but also differentially expressed between participants with and without asthma. The analysis indicated that these metabolites collectively participated in an interrelated molecular network centering on arginine metabolism, underlying the impact of traffic-related pollutants on individuals with asthma.

Conclusions: We detected numerous significant metabolic perturbations associated with in-vehicle exposures during commuting and validated metabolites that were closely linked to several inflammatory and redox pathways, elucidating the potential molecular mechanisms of traffic-related air pollution toxicity. These results support future studies of metabolic markers of traffic exposures and the corresponding molecular mechanisms.

1. Introduction

Asthma is the most prevalent chronic respiratory disease worldwide, accounting for over 495,000 global deaths annually (Roth et al., 2018). The impact of urban air pollution on asthmatic population has been a long-standing environmental health concern (Cohen et al., 2017;...
Soriano et al., 2017). Traffic related air pollution (TRAP), in particular, comprises over 25% of urban air pollution and has been linked in observational and controlled studies to asthma exacerbation and hospitalizations (Health Effects Institute, 2010). Less is known about the specific components of TRAP that may be causally responsible for these findings. Traffic emissions are highly heterogeneous, consisting of hundreds of organic and inorganic chemicals. In addition, the lack of sensitive and specific biomarkers has hindered research into the etiologic basis and acute health risk associated with exposure to TRAP in individuals with asthma.

High-resolution metabolomics (HRM), a high-throughput method involving the identification and quantitation of thousands of metabolic features associated with exogenous exposure and endogenous processes, has emerged as a powerful tool to improve exposure estimation to complex environmental mixtures (Bundy et al., 2009; Miller and Jones, 2014). Several studies have previously demonstrated that metabolomics sensitively reflect internal metabolic perturbations following exposures to urban air pollution (Huang et al., 2018; Jeong et al., 2018; Ladva et al., 2018; Liang et al., 2018b; van Veldhoven et al., 2019; Vlaanderen et al., 2017). These initial findings are promising and have led us to hypothesize that TRAP-specific metabolic markers of exposure and response may be detectable, especially in individuals thought to most susceptible to risk from this pollutant source (i.e., asthma).

To address this, we analyzed data from the Atlanta Commuters Exposure (ACE-2) study, a semi-controlled, crossover study of car commuters with and without asthma. Given their ability to provide accurate assessment of both external exposure and internal response, panel-based studies have proven to be an effective platform to investigate TRAP health effects (Delfino et al., 2006; Delfino et al., 2008; McCreanor et al., 2007; Sarnat et al., 2012). Nevertheless, results from previous targeted, panel-based studies have been inconsistent in identifying specific components of traffic that may be causally responsible for these observations (Riediker et al., 2004; Zuberbier et al., 2010) and null responses in others (Chiu et al., 2016; Wu et al., 2014). These inconsistencies are perhaps largely due to the lack of robust and specific biomarkers that accurately reflect TRAP exposure or the corresponding effects (Liang et al., 2018a; Rylance et al., 2013). In our previous ACE-2 analyses (Golan et al., 2018), we observed modest, albeit transient, increases in systemic inflammation and acute respiratory response, using several known traditional targeted biomarkers, following on-road commutes and in association with several common traffic pollutant components. In addition, perturbations of the metabolome were found to be associated with in-vehicle particulate exposure and traditional markers of inflammation (Ladva et al., 2018).

Here, we build on an initial metabolomics analysis in the ACE-2 panel, by conducting a comprehensive biological pathway analysis and chemical identification, to identify systemic metabolic perturbations associated with TRAP. We also examine how asthma status modifies these perturbations to explore potential molecular mechanisms of TRAP toxicity on people with asthma. Ultimately, we view this work as part of a larger effort to improve exposure assessment to TRAP, a critical step in the development of targeted interventions aimed at reducing the health burden associated with this pollutant source, particularly in individuals with asthma.

2. Methods

The ACE-2 study is a randomized, crossover panel study of 60 adults with self-reported, mild-to-moderate asthma (N = 30) or without asthma (N = 30). The study protocol included an extensive assessment of in-vehicle environmental exposure and repeated measurements on numerous health endpoints from 2011 to 2013 in Atlanta, GA. The study design, participant demographic characteristics, recruitment and exclusion criteria have been previously detailed (Golan et al., 2018; Krall et al., 2018). Briefly, we randomly assigned all participants to participate in two exposure sessions, scheduled 7 days apart, which consisted of a scripted 2 h highway commute and either a 2 h scripted surface street commute or a 2 h clinic visit. We conducted each exposure session during the morning rush hour (7 am to 9 am). Highway commutes took place on Interstate 285 (I-285), a relatively congested highway encircling Atlanta (2016 annual average daily traffic (AADT): 203,000). We conducted the surface street exposure session on side streets within close proximity to Emory University (2016 AADT, 10,400-25,800). The clinic visit sessions were conducted in a dedicated, internal examination room within the Emory Environmental Health Laboratory. The highway and surface street commute sessions spanned driving distances of approximately 50 km and 30 km, respectively, with participants driving their personal vehicles, and study staff as passengers. During the clinic visit, participants were seated in the clinic for the duration of the session. The study protocol and all aspects of human subjects’ participation were approved and supervised by the Emory University Institutional Review Board.

2.1. Exposure assessment

Fifty-eight pollutant species were measured in all exposure sessions using a specially-designed mobile monitoring platform (Golan et al., 2018; Ladva et al., 2018). For this analysis, however, we restricted the pollutant analytic database to 3 groups or factors, a priori, based on a published ACE-2 analysis of pollutant source attribution (Krall et al., 2018). These source-specific pollution factors were comprised of particle-bound polycyclic aromatic hydrocarbons (pb-PAH), particle number concentration (PNC), fine particulate matter (PM2.5), and noise, using both time-integrated and continuous monitors. In addition, we examined a range of size- and chemically-resolved particulate components, including 19 elements that were detected in least 60% of all collected filter samples, as well as black carbon (BC), elemental carbon (EC), organic carbon (OC), and water soluble organic carbon (WSOC). Although each of these pollutants examined may be present in and indicative of other pollution source categories, we found each to be enriched in several traffic-related sources including resuspended crustal, primary tailpipe traffic, and non-tailpipe traffic sources.

2.2. Biomonitoring and high-resolution metabolomics

Among the 60 adults that participated in the scripted commutes, 45 (21 non-asthmatic and 24 asthmatic) contributed venous blood prior to and after each sampling session. Blood was drawn at baseline and 10 h later (8 h post commute). Participants were allowed to conduct normal daily activities between each biomonitoring sessions, but they were asked not to leave the campus vicinity surrounding the laboratory facilities. In addition, they were restricted from eating 30 min prior to each health measurement session. In total, we collected and analyzed 140 plasma samples using established protocols (Ladva et al., 2017; Liang et al., 2018b). We treated each sample with two volumes of acetonitrile and analyzed in triplicate using liquid chromatography-high-resolution mass spectrometry (LC-HRMS) techniques (Dionex Ultimate 3000; ThermoScientific Q Exactive). We used C18 hydrophobic reversed-phase chromatography with positive and negative electro-spray ionization (ESI) modes, at 70,000 resolution over a mass-to-charge ratio (m/z) range of 85 to 1250, to enhance the coverage of metabolic feature detection. We applied two quality control pooled reference plasma samples, which included NIST 1950 (Simon-Manso et al., 2013) and pooled human plasma purchased from Equitech Bio, at the beginning and end of each analytical batch of 20 samples for normalization, control of background noise, batch evaluation, and post hoc quantification. Following instrument analyses of all samples, we converted raw data files into .cdf files using ProteoWizard and extracted metabolic signals using apLCMS with modifications by xMSanalyzer, which were specifically configured to enhance data quality control and correct for batch effects (Uppal et al., 2013; Yu et al., 2009). Detected
signals (‘metabolic features’) were uniquely defined by their mass-to-charge ratio (m/z), retention time and ion intensity. For further analyses, we only included metabolic features detected in > 20% of all plasma samples, with median coefficients of variation (CV) among technical replicates < 30% and Pearson correlation > 0.7. Following quality assessment, replicate samples were averaged and averaged intensities were log2 transformed.

2.3. Statistical analysis

We then conducted linear mixed effect modeling to examine associations between post-minus-pre-changes in metabolic feature intensity (i.e., relative concentration) and corresponding pollutant concentrations during the exposure session. This modeling approach is often referred to as a Metabolome-Wide Association Study (MWAS), where metabolic features were analyzed without prior knowledge of their chemical identity. We used the mean concentration of each of the measured air pollutants, during each sampling session, as the primary predictor in single-pollutant models. Models testing the main effect for each metric had the following form:

$$\Delta \log_{10} Y_{ijt} = \mu + \delta_{ij} + \beta_{1j} Pollutant_{at} + \beta_{2j} Asthma + \beta_{3j} Week_{at} + \beta_{4j} Age_{i} + \beta_{5j} Sex_{i} + \beta_{6j} Race_{c} + \beta_{7j} BMI + \beta_{8j} Baseline \log_{10} Y_{ijt} + \epsilon_{ijkt}$$  

(1)

where $\Delta \log_{10} Y_{ijt}$ refers to the log post- and pre- exposure changes in intensity for metabolic feature $j$ for participant $i$ on sampling date $t$. Separate models were conducted for each metabolic feature, from each ionization mode (plasma C18 positive ionization column, and plasma C18 negative ionization column), $\mu$ is the fixed-effect intercept and a random intercept $\theta_i$ is included to control for unspecified between-participant heterogeneity. $Pollutant_{at}$ refers to the average concentration of the traffic related pollutant $k$ for participant $i$ during the sampling session on sampling date $t$. $Asthma_i$ refers to whether participant $i$ had self-reported mild-to-moderate asthma or not. We included other covariates to control for potential between-participant differences, including age (continuous), sex (categorical), body mass index (BMI; continuous), and race (categorical, White, Asian, and Other). We also controlled for $Week_{at}$, referring to whether the exposure session corresponded to the first or second session of the participant's completion of the protocol; and $Baseline \log_{10} Y_{ijt}$, the baseline intensity for metabolic feature $j$ for participant $i$ prior to the commute on sampling date $t$. $\epsilon_{ijkt}$ represents residual random Normal error.

To further examine potential modification of the pollutant-metabolic feature associations by asthma status, we stratified the cohort into 1) participants with asthma and 2) participants without asthma. The main effects of each of the traffic exposure metrics on the metabolomic profiles for each subgroup were examined using the following model, run for each asthma status subgroup separately:

$$\Delta \log_{10} Y_{ijt} = \mu + \delta_{ij} + \beta_{1j} Pollutant_{at} + \beta_{2j} Asthma_{i} + \beta_{3j} Pollutant_{at}$$

* $Asthma_i + \beta_{4j} Week_{at} + \beta_{5j} Age_{i} + \beta_{6j} Sex_{i} + \beta_{7j} Race_{c}$

+ $\beta_{8j} BMI + \beta_{9j} Baseline \log_{10} Y_{ijt} + \epsilon_{ijkt}$  

(2)

Finally, we tested effect modification by asthma status formally by including an interaction term between the pollutant exposure and asthma status:

$$\Delta \log_{10} Y_{ijt} = \mu + \delta_{ij} + \beta_{1j} Pollutant_{at} + \beta_{2j} Asthma_{i} + \beta_{3j} Pollutant_{at} + \beta_{4j} Asthma_{i} + \beta_{5j} Week_{at} + \beta_{6j} Age_{i} + \beta_{7j} Sex_{i} + \beta_{8j} Race_{c}$$

+ $\beta_{9j} BMI + \beta_{10j} Baseline \log_{10} Y_{ijt} + \epsilon_{ijkt}$  

(3)

We corrected hypothesis testing for multiple comparisons, to identify differentially expressed features associated with specific traffic-related pollutant levels (by each ionization mode), using the Benjamini-Hochberg false discovery rate (FDRBH) procedure at a 5% false positive threshold.

2.4. Metabolic pathway enrichment analysis and annotation

To categorize metabolic feature functional activity from the LC-HRMS output, we used mummichog (v. 1.0.5), a biostatistical application that infers biological pathway elicitation based on the concurrent enrichment of detected metabolite features, without requiring prior identification (Li et al., 2013). We conducted pathway analysis separately for each set of significant features (FDRBH < 0.05) from each of the 27 air pollutants-specific linear mixed models, by each ionization mode. To minimize false positive discovery during this phase of the analysis, we re-ran the pathway analysis using a subset of the 6 most common standard adduct forms (of the 16-total included in mummichog). Each cell in the derived metabolic pathway-TRAP heat map represents a statistical association between each of the metabolic pathways and each traffic indicator. We compared the top TRAP-associated pathways observed among the asthma subgroup with those in the non-asthma subgroup using a Venn diagram.

Each of the metabolic features that were significantly associated with TRAP (FDRBH < 0.05) in the whole population or in either subgroup, and also significantly enriched in a metabolic pathway (p < 0.05, mummichog) was annotated by matching m/z value for commonly formed adducts to the METLIN (https://metlin.scripps.edu/index.php), ChemSpider (http://www.chemspider.com/), Human Metabolome Database (HMDB), and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genomel.jkJpeg pathway.html) databases, using a mass error threshold of 10 ppm (Uppal et al., 2016). To reduce the possibility of false positive discovery, we screened each of the pollutant-driven metabolic features for spectrum peak quality and purity by manual examination of their respective extracted ion chromatograms (EICs). Finally, we confirmed a selected number of annotated metabolites by comparison of m/z, retention time and ion dissociation patterns to authentic chemical reference standards analyzed in our lab using the identical method and instrument parameters via tandem mass spectrometry.

3. Results

Among the 60 adults that participated in the scripted commutes, 45 (24 with asthma and 21 without asthma) contributed paired venous blood samples prior to and approximately 8 h after each sampling session. There were generally similar demographic characteristics among participants with or without asthma, except for age (p < 0.05, Table S1). As we previously reported (Golan et al., 2018), mean levels of the predominant particulate pollutants, pb-PAH, PNC, PM2.5, as well as noise were significantly higher during highway commute sessions as compared to surface street commutes and clinic visits. With the exception of WSO, mean levels of all organic components of PM2.5, along with several metal components, were also significantly higher during the highway commute sessions (p < 0.05, Table S2).

Using both negative and positive ionization LC modes, we extracted 17,856 and 9087 metabolic features from plasma, respectively. The median CV across the triplicate samples for each feature was 23.5% and 25.4% in positive and negative ionization modes, respectively (Li et al., 2013). We conducted pathway analysis separately for each of the MWAS models (27 individual pollutants of metabolic features in two ionization modes). For a given pollutant, the number of significant metabolic features among the MWAS models ranged from 45 to 576 features (FDRBH < 0.05 in Eq. (1), Table S3). Elemental carbon, a traditional marker of diesel engine emissions, and vanadium, a transition metal shown in our previous ACE-2 source apportionment analyses to originate primary tailpipe sources (Krall et al., 2018), exhibited the largest number of associations with individual features. In total, 494 and 220 unique metabolic features were associated with at least 3 or more of the 27 pollutants in the negative and positive ionization modes, respectively (FDRBH < 0.05, Eq. (1)). We found a similar number of
Fig. 1. Metabolic pathways associated with ≥ 5 air pollutants in all ACE-2 participants. Cells were shaded according to the strength (i.e. p-value) of the association between each of metabolic pathways and significant metabolic features (FDR_{\text{BH}} < 0.05) that were associated with each single air pollutant. Pathways are ordered according to the total number of the significant pathway-traffic pollution associations (p < 0.05) in the C18 column negative ionization mode and positive ionization mode. Pollutant indicators were grouped into traffic-related sources, including resuspended crustal, primary tailpipe traffic, and non-tailpipe traffic sources.

*For HILIC positive ion mode, only the following adducts were considered: M^{+1}, M + H^{+1}, M + H2O^{+1}, M + Na^{+1}, M + K^{+1}, M + 2H^{+1}, and M(C13) + 2H^{+1}.

For C18 negative ion mode, the following adducts were considered: M-H^{-1}, M + Cl^{-1}, M + ACN-H^{-1}, M + HCOO^{-1}, M(C13)-H^{-1}, M-H2O-H^{-1}, and M+ Na-2H^{-1}.

Acronym: FDR_{\text{BH}}, Benjamini-Hochberg false discovery rate; PNC, particle number concentration; pb-PAH, particle-bound polycyclic aromatic hydrocarbons; BC, black carbon; EC, elemental carbon; OC, organic carbon; WSOC, water soluble organic carbon; As, arsenic; Cr, chromium; Ni, nickel; V, vanadium; Al, aluminum; Ca, calcium; Ce, cerium; Mg, magnesium; Ba, barium; Cd, cadmium; Co, cobalt; Fe, iron; K, potassium; Mn, manganese; P, phosphorus; Pb, lead; Sb, antimony; Ti, Titanium; Zn, zinc.
significant features associated with the pollutants in both the subgroup with and without asthma (Eq. (2)), 40.1% of which were shared by both subgroups, while 36.6% were observed only in the subgroup with asthma and 23.3% observed only in the subgroup without asthma. The number and strengths of the TRAP metabolic-features associations differed, significantly, by asthma disease status, indicative of effect measure modification by this factor (Eq. (3), Table S3).

Five metabolic pathways consistently appeared to be significantly perturbed across the varying pollutant models (adjusted \( p < 0.05 \) in *mummichog*, Fig. 1), in both ionization modes. These included pathways predominantly associated with xenobiotic-mediated oxidative stress and acute inflammatory response, such as leukotriene metabolism, vitamin E metabolism, cytochrome P450, pyrimidine metabolism, and tryptophan metabolism. Pathways involved in leukotriene and vitamin E metabolism, in particular, were consistently those most strongly associated with the measured pollutants. When stratified by asthma status (Figs. 2 and S1), pathways found to be perturbed in the participants with asthma exclusively tended to be those heavily related to acute inflammatory processes, including arginine and proline metabolism, as well as the tyrosine metabolism (\( N = 11 \)).

Based on the pathway analysis, we targeted annotation of constituent metabolic features within pathways that were perturbed by the measured pollutants, with the aim of informing the untargeted metabolomics observations. In total, we confirmed the chemical identity of 45 metabolic features (Table 1, Figs. S2 and S3), 92% of which were endogenous metabolites related to oxidative stress, inflammatory responses, and nucleic acid damage and repair. For these validated metabolites, we observed consistent significant and negative associations (\( \beta < 0 \)) between anti-inflammatory molecules and corresponding pollutant levels and significant and positive associations (\( \beta > 0 \)) between oxidative or pro-inflammatory metabolites and corresponding pollutant levels (Table 1). Notably, 25 validated metabolites were found exclusively to be associated with pollutant exposures among participants with asthma, while 7 unique metabolites were found among participants without asthma. Specifically, inflammatory and oxidative stress related amino acids, including arginine, histidine, and methionine, were consistently associated with vanadium, elemental carbon, cobalt, cerium, and pb-PAH in both negative and positive ionization mode. Several other amino acids related to inflammation and oxidative stress were also identified, including glutamic acid, serine, proline, valine, leucine, lysine, phenylalanine, and tyrosine, each showing strong and biologically-plausible associations with numerous air pollutants.

Moreover, the direction and strength of these verified-metabolite-TRAP-associations differed substantially by asthma status, indicative of effect modification of pollutant exposures on the inflammation and oxidative stress-related metabolic responses by asthma status (Fig. 3). Further, these validated metabolites were closely linked and connected in several inflammatory and redox pathways, which collectively implicate these mechanisms as part of the impact of TRAP toxicity in individuals with asthma (Fig. 4).

### 4. Discussion

Using a randomized, crossover small panel study design and HRM analytical platform, we detected numerous significant metabolic perturbations associated with in-vehicle exposures during commuting and verified metabolites that were closely linked and connected in several inflammatory and redox pathways. Collectively, we believe these findings point to several potential molecular mechanisms of traffic-related air pollution toxicity. ACE-2 was a prospective, repeated measure design study, with individual-level exposure and health measurements, and a pronounced environmental exposure contrast. As such, this study was ideal to test HRM as an environmental molecular epidemiology platform.

A key finding from the current analyses was the identification of several biological pathways that were consistently associated with elevated pollutant exposures. Many of these identified pathways,
Table 1
Chemical identity* of the metabolic features significantly associated with TRAP.

| m/z   | RT   | Chemical identity          | ESI | Group                        | Pathway                                | Associated TRAP                   |
|-------|------|----------------------------|-----|------------------------------|----------------------------------------|-----------------------------------|
| 103.0385 | 82.8 | alpha-hydroxyisobutyric acid | ESI* | asthma propanoate metabolism | As (β = −1.14); Ba (β = −0.07); P (β = −0.12); Pb (β = −0.91); Sb (β = −0.47) |
| 109.0280 | 90.8 | catechol                   | ESI* | all PAH degradation;         | EC (β = 0.07); Go (β = 35.5); K (β = 0.046); P (β = 0.12); Zn (β = 0.12) |
| 115.0021 | 78.7 | maleic acid; fumarate      | ESI* | all tyrosine metabolism;     | OC (β = 0.46); Co (β = 12.3); Zn (β = 0.05) |
| 128.0339 | 85.6 | 5-oxo-proline              | ESI* | all glutathione glutamine and glutamate metabolism | Ni (β = −0.22); As (β = −0.64); Zn (β = −0.13); Ba (β = −0.07); OC (β = −0.15); PM2.5 (β = −0.04) |
| 129.0542 | 105.8 | 4-methyl-2-oxopentanoic acid | ESI* | all valine, leucine and isoleucine degradation | Ce (β = 0.37); Cr (β = 0.11) |
| 130.0859 | 94.5 | leucine                    | ESI* | asthma valine, leucine and isoleucine degradation | EC (β = −0.24); OC (β = −0.98) |
| 131.0448 | 135.6 | 3-ureidopropionate         | ESI* | asthma pyrimidine metabolism | EC (β = −0.74); V (β = −0.92) |
| 146.0446 | 114.1 | glutamic acidβ             | ESI* | all Glutathione metabolism   | EC (β = −0.51); OC (β = −0.26); CPC (β = −0.05) |
| 147.0231 | 81.4 | ascorbate                  | ESI* | no asthma ascorbate and aldarate metabolism; | F (β = −0.16); F (β = −0.91) |
| 151.0250 | 89.6 | xanthine                   | ESI* | asthma purine metabolism     | EC (β = −0.21); EC (β = −0.58) |
| 153.0180 | 88.2 | dihydroxybenzoic acids     | ESI* | all benzene degradation;     | EC (β = −0.38); CO (β = −0.518); |
| 154.0609 | 112.1 | histidineβ                 | ESI* | asthma histidine metabolism  | EC (β = −0.73); V (β = −0.65) |
| 155.0105 | 69.0 | orotate                    | ESI* | asthma pyrimidine metabolism | V (β = −1.18) |
| 161.0440 | 110.3 | 3-hydroxy-3-methylglutarate | ESI* | asthma leucine degration     | EC (β = −0.71) |
| 165.0546 | 83.4 | 3–2-hydroxyphenyl-propanoate | ESI* | asthma phenylalanine metabolism; PAH degradation | pb-PAH (β = −0.01) |
| 167.0199 | 76.2 | urate                     | ESI* | asthma purine metabolism     | EC (β = 0.22) |
| 173.0131 | 145.2 | arginineβ                  | ESI* | asthma arginine and proline metabolism | EC (β = 0.91); V (β = −0.81) |
| 175.0237 | 81.4 | ascorbate                  | ESI* | no asthma ascorbate and aldarate metabolism; | F (β = −0.16) |
| 180.0655 | 88.0 | tyrosine                   | ESI* | asthma tyrosine metabolism   | Go (β = 0.56); EC (β = −1.30, 0.27) |
| 187.1329 | 92.1 | 10-hydroxydecanoate        | ESI* | no asthma saturated fatty acids metabolism | Ni (β = −0.25) |
| 209.0294 | 161.3 | d-saccharic acid; galactarate | ESI* | asthma ascorbate and aldarate metabolism | ph-PAH (β = −0.01); EC (β = −0.49) |
| 223.0739 | 78.5 | 3-hydroxyxynurenine        | ESI* | no asthma tryptophan metabolism | Cr (β = 0.41) |
| 327.331 | 564.5 | docosahexaenoic acid       | ESI* | asthma biosynthesis of unsaturated fatty acids | Zn (β = 0.24) |
| 346.0554 | 147.2 | adenosine 5’-monophosphate | ESI* | all purine metabolism        | Go (β = −18.0); WSO (β = −1.39) |
| 391.2849 | 104.8 | chenodeoxycholate deoxylolate | ESI* | all bile acid biosynthesis   | EC (β = 0.15); Al (β = 0.011); K (β = 0.02) |
| 464.3014 | 85.2 | glycolcholate              | ESI* | all bile acid biosynthesis;  | pb-PAH (β = −0.02); Ce (β = −0.85) |
| 94.0655 | 447.4 | aniline                    | ESI* | all aminobenzoate degradation | Ce (β = −0.29); |
| 96.0487 | 427.0 | 2-hydroxypridine          | ESI* | all nicotinate and nicotinamide metabolism | Go (β = 0.62); Zn (β = 0.04) |
| 104.1073 | 76.4 | choline                    | ESI* | all glycine, serine and threonine metabolism | Go (β = 0.48); Al (β = 0.48) |
| 105.0554 | 71.0 | 2,3-diaminopropionic acid  | ESI* | no asthma g-glutamine and d-glutamate metabolism | Gr (β = 0.07); WSO (β = 0.06) |
| 106.0501 | 118.9 | serine                     | ESI* | asthma glycine, serine and threonine metabolism | EC (β = −0.88); V (β = −0.74) |
| 114.0664 | 84.0 | creatinine                 | ESI* | asthma arginine and proline metabolism | EC (β = 0.32) |
| 116.0708 | 63.5 | proline                    | ESI* | asthma arginine and proline metabolism | EC (β = 0.45) |
| 118.0863 | 95.1 | valine                     | ESI* | all valine, leucine and isoleucine degradation | Gr (β = −0.23); Ge (β = −0.29) |
| 132.0767 | 104.0 | creatine                   | ESI* | asthma arginine and proline metabolism | EC (β = −1.10) |
| 132.1018 | 71.1 | leucineβ                   | ESI* | asthma valine, leucine and isoleucine degradation | Ni (β = 0.29) |
| 137.0546 | 83.6 | hypoxanthine               | ESI* | asthma purine metabolism     | Ba (β = −0.07); Cd (β = −297.9); Mn (β = −0.51); |
| 142.0264 | 139.2 | ethanolamine phosphate     | ESI* | all glycerophospholipid metabolism | CPC (β = 0.03); WSO (β = −0.37); |
| 144.0807 | 88.9 | naphthylamine              | ESI* | all xenobiotics by cytochrome p450 | OC (β = −0.07); Ge (β = −0.37) |
| 147.1127 | 78.7 | lysine                     | ESI* | asthma lysine biosynthesis and degradation | EC (β = −0.73); V (β = −1.14) |
| 148.0603 | 120.4 | glutamic acidβ             | ESI* | no asthma glutathione metabolism; | CPC (β = −0.04) |
| 150.0582 | 111.2 | methyl-d-aspartic acid      | ESI* | all tryptophan metabolism;   | Go (β = 6.11) |
| 154.0948 | 131.0 | 3-hydroxyanthranilic acid  | ESI* | 3-amino-4-hydroxybenzoic acid | aminobenzoate degradation |
| 156.0766 | 111.5 | histidineβ                 | ESI* | asthma histidine metabolism  | V (β = −1.06) |
| 166.0861 | 84.5 | phenylalanine              | ESI* | no asthma phenylalanine, tyrosine and tryptophan biosynthesis | AI (β = −0.01); Ce (β = −0.38); Co (β = −0.41) |
| 171.0054 | 107.9 | glyceraldehyde 3-phosphate | ESI* | all fermentation and glycolysis of carbohydrates | Pb (β = 0.16) |
| 175.1188 | 130.4 | arginineβ                  | ESI* | asthma arginine and proline metabolism | EC (β = −1.13); V (β = −0.97) |
| 176.1028 | 96.1 | citrulline                  | ESI* | asthma arginine biosynthesis | EC (β = −1.07) |

(continued on next page)
including leukotriene, cytochrome P450, vitamin E, tyrosine, methionine, and tryptophan metabolism, have been closely linked to TRAP-related acute oxidative stress, inflammatory responses, and acute cardiorespiratory effects (Capuron et al., 2011; Chow, 1991; Dahlén et al., 1981; Gonzalez, 2005; Henderson, 1994; Hotamisligil, 2006; Mackay et al., 2006; Morgan, 1997; Singh et al., 2005; Stoy et al., 2005). The identification of these specific pollution-mediated pathways mirror results reported recently (Liang et al., 2018b; Vlaanderen et al., 2017). In an analysis of 54 healthy college students living close to a major urban highway (Liang et al., 2018b), we identified several oxidative stress and inflammation-related pathways that were significantly associated with TRAP using high-resolution metabolomics; specifically, leukotriene, vitamin E, cytochrome P450, and methionine metabolic pathways showed the strong associations with multiple TRAP indicators such as BC, NO, and PM2.5. Similarly, in a small panel of 31 healthy volunteers exposed to ambient air pollution for 5 h, Vlaanderen et al., reported metabolic perturbations within 8 pathways, including tyrosine and tryptophan metabolisms (Vlaanderen et al., 2017).

In particular, leukotriene metabolism, an active inflammatory mediatory pathway, was consistently the most prominent pathways found among participants showing strongest associations with many of the measured air pollutants. As a family of active eicosanoid mediators synthesized from the oxidation of arachidonic acid endogenously, leukotrienes are thought to be a major driver of inflammation in asthma and allergic rhinitis (Nelson et al., 2008; Salmon and Higgs, 1987). In another panel of healthy participants living in close proximity to a...
major urban roadway (Liang et al., 2018b), we observed perturbations in leukotriene related metabolites linked to longer-term exposure to elevated TRAPs, including BC, CO, NO and PM_{2.5}, occurring over 3 months. In current study, there were consistent and robust associations between features putatively matched with leukotriene metabolism and numerous air pollutants, including those from the primary tailpipe (e.g. PAH, BC, and OC), crustal (e.g. Al, Ca and Mg), and non-tailpipe source (e.g. Co, Fe, and Mn). Along with inflammation, oxidative stress-related pathways were also strongly associated with in-vehicle pollution. Key oxidative stress mediators within these related metabolic pathways included cytochrome P450s, terminal oxidase enzymes in electron transfer chains (Gonzalez, 2005); vitamin E, a potent fat-soluble antioxidant that protects cells from oxidative damage (Singh et al., 2005); and tryptophan, an α amino acid and essential precursor to the neurotransmitter serotonin and the hormone melatonin (Stoy et al., 2005). Notably these proteins and amino acids have been linked to the neurotransmitter serotonin and the hormone melatonin (Stoy et al., 2005); and tryptophan, an amino acid and essential precursor to the neurotransmitter serotonin and the hormone melatonin (Stoy et al., 2005). Notably these proteins and amino acids have been linked to the neurotransmitter serotonin and the hormone melatonin (Stoy et al., 2005).

The results also point to substantial differences in pollutant-metabolic responses modified by asthma status. For putatively matched features enriched in several inflammatory pathways, including leukotriene, arginine, and proline metabolism, the strength and magnitude of their associations with air pollutants significantly differed among participant with and without asthma, indicative of a biologically plausible role of pre-existing condition in the modification of inflammatory responses to TRAP exposure. Similar patterns of differentiated responses were also observed among features matched within the tryptophan and vitamin E metabolic pathways, highlighting a potential mechanistic basis for asthma as a factor enhancing susceptibility to pollutant-mediated oxidative stress response.

The metabolic feature annotation and validation further lent coherence to the pathway analysis. Importantly, most of the metabolites we validated were endogenous molecules involved in several acute inflammatory response, oxidative stress, and DNA damage and repair processes. These validated metabolites were prevalent and detectable in most (i.e. at least 85%) of the biosamples, had triplicate CVs < 5%, and exhibited relatively pure EIC peaks, indicating relative great data quality. Together, these characteristics support their use as potential, sensitive biomarkers of pollution associated with traffic pollution.

Several validated amino acids, in particular arginine, were robustly associated with the same suite of air pollutants, including EC, vanadium, and cerium, in both negative and positive ionization modes, with differential response by asthma. We find the results involving arginine to be worth highlighting, in particular. Arginine is an essential α-amino acid related to endothelial function, inflammation, and airway hyperresponsiveness, and has been previously reported to be inversely associated with elevated level of air pollution (Silkoff et al., 2000). Here, we also observed consistent negative associations between arginine intensity and concentrations of EC and vanadium and difference in response by asthma status. Over 90% of the participants with asthma exhibited decreased arginine levels with increasing exposure to vanadium. Conversely, participants without asthma exhibited increased arginine levels with increasing vanadium.

We also identified similar trends for other inflammatory amino acids, including histidine and methionine. Histidine is a well-known inflammatory agent involved in immune responses, including airway hyperresponsiveness (Hospers et al., 2005; Liu et al., 1990). Previously, decreased levels of histidine were found to be significantly associated with inflammation and oxidative stress among groups with pre-existing conditions, such as obesity (Niu et al., 2012). In our study, we found a negative association between histidine and levels of vanadium, specifically among the asthmatic participants, while the responses of histidine were generally positively associated with other pollutant concentrations. We also identified methionine in both ionization modes, with differential responses to TRAP exhibited between asthmatic and

Fig. 4. Potential molecular mechanisms underlying the effects of traffic-related air pollution toxicity on individuals with asthma elucidated using untargeted high-resolution metabolomics on the study participants. Molecules in green denoted the metabolites detected and confirmed in the study samples. Negative associations with elevated traffic related air pollutants were presented in blue arrows and positive associations were presented in red arrows.

Acronym: TRAP, traffic-related air pollution; ROS, reactive oxygen species; NOS, nitric oxide synthases; XOR, xanthine oxidoreductase; IL-4, the interleukin 4; IL-10, the interleukin 10; TNF α, tumor necrosis factor alpha. (For interpretation of the references to colour in this figure legend, reader is referred to the web version of this article.)
healthy participants. Methionine is an essential amino acid that promotes reactive oxygen species (ROS) production endogenously. A recent study found that increased methionine supplementation in diet increases mitochondrial DNA oxidative damage in an animal model, indicative of its hepatotoxicity (Gomez et al., 2009). In ACE-2 asthmatic participants, we observed increased level of methionine associated with elevated cobalt and cerium concentrations during the commute.

Based on these collective results, we posit that the untargeted, concomitant elicitation of these metabolic features, may be related to systemic changes in a broader interrelated molecular network, one potentially directly associated with acute traffic-related air pollution toxicity. We further hypothesize that at the center of this network are perturbations in the arginine metabolome, where elevated TRAP levels induce augmentation of arginase, leading to increased observed levels of proline and polyamines converted from arginine, and decreased intensities in arginine and its precursors, including citrulline, glutamate, and 5-oxoproline. As reported in numerous in vitro and in vivo models (Morris et al., 2004; Newaskar et al., 2011; North et al., 2013; Wood et al., 2007), increased levels of proline and polyamines may eventually lead to airway hyperresponsiveness and remodeling, as well as asthma. Meanwhile, we also observed increased pollutant levels to be associated with decreased intensities of norvaline, a key inhibitor of arginase to correct endothelial dysfunction (Pokrovsky et al., 2011). Moreover, it is possible that enhanced arginase activity would compete with nitric oxide synthase (NOS) in arginine metabolism (Kim et al., 2009; Xu et al., 2004), leading to reduced bioavailability of endogenously produced nitric oxide. Correspondingly, we also observed the amount of creatine produced from arginine, a widely studied anti-oxidant (Guoyao and Morris, 1998; Lawler et al., 2002), to be decreased in ACE-2 participants following elevated in-vehicle exposures. Future, targeted in vitro and in vivo studies may further establish the veracity of these findings.

A concurrent mechanism, also potentially identifiable within our findings, is the increased levels of ROS and inflammatory mediators (i.e. leukotrienes) induced by TRAP exposure leading to redox imbalance, contributing to NOS uncoupling and inhibition. The oxidative inactivation of NOS may, over repeated longer term exposures, result in increased nitric oxide bioavailability and NOS-NO signaling dysregulation (Farah et al., 2018), leading ultimately to multiple adverse health effects including myocardial remodeling (Burger et al., 2009), endothelial dysfunction (Forstermann et al., 2017), and chronic lung disease (Holguin, 2013). Throughout this process, in-vehicle pollutant exposure might also diminish the antioxidant effect of NOS on xanthine oxidoreductase (XOR) inhibition. XOR catalyzes the oxidation of hypoxanthine to xanthine and the oxidation of xanthine to uric acid, generating potent ROS such as superoxide (Kelley et al., 2010). Under homeostatic conditions without abnormal environmental stress, neuronal NOS (nNOS) inhibits XOR to maintain NOS activity (Farah et al., 2018), while the loss of nNOS inhibition of XOR-derived ROS would lead to oxidative-stress-mediated uncoupling of the residual endothelial NOS (eNOS). In our results, we consistently observed elevated pollutant exposure associated with decreased intensities of key components in the XOR pathways, including AMP, hypoxanthine and xanthine. In addition, several precursors of glutathione, an essential antioxidant of preventing damage to important cellular components caused by reactive oxygen species (Ceballos-Picot et al., 1996), were found to decrease as TRAP levels increased in the ACE-2 study, including choline, cystine, 5-oxoproline, and glutamate.

Although these findings are biologically-plausible and statistically robust, specific attention should be given to caveats inherent in many omics-based analyses and small panel designs, including our own. The first concerns identifying a causal agent. In conducting our statistical modeling, we used each of the 27 single air pollutants, independently, as surrogates of exposure to primary traffic pollution, a highly heterogeneous mixture. Thus, an observed metabolic perturbation associated with a particular air pollutant may not necessarily indicate a causal association between the metabolic feature with that specific modeled indicator. Instead, such change may be more likely associated with multiple, correlated pollutants within a complex traffic mixture, which is an interpretation supported by the fact that over 60% of the significant features in the statistical models were jointly associated with at least three or more individual pollutants. Nevertheless, we did observe that some pollutants were more strongly associated with metabolic perturbations, including EC, pb-PAHs, and vanadium.

The post-commute blood draw was collected 8 h after each commute session. During this period, participants were allowed to conduct normal daily activities between each biomonitoring sessions, but they were asked not to leave the campus vicinity surrounding the laboratory facilities. Nevertheless, we were unable to either control or measure all aspects of an individual’s environmental exposures during the course of their daylong participation in the protocol. Therefore, unmeasured exposures that may be present in-vehicle such as gaseous co-pollutants or dietary, or products of endogenous biochemical processes related to an individual’s complex internal biological processes, may have introduced confounding or other forms of bias to our analysis.

Additionally, although the demographic characteristics among participants with or without asthma were generally similar, there may have been unmodeled factors, including asthma control status and medication usage, which partially contributed to some of the observed metabolomics differences. To address this, we conducted sensitivity analyses on model specification and inclusion of these factors, with results that were consistent and robust to model specification and inclusion of these covariates. Finally, given the highly multidimensional nature of this analysis, there is an inevitable increased risk of false positive discovery (i.e. Type 1 error) due to the multiple comparisons. Along with using the Benjamini-Hochberg procedure to correct for multiple testing, we screened each of the candidate significant features based on the quality of their spectral peak by manual examination to minimize false positive results.

In summary, the observed differential metabolic perturbations among ACE-2 participants with and without asthma further point to increased risk of inflammation and oxidative stress for commuters with asthma when exposed to elevated TRAP. Collectively, we believe these findings support the further use of metabolic markers in assessing TRAP exposures, corresponding acute response, and potential as a sensitive tool for elucidating molecular mechanisms underlying TRAP toxicity and its health effects related to this ubiquitous source of environmental pollution. Moreover, the ability to establish a molecular basis that explains these observations may eventually lead to the development of new therapeutic or behavior interventions for mitigating potential health risk.

Sources of support

The presented research was supported by the Clean Air Research Center grant to Emory University and the Georgia Institute of Technology from the US Environmental Protection Agency (USEPA, RD834799) and the Emory Human Exposure Research Center (HERCULES), supported by the National Institute of Environmental Health Sciences of the National Institutes of Health under Award Number P30ES019776.

Declaration of interests

None.

Acknowledgments

The authors extend their gratitude to the participants and field team members of this research. The study was supported by a Clean Air Research Center grant to Emory University and the Georgia Institute of Technology from the US Environmental Protection Agency (USEPA,
RD834799) and the Emory Human Exposure Research Center (HERCULES), supported by the National Institute of Environmental Health Sciences of the National Institutes of Health under Award Number P30ES019776. The content of this publication is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the USEPA. Further, USEPA does not endorse the purchase of any commercial products or services mentioned in the publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.04.003.

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