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Traffic Exposures and Metabolic Responses in Commuters

By

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Doctor of Philosophy

Environmental Health Sciences

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TRAFFIC EXPOSURES AND METABOLIC RESPONSES IN COMMUTERS

By,

Chandresh Nanji Ladva
B.A., University of Pennsylvania, 2007
M.P.H., Yale University, 2010

Advisor: Jeremy A. Sarnat, Sc.D.

An abstract of
a dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
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in

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Abstract

Atlanta commuters, more than 78% of the working age population, are spending an estimated 31 minutes on the road each way to work. The resulting traffic air pollution exposure has become the subject of extensive research. Air pollution exposures elicit mixtures of biological responses across many scales, from organ-scale wheezing, asthma, and lung cancer to molecular-scale systemic inflammation. Complexity also extends to the exposures themselves. Traffic pollution components, like black carbon, polycyclic aromatic hydrocarbons, fine particulate matter or $PM_{2.5}$, and metals, contribute to toxicity through particle size or oxidative potential. To capture the complexity of both exposure and response, new scales of measurement and data integration with established methods are necessary. Traditional exposure assessment methods married to tools for analyzing the exposome—an accumulation of environmental factors and corresponding biological responses over a lifespan—may aid in the investigations of traffic pollution related human health responses.

The present body of work explores the high-dimensional natures of traffic pollution and human biology with the expectation of elucidating the connections between them. The first aim assessed the short-term covariance between two important aspects of traffic exposure: particle pollution and noise. High temporal resolution measures of exposure revealed varying patterns of covariance of PM2.5, BC, and pb-PAHs with noise that changed depending on the roadway environment being sampled while PNC and noise had stable covariance. The second aim turned to internal mixtures and was the first examination of metabolomic profiling of plasma, saliva, and exhaled breath condensate together. There was good correlation between features shared between matrices, and those correlations were stable over time. This aim opened potential for identifying markers of traffic pollution exposure in these three matrices using a newer chemical measurement tool called high-resolution metabolomics. Finally, the third aim used a panel study of commuters to examine whether in-vehicle traffic pollution exposures were associated with changes in inflammation and metabolomic profiles. Particulate metal exposures were found to induce changes in plasma metabolism consistent with inflammation.

The dissertation demonstrates that high-resolution measurements can capture the complexity of both traffic exposure and human response for new insights on associations between them.

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While the body of work enclosed here is the product of over 5 years of effort, the body that did the work is the product of over 30 years of effort by those around me. The energy, love, guidance, and support given to me by my friends, mentors, and family are responsible for my successes. These people endured my failures, and insisted I press on. Although uncharacteristic of me, I have trouble finding the words to convey my gratitude and humility for every one of them.

I will still try.

My parents, Nanji and Sumita, faced many hardships over their lives. Their undying hope for and determination to building better futures provide the inspiration for pushing my own limits. My mother told me long ago, “never stop learning.” She was precluded from pursuing education despite her immense thirst for knowledge. And so, I learned and studied, from high school, through college and graduate school, and more graduate school—trying my best to not take for granted the opportunities that were available to me. Staying on track in this journey was only possible through the guidance of older siblings, Nimisha and Rajiv. Their experiences of education and building lives in a new country were vastly different from my own: They knew how much was at stake. I leaned on them, time and time again, to help me through life’s challenges and wonders. I will not dare forget my first day on my college campus. My sister spent a quiet moment with me, gave me a hug, and gave her final instruction: “Don’t f*** up.”

So far, so good (mostly).

Some of my best choices have been the people I have in my life. Akshara, an intern I met in Switzerland, let me chase her around for a few months…then a few years. We married in the middle of my doctoral training, and, nevertheless, she persisted. Every day, she lifted me up, kicked me out the door, and made sure I had the strength to do what I had set out to accomplish. She sacrificed her rest to ensure I was up hours before dawn to make it in for

sampling during my data collection. She heard my insecurities and doubts, and brushed them aside for me. She has had more faith in my abilities than any one I have ever known. I hope I can repay her for that strength and love.

At Emory, I have been very lucky. I found a friend and mentor in Jeremy, who eventually became the chair of my dissertation committee. His enthusiasm, curiosity, and cautiousness has been instrumental in my own development as a scientist. The kindness with which he mentored, maybe unbeknownst to him, primed one of the most important lessons I learned: inspiring the best work from people may come from letting those curious minds explore. Jeremy's support and faith in me have been essential in bringing this dissertation to completion, and gave me a great honor to be his first Ph.D. student. He also chaired a "dream team" of a committee, which has given so much guidance over the years. Dana and Tianwei have fielded barrages of questions for analytics, but, more so, inspired me to push my critical thinking. Dean made certain every precious meeting I had with him left me with advice to last me through my career. Finally, Gary reminded me to take a step back from the minutia and critically think about the foundations of my thinking. He also, and I can say it here, was one of my biggest cheerleaders. I have learned so much and been given so many opportunities from my teachers.

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Chapter 1: Introduction

Commuters employ a variety of transportation modes, but a disproportionate time is spent on roadways compared to walking or using public transit in the United States. The 2015 American Communities Survey found that workers, in the Atlanta Metropolitan Statistical area, spent a mean travel time of 31.3 minutes each way between home and work, up from the 2010 estimate of 25.4 minutes [1]. In spending more time on the roadway, Atlantans are potentially exposed to high levels of traffic-pollutants [2, 3], which have been shown to modify heart rate variability [4, 5], lung function [6-8], airway inflammation[9, 10], and oxidative stress[11-13].

From a health effects standpoint, roadway commuting constitutes a highly complex exposure and health response setting. Commuters are exposed to a variety of chemical [14-16] and non-chemical [17] pollutants—an exposure milieu—that collectively has the potential to initiate a plethora of health responses via multiple biochemical pathways. External mixtures are comprised of measurable pollutant components outside of the human body, which include chemical air pollutants, noise, and bioaerosols. Internal mixtures are comprised of measurable compounds within the human body of both endogenous and exogenous origin. Both internal and external mixtures share similar features with respect to analytical challenges and are highly variable over space and time. Additionally, both external and internal mixtures contain components that covary and interact with one another.

Pollutant exposures experienced during commuting periods consist of a complex mixture of particles, gases, organic and inorganic components that vary substantially over space and time[18]. This traffic pollution exposure milieu, as well as its multiple potential mechanisms of toxicity, provides challenges to understanding the impacts of daily commuting on human health. Methods have been developed to better characterize the on-road multi-pollutant environment in time and space. Furthermore, novel advances in high-resolution approaches have been proposed to examine pervasive, biological responses to traffic pollution [19, 20]. These approaches, including high-resolution metabolomics, may foster opportunities to identify new biomarkers of exposure or effect. Both external, ‘bottom-up’ environmental measurements and

internal, ‘top-down’ biomonitoring share a primary objective of characterizing and interpreting highly dimensional, dynamic data; they have the potential to substantially advance etiological understandings of air pollution health effects. To date, information is limited regarding these bottom-up and top-down methods with respect to sources of variability and the capability to resolve exposures to specific environmental stressors.

Similar to findings from other panel-based commuter studies [21-25], initial findings from a recently conducted pilot commuter exposure study (ACE-1) suggest that modeling associations between single pollutants or classes and corresponding health response typically does not yield consistent epidemiologic results. Understanding exposures to traffic pollutant mixtures by how their components correlate with one another may help identify the key drivers of health responses. For example, traffic related particle pollution and noise have separately been associated with cardiovascular disease. Yet, both share mobile vehicle sources and may operate on related pathways progressing to disease in the long-term. While some studies have examined this in chronic exposure settings and found little evidence of confounding[26, 27], the potential for confounding in short-term exposures and response are not well understood. Characterizing the short-term covariance of chemical and non-chemical exposures, then, provides information to epidemiologists of the research needs to assess short-term response to traffic exposure.

Sensitively detecting responses to traffic pollution exposures may be achieved through newer analytical technologies, such as high-resolution metabolomics. This method has demonstrated its ability to measure endogenous metabolism and exposure to xenobiotics [28, 29], especially in blood. In studies targeting air pollution exposures, perturbations in human metabolism from polycyclic aromatic hydrocarbons [30] and airborne metals [31] are quantified using advanced statistical methods and contrasting exposures. However, as an exposure assessment tool, high-resolution metabolomics is a nascent entrant in the field of exposure science. While plasma, serum, breath, and urine metabolomics are increasingly being used to

study bioeffect at different time scales, other potential biofluids useful in air pollution exposure are not well characterized nor are they compared. One specific gap lies in the comparability of plasma—a gold standard matrix—and either saliva or exhaled breath condensate with respect to their metabolomic profiles. These less-invasive biological matrices have already been useful in detecting specific, inflammatory changes attributed to traffic exposures [32-35]. And while investigations have shown that metabolomics can be used in each, the direct comparison of these three has yet to be established.

The current dissertation addresses these knowledge gaps by leveraging data and resources at two large academic centers, the Southeastern Center for Air Pollution Epidemiology (SCAPE) and the Health and Exposome Research Center: Understanding Lifetime Exposures (HERCULES) at Emory University and Georgia Tech. Through SCAPE's Atlanta Commuters Exposure (ACE-2) study, 60 participants participated in rush hour commutes and controlled exposure conditions. In-vehicle air pollution, specifically fine particulate matter ($PM_{2.5}$), ultrafine particles, trace metals, and organic carbon, were collected using both time-integrated and continuous methods, while biological samples, including exhaled breath condensate and plasma, were collected at regular intervals throughout the sampling protocol, before, during and after the commutes.

Our present ability to reliably measure the variety of internal and external components leads generates large amounts of data with high dimensionality. Extreme dimensionality in the data frequently precludes the use of standard statistical inference methods. Methods have been developed in disciplines such as genomics and psychology to impose dimension reduction in the data to allow inferences. In genomics, microarray analyses generate large amounts of data over large sample sizes [36-40]. Addressing this required developing statistical methods to reorganize large amounts of data. Psychology explores latent, unobserved variables by grouping measured variables using their correlation structures [41, 42]. Combined, these

disciplines have created an opportunity for exposure science, epidemiology, and toxicology to capture the complexity of air pollution health effects through adoption of these techniques.

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Overview of Dissertation

The present dissertation investigates the complexity of traffic mixtures and the feasibility of novel and emerging exposure assessment tools in aiding the health effects study of traffic-related air pollution exposures. Generally, the chapters are each a proof-of-concept study exploring fundamental questions including: 1) particulate matter and noise interactions in environmental mixtures; 2) correlations of metabolomic profiles within individuals across blood plasma, exhaled breath condensate, and saliva; and 3) associations of traffic-related exposures with changes in inflammatory signaling and metabolomic perturbations.

Chapter 2 begins with examining the associations between noise and particulate matter pollution in different roadway environments. Near-roadway, outdoor monitoring is typically used in both noise and pollution monitoring networks. Linking true exposures to these monitoring networks is important for understanding the presence of exposure measurement error in health effects settings. In this analysis, two in-vehicle environments, along with a near roadway setting, were sampled to capture exposures on-road during realistic commuting conditions and to specifically examine the effects of the vehicle shell on modifying the associations between noise and PM. Exposures in near-roadway and in-vehicle environments were measured continuously to provide temporally-resolved examination of the associations for different $PM_{2.5}$ components. Together, the study quantifies associations among different chemical and physical pollutants within the external, environmental mixture experienced by road commuters.

Chapter 3 examines the suitability of high-resolution metabolomics for air pollution exposure assessment in three biological matrices for bioeffect monitoring: plasma, saliva, and exhaled breath condensate. Air pollution exposure science has increasingly relied on the measurements of both environmental exposure and markers of acute biological response to ascertain the underlying mechanisms driving health effects. However, this approach is costly and limited in capturing the hypothesized pervasive response to air pollution mixture exposures.

This study assesses the comparability of metabolomic profiles across three biofluids typically used in studies of acute effects that have not previously been studied jointly.

Finally, Chapter 4 investigates inflammatory and metabolic responses in a panel of car commuters. The Atlanta Commuter Exposures (ACE) study explored the associations of well-characterized traffic-related pollution with acute cardiorespiratory changes in repeated measures, crossover design of asthmatic and non-asthmatic car commuters. Integration of high-resolution metabolomics allows a structured examination of metabolic response over time in the same population while paralleling the ACE study's targeted, cardiorespiratory analyses: a combination of hypothesis-generating and hypothesis-driven techniques. Traffic-related pollutants are examined in turn for their associations with biological changes in the population to provide information of which components of the traffic pollution mixture are driving phenotypes.

The present dissertation utilizes several advanced tools of exposure measurement, chemical analysis, and bioinformatics to characterize the traffic mixture and corresponding human response. It demonstrates that the complexity of traffic mixtures and human response can be disentangled to the extent where their dynamic states are quantifiable, provided quality, high-resolution measurements and appropriate statistical methods.

Chapter 2: Assessing Short-Term Covariance between In-Vehicle Particulate Pollution and Noise

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KEYWORDS: noise, traffic, pollution, covariance, short-term exposures

Abstract

Traffic exposures are complex and include chemical and physical pollutants that have independent associations with human health in chronic exposures. While co-exposures to noise and traffic air pollution have been studied in chronic health effects, far less has been studied in the sub-chronic, acute setting. This study compares the association between noise and air pollution in near-road and on-road microenvironments using fine-temporal measurements of both exposures.

Methods: Three microenvironments--near-roadway (NR), stationary in-vehicle (IVS), in-vehicle mobile (IVM)—were continuously sampled for noise (dBA), particulate matter less than 2.5microns in diameter ($PM_{2.5}$ mass), particle number concentration (PNC), particle-bound polycyclic aromatic hydrocarbons (pb-PAHs), and black carbon (BC). Each environment was sampled on 6 different days for 2 hours each during morning rush hour conditions. Associations between noise and each pollutant were examined at two temporal scales representing sub-chronic and acute exposures using correlations and mixed effects regression.

Results: Exposures to pollutants were, on average, highest IVM and lowest IVS, except for $PM_{2.5}$ mass concentrations [Mean (SD): 29.2 (6.6) $\mu g \cdot m^{-3}$] and noise levels [73.8 (3.3) dBA] were highest NR. Associations to PNC and noise were positive and consistent in direction across microenvironments. Other pollutant associations with noise were more variable and could be modified by vehicle window status in a pollutant-dependent manner.

Conclusions: Roadway environments are dynamic and can have different associations between noise and particle pollution exposures depending on microenvironments, their ventilation conditions, and the pollutants considered. PNC and noise have robust, short-term covariance regardless of roadway environments sampled, while other pollutants suggest complex, variable associations that may be important to assessing joint associations with acute human health responses.

Introduction

Multipollutant air pollution health effects have emerged as a primary area of research, designed both to inform future regulatory interventions and elucidate complex biological pathways attributable to observed risk [1-3]. This interest has led to recent efforts to jointly characterize exposure to physical and chemical stressors associated with road traffic, including noise and mobile source pollutant species [4-13]. Human exposures to both traffic air pollution and traffic noise have independently been shown to have adverse health impacts[14-16]. Disentangling the health risks attributable from each has been, until very recently, poorly studied because of a lack of standardized urban noise monitoring. In the United States, limited noise monitoring is variably conducted by local jurisdictions, while air pollution monitoring is federally mandated, standardized, conducted at the state-level. A recent interest in examining joint exposures and corresponding health response to multiple air pollution components [1, 17-20], is based largely on the understanding that individuals are typically exposed to complex pollutant mixtures concurrently rather than a single pollutant in turn. Numerous chemical, physical, and biological agents may also be causally linked to a suite of adverse health endpoints entangled by overlapping biological responses. Epidemiology examining joint exposures has centered on several multivariable modeling approaches [17, 21-23] of primarily gaseous and particulate air pollution with less attention given to joint exposures of chemical and non-chemical traffic pollutants, like noise.

Traffic exposures are complex mixtures, which include numerous chemical and physical stressors which may adversely affect human health via several biological pathways [4, 14, 24-27]. While the impact of joint exposure to noise and TRP over long-term exposure period and corresponding chronic disease incidence has been studied to an extent [4, 5, 7], there is a paucity of published findings examining joint exposures over short-term periods, which have specific relevance for studying sub-chronic and acute health response [9]. Notably, the in-

vehicle microenvironment is known to expose commuters to very high levels of traffic-related pollutants (TRP) and noise with demonstrated associations to subclinical responses [24, 25]. It is important, then, to study the association between these co-exposures in-vehicle as they may differ from near-roadway monitoring.

A large body of evidence links noise and air pollution to a variety of cardiovascular outcomes. Air pollution exposures to PAHs, black carbon, and other $PM_{2.5}$ components likely operate through oxidative stress and inflammatory processes to ultimately lead to these measurable associations [28-30]. Possibly operating through cognitive, stress-mediated responses, noise exposures have also been shown to also be associated with cardiovascular mortality and atherosclerosis in populations in Europe and the US in long-term exposures [5, 8, 11]. Studies in animals and humans have associated exposures to particulate matter with an aerodynamic diameter less than 2.5 microns ($PM_{2.5}$) and diesel exhaust to acute modulation of sympathetic control of the heart [26, 31]. Long-term exposures to TRP through residential exposure assessments and cross-sectional analysis demonstrate increased risk of atherosclerosis [6, 10, 15], although two prospective cohort studies of similar exposure characterization showed no significant association [12, 32].

Chronic disease epidemiology has approached potential confounding by utilizing maps of modeled or interpolated estimates of noise for long-term, average exposures in locales where roadside noise monitoring networks are available, as in the European Union [4-6, 8]. Questions remain about short-term co-exposures of noise and air pollution for acute health outcomes. Few studies have examined the effects of both noise and other pollutants by controlling for the effect of averaged noise exposure with daily concentrations of PM in chronic health outcomes [11, 12]. Acute response, specifically changes in heart rate variability (HRV), has also been examined over shorter temporal scales, using continuous measurements of noise and TRP. Huang et al. (2013), for example, examined changes in HRV associated with corresponding daily air pollution concentrations, modified by noise intensity. Their results showed that increased exposures in

either $PM_{2.5}$, BC, or carbon monoxide and noise levels above 65.6 dBA resulted in marked reductions in HRV parameters. These effects were weaker at noise co-exposures < 65.5 dBA. The case for further analysis examining the joint effect of both exposures on health is strong, but the physical characteristics of the exposure are still not fully understood.

The present study examined microenvironmental and temporal effects on the association between noise and air pollution, through a small, targeted sampling campaign of $PM_{2.5}$ traffic-related pollution (TRP) and noise. The goal was to quantify the TRP-noise association near-roadway and on-roadway while considering the potential modification of associations by window status and speed. It fills an important knowledge gap of the potential for confounding by capturing the physical properties of these co-exposures in different traffic microenvironments.

Methods

Measurements of $PM_{2.5}$, select particulate constituents, and noise were collected in three on-road and near-road microenvironments: 1) an outdoor, near-roadway setting (NR); 2) an in-vehicle, stationary setting (IVS), with the vehicle engine off; and 3) an in-vehicle, mobile (IVM) setting. The NR scenario was designed to examine outdoor, near roadway associations between noise and corresponding particulate pollutant measures in a setting comparable to existing roadside monitoring networks. The in-vehicle stationary and mobile setting were conducted using a dedicated, instrumented test vehicle: a 2008 Honda Civic. IVS sampling afforded the chance to examine the impact of the vehicle shell on associations between the particulate pollution metrics and noise within an indoor microenvironment, while removing contributions of noise and other self-emissions generated from the test vehicle. Finally, IVM sampling examined in-vehicle pollution and noise associations experienced by drivers and passengers during realistic commuting settings. For all the settings, $PM_{2.5}$ pollutant concentrations were compared with simultaneously measured noise levels using correlation and

mixed effects regression analyses across two temporal scales. In total six, approximately 2-hr sampling sessions were conducted in each microenvironment.

Routes, locations, vehicle conditions, and timing were tightly controlled for the pilot study. The NR and IVS scenarios were conducted in a parking lot adjacent (within 10 meters) to Interstate 75/85 in Atlanta, GA, an oft congested thoroughfare connecting the northern and southern suburbs through the center of the city. IVM sampling routes were mainly restricted to the network of highways within and around Atlanta, specifically I-285, I-75, I-85, and I-20. For this study, recirculation was set to fresh air and fan speed was left on a medium setting. Window status (opened or closed) was alternated at 30-minute intervals during all in-vehicle sampling sessions to capture variability in exposure shown previously [33]. When the windows were open, the fan was turned off. All sampling took place in the mornings, from approximately 7AM to 9AM, to measure exposure levels during a rush hour period. In total, 18 sampling sessions, 6 for each scenario, comprising 2,170 minutes of sampling, were conducted to generate the results reported.

Pollutant sampling. Pollutants measured in the study were selected for the availability of well-characterized, continuous instrumentation, and for their reported associations with health effects [9, 28, 34, 35]. PM mass concentrations of particles with aerodynamic diameters less than 2.5 microns ($PM_{2.5}$ mass) were sampled with an Aerotrak™ (TSI; Shoreview, MN) for particulate matter volume ($\mu m^3 \cdot cm^{-3}$). Volumetric concentrations were converted to mass concentration (in $\mu g \cdot m^{-3}$) using an assumed, empirically-derived synthetic density [22]. A P-Trak™ (TSI; Shoreview, MN) was used to measure particle number concentration (PNC) (in $\# \cdot cm^{-3}$) of particles between 20 and 1000 nm in diameter at 1-second intervals. PNC is frequently used as an indicator of ultrafine particulate matter concentration. A micro-aethalometer (AethLabs; San Francisco, CA) was used to collect black carbon (BC) concentration data ($\mu g \cdot m^{-3}$) for every minute. A photoelectric aerosol sensor (PAS) (EcoChem Analytics; League City, TX) collected particle-bound polycyclic aromatic hydrocarbon (pb-PAH)

concentrations ($ng \cdot m^{-3}$) every second. Noise was collected using an HD600 audiometer (ExTech; Nashua, NH) recording sound levels in A-weighted decibels (dBA) every second. The choice of dBA sampling arose from its use in prior studies that focused on noise exposures and its use by the National Institute for Occupational Safety and Health for a Recommended Exposure Limit for occupational injury. All data measured at resolution less than 1-min were averaged to create 1-min concentrations. The 1-min average permitted sufficient resolution to observe fine-scale temporal trends in the associations between noise and other vehicle emissions. Median 1-min concentrations and noise levels from a sampling session were used to represent exposures at 2-hr resolutions.

This study was conducted as a sub-analysis within a more extensive study, the Atlanta Commuters Exposure (ACE) Study [24, 25]. All sampling instrumentation was contained in a sampling tray secured with neoprene foam used to secure each device. Specific information pertaining to data quality and sampling design has been published [25].

Data Analysis. Particulate pollutant concentrations and corresponding noise levels were compared at the 2-hr and 1-min time scales using descriptive, graphical, correlational and regression modeling approaches across microenvironments. In the IVM setting, a further analysis of the interaction of speed and noise on particle pollution was also investigated. First, our examination of the various microenvironments focused on overall patterns of medians and coefficients of variation (CoV) for pollutant concentrations and noise levels. These were compared across sampling microenvironments using Kruskall-Wallis tests by ranks. Next, the association between noise and particle pollution was measured at the 2-hr, sampling session level time scale. Within-session medians of pollutants were plotted against median noise levels to capture associations over aggregated time periods. At the 1-minute resolution, Spearman's rank correlation coefficients (r_S) were calculated within sampling sessions to explore the monotonic associations of non-normally distributed pollutants with normally distributed noise data.

One-minute temporally-resolved associations were examined in R (v. 3.0.1) using the "lme4" package—for fitting generalized linear mixed effects regression models. The pollutants exhibited right-skewed distributions that were approximately log-normal, satisfying generalized linear model assumptions after log transformation. Regression diagnostics conducted on the univariate relationships of TRP and noise at the 1-minute resolution identified three distinct observations of extreme concentrations that exerted high influence, as assessed using Cook's distance and leverage techniques; therefore, one observation was removed from each of the $PM_{2.5}$ mass, BC, and pb-PAHs datasets.

Each pollutant parameter was examined for autocorrelation within a sampling session using the autocorrelation function in R through a lag of 25 minutes. Generally, autocorrelation was moderate for lag-1 through lag-2 measurements with weaker autocorrelation through lag-25 for all PM measures. Noise had a strong autocorrelation at lag-1 with a linear attenuation to lag-10. There was variability in the strength of temporal autocorrelation across sampling microenvironments and sampling sessions. In lieu of defining different models to include these differences for each microenvironment and sampling session, the linear mixed effects regression models included control for the prior two minutes of natural-logged air pollution and prior 1-min noise measurements to account for autocorrelation of minute by minute measurements. A five-factor interaction variable (NR, IVS-Windows Down, IVS-Windows Up, IVM-Windows Down, and IVM-Windows Up) was created to estimate the effects of each sampling condition without having to stratify the models further. The NR scenario was the reference category. The interaction models were expressed, in generalized form, as:

$$(1) \qquad TRP_{jt} = (\beta_0 + \theta_j) + [\beta_1 Noise_t + \beta_2 Noise_{t-1} + \beta_3 TRP_{t-1} + \beta_4 TRP_{t-2}] * Interaction + \varepsilon_{jt},$$

where the outcome TRP_{jt} is the measured particulate pollutant, at time, *t*, within each *j* sampling session. Noise is included at time *t*, $Noise_t$, and at a 1-min lag, $Noise_{t-1}$. Also included were 1- and 2-minute lags of each pollutant, TRP_{t-1} and TRP_{t-2}, respectively. Including time-lagged variables for both noise and TRP permitted examination of temporal trends in the joint associations. For instance, $Noise_{t-1}$ allowed the authors to see whether a previous noise event would result in a later association with a specific TRP. The model error, ε_{jt}, and random error, θ_j, are both normally distributed with mean of zero and standard deviations of σ^2 and τ^2, respectively. The model was run separately for each TRP-noise pair and resulting estimates were scaled by the interquartile range (IQR) of the logged concentrations, for comparability across models. Given the varying units of measure in the pollutant parameters, the log-transformation, and the scaling, the model coefficients should not be interpreted as physical units of change in either noise or pollutant concentration, but rather as an indication of the strength and direction of association.

Studies have demonstrated that in-vehicle particle concentrations vary on speed—operating through altered air exchange rates and resulting particle deposition—making it a potential modifier of the association between noise and pollution [36, 37]. A secondary analysis examined the interaction between vehicle speed and noise levels on in-vehicle pollutant concentrations using local regression (LOESS) on the complete cases. The interaction-only model plotted in 3-dimensional space provided the additional pollutant exposure predicted by vehicle speed and in-vehicle noise as joint contributors. TRP concentrations were standardized by respective IQRs for comparability across pollutants. This graphical approach allowed for a descriptive analysis of how associations between noise and the TRP measures varied within IVM scenario by changing vehicle speeds.

Results and Discussion

We conducted continuous, extensive air pollution and noise sampling during 18 sessions in NR, IVS, and IVM exposure scenarios. In total, we collected and analyzed 2,170 minutes of multi-pollutant measurements for the current analyses. Mean session durations, for the NR, IVS, and IVM scenarios were 114 (± 4), 118 (± 1), and 129 (± 12) minutes, respectively. Overall, sample completeness for each of the measured parameters was high, with minor data loss (19.2%) in the IVM setting for each of PNC and pb-PAH measures due to an instrument-specific malfunction during a single sampling session.

Scenario-Specific Differences in Noise and Pollutant Concentration. Pollutant and noise levels differed across the three sampling scenarios (Table 1). Broadly, for all the pollutants we measured, mean concentrations were highest while conducting IVM sampling, followed by NR, then IVS sampling. For PNC and pb-PAHs, these levels were significantly higher than corresponding concentrations in the IVS and NR scenarios (Kruskal-Wallis p-values: <0.001). The exceptions to this trend were $PM_{2.5}$ mass and noise, which were highest during the NR sampling protocol compared to IVM ($PM_{2.5}$ mass: 29.2 vs. 14.2 $\mu g \cdot m^{-3}$; noise: 73.8 vs. 69.8 dBA, respectively). Like the mean values, maximum short-term peak pollutant concentrations (1-min) were also highest during IVM sampling for all pollutants, as compared to sampling conducted in the other scenarios.

The in-vehicle environment is widely studied for gaseous or PM pollution [38-44], but has rarely captured both noise and TRP concentrations. Generally, noise and pollutant levels measured in the current study were comparable, if not slightly higher, than those reported in previous studies involving continuous roadside monitoring [45, 46] (Table 1). Morelli et al. (2015) and Can et al. (2011), for example, reported median noise levels between 62-67 dBA and median PNC concentration ranging from 9,870-20,800 particles/cm^3 in European urban or near-road environments. The siting of traffic monitors had differing requirements for both

studies. Can et al. (2011) placed monitors 2 meters from the façades of buildings in the street canyon, while the network of monitors Morelli et al. (2015) used defined a traffic monitor by distance to major road, street density, building density, and population density. Distances to nearest road for the traffic monitors alone were not provided. Previous studies examining the differences in pollutant concentrations within roadway microenvironments have also shown elevated in-vehicle BC, $PM_{2.5}$, and PNCs compared to outdoor measurements [22, 47-51]. Geiss et al. (2010), for example, measured in-vehicle, average $PM_{2.5}$ mass and average PNC at 26.9 $\mu g \cdot m^{-3}$ and 16,391 particles$\cdot cm^{-3}$ during rural driving conditions, which was 3.8 and 9.8 times greater than the car in parked conditions, respectively.

Comparisons between the two stationary sampling protocols, conducted at the same location and distance from the roadway, showed that IVS pollutant levels were consistently lower than those measured during outdoor, NR sampling. Differences between the scenarios were especially pronounced for noise levels, which were roughly 10-fold lower during IVS sampling as compared to NR. These differences in measured pollutant levels are likely attributable to infiltration attenuation associated with the sampling vehicle shell[27, 36], although attenuation strength varied by pollutant. IVS concentrations of the particulate pollutants, for example, were no more than 3% lower than corresponding NR levels. Continuous monitoring also indicated that the pollutant measurements exhibited moderate-to-high minute-to-minute variability, expressed as coefficient of variation (CoV), ranging from 23% for $PM_{2.5}$ mass within NR to 93% for BC within IVM sampling scenarios (Table 1). Noise, in contrast, was relatively invariable (4 - 10% CoV) across all sampling scenarios. Generally, as with the mean 2-hour pollutant values, observed variability was greatest during IVM sampling for every pollutant, with the exception pb-PAHs, which were most variable during NR sampling.

Direct pollution and noise comparisons among the sampling scenarios should be viewed cautiously, given the non-concurrent sampling design. To address this limitation and better contextualize between-scenario comparisons, we examined differences between our measured

ambient $PM_{2.5}$ and BC pollutant levels and those from a background stationary ambient monitoring site (the Jefferson Street (JST) monitoring site, located approximately 2.5 km from the NR and IVS roadway sampling locations). The results showed that concurrent background $PM_{2.5}$ and elemental carbon—analogous to BC—concentration during the field sampling, within each of the scenarios were comparable, if not slightly lower during the IVM scenario (mean 2-hr background ambient $PM_{2.5}$ concentrations during NR, IVS, and IVM sampling: 15.7, 14.5, and 11.7 $\mu g \cdot m^{-3}$, respectively; mean 2-hr background ambient EC concentrations during NR, IVS, and IVM sampling: 1.3, 1.2, 1.0 $\mu g \cdot m^{-3}$, respectively). Together, the descriptive findings support between-scenario differences in TRP and noise among the near- and on-roadway microenvironments which were not reflecting corresponding differences in background pollution.

Covariance between Noise and Particulate Pollution. A primary aim of this sampling protocol was to assess pollutant and noise covariance over short-term exposure durations and, consequently, better understand their potential to serve as either confounders or effect modifiers of each other in epidemiologic models examining acute traffic-related health effects. Confounders must be associated with both the exposure and health endpoint of interest over a relevant exposure window. Independent associations between either TRP or noise on cardiovascular health have been shown[4, 15, 25-27], but less is known of the relationship between these exposures[9, 11]. Our analyses of covariance patterns point to meaningful differences in the strengths of associations between noise and the various TRP components.

Based on these results, several key findings emerged related to covariance between noise and several key particulate TRP components. First, when integrated over 2-hr periods, sampling sessions which were noisier, on average, tended also to be those with higher pollutant concentrations. Slopes from scatterplots of between noise and TRP were largely positive for all pollutants, with differences by microenvironment and pollutant (Figure 1), indicating that noisier sampling sessions were also those with highest particulate pollution levels. Notably, the plots also highlight substantially greater between-scenario differences in noise levels, compared to

within-scenario differences, which varied little from session to session. These results offer some indication, at a 2-hr temporal resolution, of the potential for confounding.

Short-term covariance among the pollutants measured in this protocol was moderate, varying by pollutant, sampling scenario, and ventilation status within a given scenario (Table 2). Broadly, the mixed model results assessed short-term covariance patterns on a minute-to-minute resolution indicated that the strongest associations were between noise and PNC. Across each of the three sampling scenarios and window status conditions, same-minute noise levels were shown to be positively and significantly associated with corresponding PNC concentrations. The findings indicated that the strength of the noise-PNC covariance was nearly instantaneous and existed primarily between same minute measurements of each pollutant. Conversely, there were weak, non-significant associations between these two pollutants when noise was assessed at lag-1-min. We believe the strength and robustness of this association points mainly to a shared scale of atmospheric residence time for both pollutants in roadway settings. Hudda et al. (2011) conducted size-resolved ultrafine particle measurements, and estimated air exchange rates in moving vehicles with closed windows, and found the particles < 50nm were removed from the environment with greater efficiency, likely through diffusion. The attenuation of the association we observed suggested that the microenvironmental characteristics impact the association between noise and PNC, possibly by removing particles that contribute to the strength of the association. This, with the previously demonstrated association between PNC and A-weighted noise [46], may reflect a fresh car source rather than a diesel or total traffic source for both noise and TRP exposures.

Overall associations were strongest between noise and TRP while sampling in a moving vehicle (i.e., during the IVM protocol), compared either near-roadway scenario, when assessed both over 2-hr and 1-min temporal resolutions (Figure 1, Table 2). Model results during IVM sampling showed significant, positive associations between $Noise_t$ and $PM_{2.5}$, BC, and PNC models during periods when windows were closed (β = 0.08 CI: 0.05, 0.12; β = 0.20 CI: 0.06,

0.35; and β = 0.19 CI: 0.08, 0.29, respectively). When windows were opened, $Noise_t$ associations were positive and significant between noise and PNC and pb-PAH models, indicating differential associations based on window status between noise and $PM_{2.5}$ mass, BC, and pb-PAHs, but not for PNC (Table 2). Examining the TRP_{t-1} and TRP_{t-2} estimates across IVM suggests longer residence times of both BC and pb-PAHs, regardless of window status.

The effect of windows on the association between noise and TRP was pronounced in the IVM setting for all TRP, except PNC. We showed that noise and both $PM_{2.5}$ mass and BC covaried only when the windows were closed and while the vehicle was in motion. In the windows closed setting, a recent experimental and modeling study by Ding et al. (2016) demonstrated the transport characteristics of $PM_{2.5}$ are impacted by vehicle speed, ventilation setting, and deposition processes[52]. Hudda et al. (2011) found comparable concentrations of PNC between in-vehicle and outdoor measurements when the stationary vehicle windows were open, suggesting that opened windows potentially introduce mixtures similar to the NR setting. Additionally, Fruin et al. (2011) observed that increased vehicle speed increased air exchange rates and, in turn, decreased particle losses in vehicles with the windows closed [36, 37]. Turning to noise, Li et al. estimated a 20dB difference in noise transmission loss between windows closed and windows opened in their study of in-vehicle noise[27]. Together, with our observation that window status did not modify the association between noise and PNC, these studies provide support for the supposition that closed windows stabilize the mobile, in-vehicle environment by limiting air exchange to the ventilation system and gaps in the shell. This process may select for particle fractions of $PM_{2.5}$ mass and BC that appeared to covary with noise. With the windows opened, the mobile, in-vehicle microenvironment became more turbulent and resembled the NR scenario with respect to the association between noise and certain TRP.

Park et al. (1998) and Ott et al. (2008) both demonstrated increased air exchange rates in a stationary vehicle with fresh air mechanical ventilation relative to a fan off and windows

closed setting [50, 51]. These rates are known to contribute to particle losses and to specific size fractions[36]. The lowest attenuation in particle losses were experienced by particles sized 200-400nm, while smaller particles were thought to be lost to diffusion[53]. The losses were not limited to TRP, as Li et al. (2016) measured sound level losses in a stationary vehicle due to manufacturer-installed sound proofing, with sound reductions from 25 to 50 dB [27]. The effect of the vehicle shell impacted both noise and TRP exposures, and modified the association between the two.

Within IVS sampling, we observed moderate to strong session-specific associations between noise and $PM_{2.5}$ mass, PNC (Table 2). Our sampling in the IVS setting did not indicate that windows modified the association between noise and TRP. Associations between $Noise_t$ and TRP were significant and positive for $PM_{2.5}$ mass and PNC when windows were either opened or closed, despite substantially lower median TRP concentrations during periods where the windows were closed ($PM_{2.5}$ mass: closed – 16.2 μg/m^3, opened – 31.6 μg/m^3; PNC: closed – 6,601 #/m^3, opened – 9,287 #/m^3) (Table 2). For $PM_{2.5}$ mass, associations with noise were similar during periods when the windows were closed (β = 0.46 CI: 0.38, 0.54) and opened (β = 0.48 CI: 0.38, 0.59). For both BC and pb-PAH, during periods when the windows were opened, associations with noise were non-significant. Finally, the NR setting showed current minute noise levels ($Noise_t$) to be a significant, positive predictor of PNC (β = 0.37 CI: 0.14, 0.60) and pb-PAHs (β = 0.62 CI: 0.20, 1.03), but not $PM_{2.5}$ mass or BC. Noise levels occurring during the previous minute ($Noise_{t-1}$), were not significant predictors of any TRP in the NR setting.

Vehicle speed served as a potential modifier of the associations between noise and TRP. In this field study, speed was highly correlated with noise and mild-to-moderately correlated with all measured TRP (r_S = 0.19 - 0.41) in our IVM setting. These corroborated previous observations of the respective associations [36, 54]. The authors did find, however, evidence of null associations between speed and in-vehicle $PM_{2.5}$ in the literature [48]. Higher speeds were associated with greater TRP infiltration in the vehicle cabin when the windows

were closed and with no apparent association when the windows were open [55]. With noise, higher speeds increased the combined noise (engine, exhaust, tires, etc.) generated from movement across the road [54]. As speed was known to associate with our exposures of interest independently, we examined the interaction effect of speed and noise on pollutant concentrations.

Generally, we found high speed and noise jointly increased pollutant levels, with minor differences between specific TRP (Figure 2). In the case of PNC, the interaction was particularly pronounced at the highest levels of both noise and speed. We demonstrated that the interaction between speed and noise, as it impacted pollutant concentrations, was not homogenous across the range of vehicle speed and that it contributed to the dynamic nature of exposure within a vehicle cabin. Although the overall pattern of the interaction was shared across all measured TRP, BC and PNC appeared to have different strengths of the interaction effect as compared to $PM_{2.5}$ mass and pb-PAHs. Examining the scatterplots (results not shown) indicated the modeled extreme highs for BC, pb-PAHs, and PNC are influenced by a small number (N = 3-4) of 1-min observations clustered at high speeds (70-95 km/h) and high noise levels (~82 dB), while the $PM_{2.5}$ mass maximum is driven by many observations at lower noise levels (~77 dB) and higher speeds (~115 km/h). At the IVM median noise level (71 dB), standardized interaction TRP concentrations changed minimally as speed increased, with percent changes between minimum and maximum concentrations of 96-97% for $PM_{2.5}$ mass and BC; lesser changes (31-38%) were observed for pb-PAHs and PNC.

There were key limitations to the current study design and generalizability of our findings. As noted, all in-vehicle results were conducted using a single, dedicated test vehicle, which limited inferences related to associations for other vehicle makes and models. Sampling within a dedicated car at the same location as a near-roadway location allowed us to evaluate the impacts on measurement by the vehicle shell. Thus, the variability of physical processes associated with noise and chemical fate and transport could be examined closely without the

variability introduced by multiple vehicles. Conducting a similar campaign on a broader vehicle fleet was beyond the scope of the current study, and fleet variability has been previously studied to some extent [33, 37]. Despite this, we believe that the physical and correlational characteristics observed in each of the exposure scenarios to be broadly applicable to other settings.

Another potential limitation was the small number of sampling sessions within each sampling scenario. The statistical methods employed here, such as the mixed model, adeptly maximized efficiency and still provided reliable estimates. Nevertheless, greater numbers of sessions may have improved characterization of microenvironment-specific associations with simpler techniques. Finally, our study measured A-weighted noise only, which has previously been shown to bias against low frequency sources like heavy trucks and diesel vehicles[46]. Future work should follow Morelli et al. (2015) and Wang et al. (2016) in examining frequency resolved, high-resolution measures of noise[46, 56]. Doing so may capture stronger associations between low- or medium-frequency noise and markers of heavy vehicles, such as BC [57].

Continuous, high resolution sampling in a closed environment provided an improved assessment of the relationship between noise and TRP. The differences in concentrations across sampling scenarios suggested that the association between air pollution and noise may also be different across these conditions. The session-specific correlation coefficients demonstrated changes in strength of the association with noise by pollutant. With the vehicle shell filtering both noise and pollution, the estimates of association were more consistent. It is likely that the selection imposed by the vehicle shell on both noise and pollution enhanced this correlation using our sampling methodology. The dynamic nature of the association of noise with TRP encourages the measurement of both in future analyses for short-term exposure and acute health effects.

Conclusions

Collectively, the descriptive results from this field study clearly pointed to in-vehicle particulate pollution and noise levels that were elevated, with higher peak concentrations, and greater short-term variability, relative to similar near road levels. Moreover, these findings supported the interpretation of distinct differences in exposure among various roadway microenvironments [58]. The varied chemical composition, fate, and transport properties of traffic-related air pollution provided challenges to understanding the complex relationship they have with traffic-related noise emissions. Despite this, the findings of this study suggested that associations exist between noise and several pollutants. In our quasi-controlled field experiment, we observed strengths of association ranging from moderate (i.e., $r_S > 0.60$) to weak depending on several factors such as sampling scenario, vehicle speed, and in-vehicle ventilation status. Each measured air pollutant may have numerous emission sources, even if they originated from the same active roadway.

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Tables and Figures

TABLE 1: Summary Statistics for 1-minute Average Measures by Sampling Scenario

	$PM_{2.5}$ Mass - $\mu g \cdot m^{-3}$	BC - $\mu g \cdot m^{-3}$	PNC - $\#\cdot cm^{-3}$	pb-PAH - $ng \cdot m^{-3}$	Noise - dBA
Near-Roadway (N = 685)					
% Completeness	100	98.7	100	100	100
Mean (SD)	29.2 (6.6)	4.2 (2.2)	15,533 (8,389)	54.2 (41.5)	73.8 (3.3)
Maximum	44.6	20.0	73,715	424.2	79.5
Median (IQR)	28.1 (10.0)	3.7 (2.4)	12,868 (8,644)	42.8 (43.3)	74.5 (4.5)
Minimum	17.5	0.3	5,538	4.5	64.1
% CoV	23	52	54	77	4
In-Vehicle, Stationary (N= 710)					
% Completeness	100	95.6	100	99.6	100
Mean (SD)	23.4 (10.7)	3.2 (2.9)	8,941 (4,990)	39.4 (28.8)	58.1 (5.2)
Maximum	73.9	28.6	47,854	323.7	66.7
Median (IQR)	21.8 (16.3)	2.6 (1.9)	7,661 (4,664)	32.8 (32.3)	59.2 (10.3)
Minimum	6.0	0.2	2549	4.5	49.5
% CoV	46	89	56	73	9
In-Vehicle, Mobile (N= 775)					
% Completeness	97.7	97.4	81.8	81.8	100
Mean (SD)	14.2 (8.2)	6.6 (6.1)	23,541 (15,243)	113.3 (65.3)	69.8 (7.0)
Maximum	75.5	67.0	97,736	621.7	82.1
Median (IQR)	13.2 (10.9)	5.1 (5.5)	21,342 (19,073)	107.2 (62.0)	71.2 (9.8)
Minimum	2.5	0.1	2,392	1.3	53.0
% CoV	58	93	65	57	10

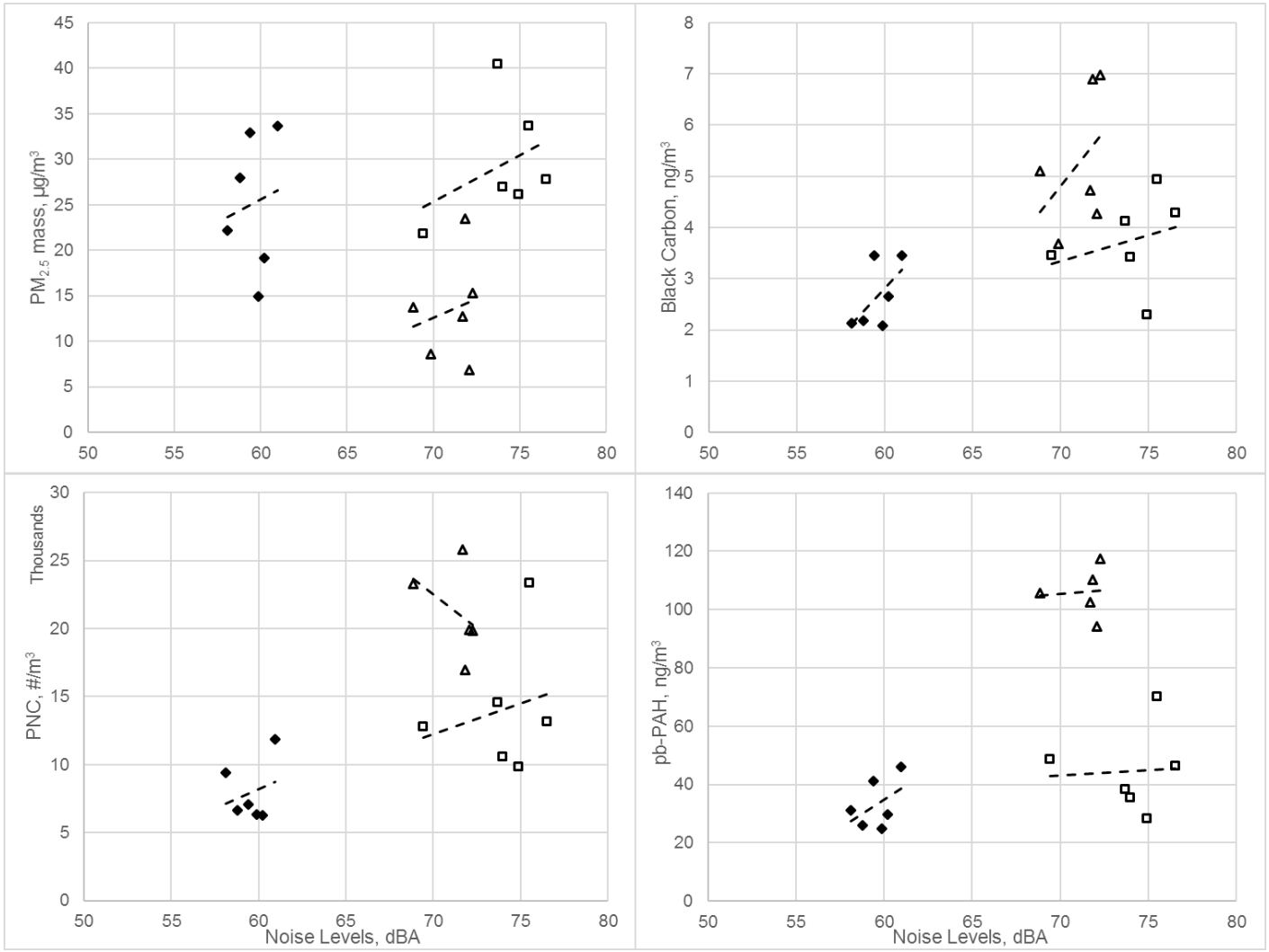

FIGURE 1: 2-hour and 1-minute resolution correlations between noise levels and particle pollution. Median TRP concentrations were plotted against median noise levels (dBA) for $PM_{2.5}$ mass, black carbon, particle-bound polycyclic aromatic hydrocarbons (pb-PAH), and ultrafine particles (PNC) (clockwise from top left). Dashed lines (- -) are regressions of median noise levels versus median TRP concentrations within a microenvironment. Filled diamonds (♦), open triangles (▲) and open squares (□) are the IVS, IVM, and NR scenarios, respectively.

TABLE 2: Scaled Linear Mixed Model Estimates and 95% CIs of Audio and Lagged Pollutant Covariates on Concurrent PM

	Minutes (Sampling Sessions)	$Noise_t$ (dB)	$Noise_{t-1}$ (dB)	TRP_{t-1}	TRP_{t-2}
$PM_{2.5}$ Mass log(μg/m^3)	2115 (18)				
Near Roadway		0.02 (-0.07, 0.12)	-0.01 (-0.11, 0.09)	**0.46 (0.34, 0.59)**	**0.22 (0.10, 0.34)**
In-Vehicle, Stationary					
Windows Closed		**0.46 (0.38, 0.54)**	**-0.19 (-0.28, -0.11)**	**0.69 (0.59, 0.80)**	**-0.12 (-0.21, -0.03)**
Windows Opened		**0.48 (0.38, 0.59)**	**-0.35 (-0.47, -0.24)**	**0.58 (0.48, 0.67)**	-0.02 (-0.10, 0.07)
In-Vehicle, Mobile					
Windows Closed		**0.08 (0.05, 0.12)**	**-0.06 (-0.10, -0.02)**	**0.62 (0.56, 0.68)**	-0.05 (-0.11, 0.00)
Windows Opened		-0.03 (-0.07, 0.02)	**0.07 (0.02, 0.11)**	**0.44 (0.20, 0.68)**	0.01 (-0.03, 0.05)
Black Carbon log(μg/m^3)	2024 (18)				
Near Roadway		0.35 (-0.01, 0.70)	-0.17 (-0.52, 0.19)	**0.43 (0.36, 0.50)**	0.01 (-0.06, 0.08)
In-Vehicle, Stationary					
Windows Closed		0.10 (-0.19, 0.39)	0.06 (-0.26, 0.38)	**0.69 (0.59, 0.80)**	**-0.15 (-0.24, -0.05)**
Windows Opened		-0.11 (-0.51, 0.28)	**0.77 (0.33, 1.20)**	**0.39 (0.29, 0.48)**	0.04 (-0.06, 0.13)
In-Vehicle, Mobile					
Windows Closed		**0.20 (0.06, 0.35)**	-0.04 (-0.18, 0.11)	**0.53 (0.46, 0.61)**	**0.20 (0.13, 0.27)**
Windows Opened		0.07 (-0.12, 0.27)	0.11 (-0.08, 0.30)	**0.51 (0.36, 0.66)**	**0.13 (0.05, 0.22)**
PNC log(#particles/m^3)	1995 (17)				
Near Roadway		**0.37 (0.14, 0.60)**	0.01 (-0.22, 0.25)	**0.49 (0.42, 0.56)**	**0.08 (0.02, 0.15)**
In-Vehicle, Stationary					
Windows Closed		**0.29 (0.12, 0.47)**	-0.06 (-0.25, 0.14)	**0.86 (0.73, 0.99)**	**-0.26 (-0.38, -0.14)**
Windows Opened		**0.27 (0.02, 0.53)**	0.02 (-0.26, 0.30)	**0.65 (0.55, 0.75)**	-0.07 (-0.17, 0.03)
In-Vehicle, Mobile					
Windows Closed		**0.19 (0.08, 0.29)**	0.01 (-0.10, 0.11)	**0.89 (0.79, 0.99)**	**-0.13 (-0.22, -0.03)**
Windows Opened		**0.18 (0.06, 0.31)**	0.05 (-0.07, 0.18)	**0.72 (0.56, 0.87)**	-0.02 (-0.11, 0.07)
pb-PAHs log(ng/m^3)	1992 (17)				
Near Roadway		**0.62 (0.20, 1.03)**	-0.23 (-0.64, 0.19)	**0.62 (0.54, 0.70)**	0.02 (-0.06, 0.11)
In-Vehicle, Stationary					
Windows Closed		0.18 (-0.13, 0.49)	-0.09 (-0.43, 0.25)	**1.11 (0.96, 1.26)**	**-0.29 (-0.44, -0.13)**
Windows Opened		-0.13 (-0.59, 0.32)	**0.87 (0.38, 1.37)**	**0.87 (0.75, 1.00)**	**-0.15 (-0.27, -0.03)**
In-Vehicle, Mobile					
Windows Closed		0.09 (-0.09, 0.27)	0.08 (-0.10, 0.26)	**0.36 (0.22, 0.51)**	**0.19 (0.04, 0.34)**
Windows Opened		**0.30 (0.07, 0.52)**	0.03 (-0.20, 0.25)	**0.45 (0.27, 0.63)**	**0.28 (0.17, 0.39)**

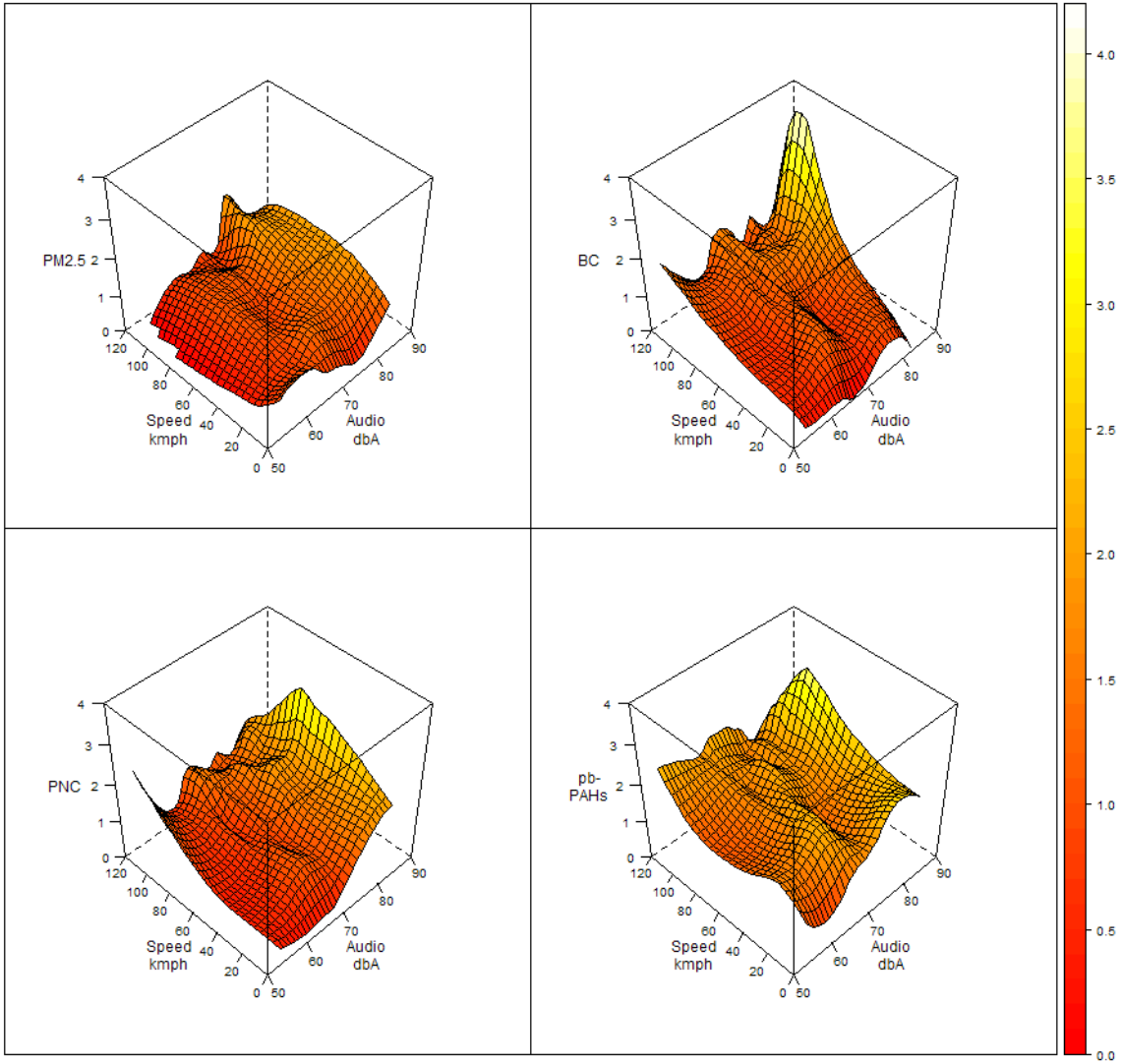

FIGURE 2: Three-dimensional interaction plots between vehicle speed and noise for each PM pollutant for the in-vehicle, mobile (IVM) scenario. Pollutant concentration axes are scaled by respective interquartile ranges and colorized red to white by increasing magnitude.

Chapter 3: Metabolomic Profiles of Plasma, Exhaled Breath Condensate, and Saliva are Correlated with Potential for Air Toxics Detection

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KEYWORDS: exhaled breath condensate, saliva, plasma, high-resolution metabolomics, traffic pollutants, air toxics, exposure assessment, correlation

Abstract

High-resolution metabolomic profiles may serve as sensitive indicators of traffic air pollution exposure and provide biological plausibility for exposure related disease etiology. Exposure assessment studies have considered blood, breath, and saliva as biological matrices suitable for measuring responses to air pollution exposures. This pilot study examined the comparability of these three matrices using high-resolution metabolomics (HRM) and explored their potential for measuring mobile source air toxics.

Methods: Four participants provided saliva, exhaled breath concentrate (EBC), and plasma before and after a 2-hour road traffic exposure. Samples were analyzed on a Thermo QExactive MS system in positive electrospray ionization (ESI) mode and resolution of 70,000 with C18 chromatography. Data were processed using apLCMS and xMSanalyzer on the R statistical platform.

Results: HRM measured 7,110, 6,019, and 7,747 reproducible features in plasma, EBC, and saliva, respectively. Correlations were moderate across all pairwise comparisons of intensity within profiles, with the strongest between EBC and saliva. The associations of mean intensities between matrix pairs were positive and significant, controlling for subject and sampling time effects. Six out of 20 features shared in all three matrices putatively matched a list of known mobile-source air toxics.

Conclusions: Plasma, saliva, and EBC have largely comparable metabolic profiles measurable through HRM. These matrices have the potential to identify and measure mobile source air toxics, though further, targeted study is needed.

Introduction

Identifying the causal agents of traffic-related health effects is complicated by the nature of the exposure mixture and by the expense and labor of measurement. The exposome is a theoretical construct that expands exposure science to include measurements of both external and internal features [1, 2]. It relies upon approaches with the potential to link exposure assessment to toxicological insights. This produces a remarkable opportunity to integrate traditional, validated exposure assessment technologies with the development of high-throughput, low-cost methods that may represent both exposure and response [3-5]. The exposome framework holds the potential to address the etiological challenges in environmental health fields, including that of traffic-related pollution (TRP) and health. The utility of one tool, high-resolution metabolomics (HRM), needs to be explored for air pollution studies, especially the biological samples often used in field research.

HRM allows for the identification and quantitation of small molecules via high-throughput, high-yield analytical chemistry and bioinformatics. HRM may serve as an ideal tool for conducting exposure assessment of complex external and internal stressors. The use of HRM within traditional environmental epidemiologic settings has been limited, but growing. Previous studies have used metabolomic approaches to characterize high and low exposure to polycyclic aromatic hydrocarbons (PAHs) or metals, as well as differential perturbations in metabolism, between high and low exposure cohorts [6-10].

The specific components of TRP that are most relevant to human health response have been difficult to identify using traditional exposure assessment methods. The toxic effects of exposure to airborne total particulate matter (PM) from primary TRP have been well-established [11-14]. These toxic effects range from increased short-term morbidity in individuals with preexisting respiratory and cardiovascular disease to excess mortality rates over life-long exposure durations [15, 16]. Given the extreme chemical and physical heterogeneity of TRP,

however, it is not clear which components or mixtures are most responsible for driving the observed adverse responses [17-20], nor which specific biological pathways contribute to PM-specific disease etiology [20-25]. Metabolomics may provide much needed insight into these gaps that traditional exposure assessments conducted to date have been unable to address.

However, answers to more fundamental questions about the utility of the HRM in air pollution health work is needed. As a first step, understanding how metabolomic profiles vary by biological matrices in the same individuals may inform future study designs in this exposure science subfield. Non-invasive sampling of exhaled breath (EBC) [12, 26] and saliva [27] may serve as viable alternatives to more invasive blood sampling. Comparative metabolomic profiling across the plasma, EBC, and saliva matrices may assist with the identification of the most relevant components of PM exposure for health. To date, studies of HRM and human health have mainly considered human blood and urine. Relatively few studies have been conducted on saliva or EBC using HRM, with even fewer examining metabolomic profiles across multiple biological matrices simultaneously [28-33]. Another question of longitudinal changes of profiles remains as few studies have examined daily changes in metabolomic profiles for humans [34-39]. The paucity in the literature HRM profile comparability for these three matrices is an opportunity to examine their utility for air pollution epidemiology.

To address these questions, a pilot commuter study was conducted in which plasma, EBC, and saliva were collected for a small set of participants. The overarching aims of this pilot study was to a) assess feasibility of conducting metabolomics analyses on these three matrices using the Emory metabolomics platform; b) compare metabolomic profiles in plasma samples with those in the two, non-invasively collected matrices, saliva and EBC, and; c) evaluate whether the metabolomics platform identified features that are expected to be associated with exposure to TRP.

Methods

The current analysis used data collected as a pilot field study, nested within two larger panel studies of daily car commuters in Atlanta, GA [40-42]. A convenience sample of four participants, who provided repeated samples of venous blood, EBC, and saliva over a multi-day period, was recruited. To mirror the larger commuter study designs, the pilot sampling was conducted before and after a scripted, 2-hr commute during morning rush hour.

The pilot protocol comprised of four repeated measures of biological samples for four participants. Two sample collections were conducted on the first day (Day 0); one pre-commute session at 7AM, and one immediately following the commute at 10AM. The subsequent sampling times occurred at 11AM on both +1 and +2 days.

Biological Sampling. For each subject, sampling sessions included whole blood collection, passive saliva and exhaled breath condensate collection [41]. Briefly, venous blood was collected in 3mL, ethylenediaminetetraacetic acid-treated (EDTA) tubes (BD Vacutainer) from either the left or right median cubital vein and centrifuged immediately for plasma retrieval. Saliva was collected passively into sterile 15mL conical centrifuge tubes (Falcon) held against the lips. Rtubes (Respiratory Research) were used to collect roughly ~2mL of exhaled breath condensate. Biological samples from each matrix was aliquoted into microcentrifuge tubes and stored at -80ºC within 20 minutes of collection. Metabolomic analyses were conducted within four months of collection.

Metabolomics and Data Processing. Analytical chemistry was performed following procedures developed by Soltow et al. (2013). For each sample, 65µL were diluted into 130µL of acetonitrile spiked with isotopic standards. After a 30-minute equilibration and protein precipitation, triplicate 10µL aliquots were placed into autoinjector vials and run across two batches through a C18 reverse phase liquid chromatography coupled with high resolution mass spectrometry (HR-MS) on a QExactive Orbitrap (ThermoFisher) [43]. The analytical platform

allows both positive and negative electrospray ionization (ESI), providing two, distinct runs per replicate. For this analysis, we focused only on positive phase features from positive ESI, since a greater number of features for this phase have been validated and annotated using spectral information available in the Human Metabolome Database (HMDB).

Raw mass spectrograms output from the analytical platform are processed to extract features, which represent individual ions detected from the mass spectrometer. Each feature is indicated by a unique combination of its mass-to-charge ratio (m/z) and retention time (RT). The relative concentration of a given feature within a replicate is indicated by the integrated ion intensity.

Feature extraction was performed using two, integrated algorithms developed by the Emory University Clinical Biomarkers Laboratory (clinicalmetabolomics.org), a research center integrating expertise in metabolomics and informatics. The two algorithms were the adaptive processing of liquid chromatography mass spectrometry (apLCMS) and the xMSanalyzer packages, which were developed for use in R (versions 6.0.1 and 2.0.6.1, respectively)[44, 45]. The apLCMS program was run in 'hybrid' mode, meaning it was supplied mass information of known metabolites [46]. The list of known metabolites comprised of monoisotopic masses from HMDB (v. 3.6) and the US Environmental Protection Agency's Master List of Compounds Emitted by Mobile Sources. The xMSanalyzer package then modifies the resulting data from apLCMS for quality and reproducibility. Complete feature extraction and data processing was done separately for each biological matrix to reduce noise introduced by mixing sample types. This resulted in three distinct feature tables for each sample type: plasma, EBC, and saliva.

The feature tables were pre-processed following Soltow et al. (2013). Briefly, the ion intensities in each of the feature tables were $\log_2$ transformed. Raw zero values were set to missing after transformation. Triplicate intensity values for a feature within a biological sample were averaged, with missing values ignored, to produce the final feature tables for statistical analysis. Metabolite levels of select features were generated using reference standardization

and compared to previously published ranges [47].

Statistical Analysis. Statistical analyses were conducted in R (v.3.1.3). To evaluate the overall associations among the biological matrices, three key data subsets were sequentially examined: 1) All features detected in a biological matrix ('All'); 2) a subset of features measured with low triplicate variability in intensity (i.e., < 10% median coefficient of variation) ('<10% CoV'); and 3) a subset of features with masses that matched compounds in the USEPA Master List of Compounds Emitted by Mobile Sources ('EPA-matched'). Feature counts across matrices were compared, descriptively in Venn diagrams using functions provided in 'xMSanalyzer.'

For this analysis, a shared feature across matrix pairs was defined as a feature with a m/z within 10 ppm and a retention time within 300 seconds of a feature in another matrix. Only shared features between matrix pairs were used for subsequent analyses, similar to the inter-biofluid analysis performed by Pinto et al. (2015).

Scatterplots of 'All' shared features across matrix pairs provided a visual assessment of the joint distributions of mean feature intensities across subjects at the pre-exposure sampling. Correlations between matrix pairs of mean feature intensities at each sampling time were examined using Spearman's rank correlation analysis (r_S), with confidence intervals estimated using the Fisher transformation [48].

To examine the impacts of subjects and sampling time on the association of average intensities across matrix pairs, linear regression was used on the EPA-matched subset. The analysis focused on this subset because its members were features potentially representing traffic-related pollution. The model constructed examined associations of a feature intensity in one biological matrix (X_{ij}) to the m/z and RT-matched feature intensity in another (Y_{ij}). The regression can be expressed as:

(1) $$Y_{ij} = \mu + \alpha_{ij}X_{ij} + \sum_{i=1}^{n-1}\beta_i Z_i + \sum_{j=1}^{t-1}\gamma_j W_j + \varepsilon_{ij},$$

where $Z_i = \begin{cases} 0, subject\ 1 \\ 1, subject\ 2 \\ 2, subject\ 3 \\ 3, subject\ 4 \end{cases}$ and $W_j = \begin{cases} 0,\ pre-exposure \\ 1,\ post-exposure \\ 2, +1\ day \\ 3, +2\ day \end{cases}$.

Here, μ represents the mean intensity of the referent group and ε_{ij} the residual error, normally distributed around 0. The model was run separately for each pair of matrices: 'EBC = plasma', 'saliva = plasma', and 'saliva = EBC'. Both the predictor and dependent variables were log2-transformed. A secondary analysis was conducted to explore the range of intensities in the sample population. The variability of specific features in the EPA-matched subset may have differential influence on observed associations and were examined using scatterplots (Supplementary Figure 1).

Annotation of shared, EPA-matched compounds. Feature annotation is the process in which a putative identification of a feature can be made through the mass querying online chemical databases like the HMDB. In this pilot study, the EPA-matched subset provided an opportunity to compare identifications with HMDB and ChemSpider, a comprehensive, chemical structure database curated from roughly 500 sources, to evaluate the feature annotation process and needs for use in environmental epidemiological applications.

As a means of conducting initial, semi-quantitative annotation of the metabolomics output, features from the EPA-matched subset that were shared in all matrices were subjected to a manual annotation using HMDB and ChemSpider [49]. First, the m/z ratios from the 20 features were used to calculate computed masses using only proton adducts and queried in HMDB. If a mass-match was successful, a semi-quantitative comparison was made between the compound entry or entries in HMDB and compound identification provided in the USEPA Master List. The number of compound matches that overlapped, if any, was recorded. Secondly, entries from HMDB and the USEPA Master List were compared qualitatively. For example, if an entry in HMDB was detected, quantified, and validated as an endogenous

metabolite, then this was determined to be a better identification than a dissimilar compound match with the Master List. However, if no match was found in HMDB, the putative identification using the Master List was used to search ChemSpider. The purpose of this step was to examine other potential sources for exposures. If the putative identification is for a compound shared in other potential exposures, such as consumer products or foods, it was no longer considered a putative TRP-related feature.

Results

We retrieved 14 of 16 plasma, 15 of 16 EBC, and 16 of 16 saliva samples across a three-day sampling period across all participants. Participant metabolic characteristics, derived from the metabolomics analysis of plasma, at baseline, were comparable to or within a range of previously reported values for selected features (Table 1) [50]. In total, raw feature extractions yielded unique 9,377-10,583 features in both plasma and saliva, and 7,688-8,565 features in EBC. After summarization and data cleaning, 7,110, 6,019, and 7,747 features were measured in plasma, EBC, and saliva, respectively. The median coefficients of variation across triplicates of samples within each biological matrix was less than 15% (Plasma: 13.5%, EBC: 14.3%, and Saliva: 13.2%). Saliva had the largest number of unique features (N=4,227) that were not present in either EBC or plasma; compared to the number of unique features in both plasma (N = 3,718) and EBC (N = 2,381). Most identified features in EBC (60.5%) were also found in the other matrices (Figure 1). Overall, the associations between metabolomic profiles from matrix pairs were consistent across repeated measures and across subjects.

Between-matrix scatterplot among ‘All’ features showed generally positive linear relationship, and prominent clustering along a 1:1 line for features shared between paired matrices (N = 3,085 – 3,255) (Figure 2). To examine linear association more closely, we used Spearman’s correlations stratified by matrix-pairings, four sampling time points, and data subsets (Figure 3). When examining ‘All’ shared features identified at each of the sampling

periods, correlations between EBC and plasma ranged from 0.51 to 0.58 across all four sampling time points (p-value < 0.0001, for all pairwise correlations). EBC-plasma correlations were comparable, albeit slightly stronger (r_S = 0.56 – 0.65; p < 0.0001), for those features measured with greater confidence (i.e., '< 10% CoV') (N = 674 – 861). EBC-plasma correlations involving the subset of features within the 'EPA-matched' subset, ranged between 0.41 and 0.52 (p < 0.0001), across the various sampling time points. Saliva-plasma and saliva-EBC pairwise correlations were also positive and moderate across all data subsets (r_S = 0.51 – 0.69; p < 0.0001 and 0.60 – 0.80; p < 0.0001, respectively). The estimates of the correlation for EBC vs. saliva were stronger across all subsets ('All': ρ=0.60-0.66; '<10% CoV': 0.72-0.80; 'EPA-matched': 0.66-0.79).

Generally, correlation patterns across all repeated measure sampling time points were very consistent within each pairwise matrix comparison and data subset and did not vary by more than 13%. In the examination of the features matching the USEPA Master List ('EPA-matched'), the correlations varied from 16% in saliva vs. EBC to 23% in plasma vs. saliva. The greater variation in correlation estimates may be explained by the relatively small number of features being compared (N = 36 – 54).

Linear regression for the 'EPA-matched' subset demonstrated that the mean feature intensities did not vary by subject or sampling time, except for +1 Day sampling time in the EBC = plasma model. The feature intensities in EBC were significantly reduced (β(SE): -0.66 (0.30)) at the +1 Day time point relative to the pre-exposure measurement holding plasma intensity and subject constant. Strengths of associations between matrix pairs across all three models were positive and statistically significant (p < 0.0001) (Table 2). For example, a 10% increase in the geometric mean of feature intensities in plasma corresponded to a 6.0% increase in the geometric mean intensities in EBC. The same increase (10%) in plasma or EBC was associated with a corresponding 7.7% and 7.4% increase in saliva for models 2 and 3, respectively.

Highlighting the EPA-matched subset permitted a semi-quantitative examination of feature annotation. Of features that were found in all three matrices (N = 20), only eight uniquely matched the curated Master List of Compounds Emitted from Mobile Sources and were not found in HMDB (Table 3). These features present new, putative identifications found in plasma, EBC, and saliva and are less likely features of endogenous metabolism. A ninth feature, matching the USEPA Master List as n-nitrosomorpholine, had two matches to drug metabolites in HMDB which are unlikely in our healthy population. Three of these features, putatively identified as n-nitrosodiethylamine, n-nitrosomorpholine, and C29H50 (i.e., 30-normoretane), had high relative concentrations across all matrices. Further examination of the usage of the eight compounds, using ChemSpider as the source, suggested that three of them may be from exposure to consumer hygiene or food products.

Discussion

Comparison of feature information across multiple, biological matrices is a critical initial step towards evaluating HRM's usefulness as a less cumbersome and less invasive method for characterizing widespread environmental exposures versus traditional exposure assessment approaches. A number of studies have previously compared metabolite features measured across matrices sampled from the same persons [30, 32, 33, 51-56]. To our knowledge, however, the results from our pilot study comprise the first to examine matrix comparability between plasma (relatively invasive) and saliva or EBC (non-invasive) using this measurement method. Our data showed moderate associations among extracted features in each of the three matrices, with the strongest association between features in EBC and saliva. These results provide general support for the use of HRM to measure metabolic profiles in matrices collected non-invasively, specifically in EBC and saliva. The number of shared features as well as the between-matrix associations among these shared features suggest that either saliva or EBC may also be comparable to plasma.

Importantly, using our bioinformatics platform for metabolomics, we were able to extract a greater number of features than previous investigations [45]. In addition, the use of xMSanalyzer and apLCMS yielded thousands of reproducible features in each matrix, with most features shared across matrices. By comparison, previous metabolomic analyses have detected features numbering the mid-to-high hundreds [54, 57]. Although saliva had the greatest absolute number of individual features extracted, EBC shared a larger proportion of detected features than either plasma or saliva.

Generally, we observed strong associations between the mean feature intensities in plasma and corresponding intensities in both EBC and saliva. As indicated by the results from the linear models, these associations were not driven by individual subject effects, nor by sampling time points. The lack of significant subject effect is contrary to a previous repeated measures study of metabolomics [57]. A possible alternative explanation may be that the sample size of our study does not reflect the true heterogeneity of metabolomes in larger populations. However, intensities of shared features between matrix pairs showed pronounced linearity in their relationships to each other in the scatterplots. The clustering around a 1:1 line showed that high intensities in one matrix was typically indicative of similar intensities in another matrix. Do et al. reported that 93.5% of total between-fluid correlations of plasma and saliva matched to the same biochemically-valid networks conferring confidence in annotation, but the average of the significant, partial correlations reported (N = 14 metabolites) was very weak between the same metabolites (r = 0.06) (2015). Our results, with respect to plasma and saliva, show a moderate correlation among feature intensities between the matrices. The difference in interpretation of the association, aside from differing statistical methods, is likely attributable to the authors exclusion of unknown, unannotated features.

In our results, the relative strength of association was similar regardless of the data subset analyzed, suggesting that the cross-matrix correlation patterns were not merely driven by features that were more reliably measured or identifiable (Figure 3). Our regression modeling

results using the 'EPA-matched' subset supported these findings, indicating positive and significant associations of mean intensities between matrices that did not vary by subject or time, appreciably. These associations echoed the patterns of correlations between biological matrices in the 'All' and '<10% CoV' subsets. These results provide promising initial support for the use of our environmental metabolomic platform for the robust detection of compounds that may be associated with potential environmental sources.

Previous studies that have examined within- and between-person variability in metabolomic profiles across a variety of analytical platforms and matrices have found that the measured metabolites had much less within-person than between-person sources of variability [58-60]. In contrast, our profiling, which captured thousands of features in each matrix, indicated negligible differences when comparing the mean feature intensities between persons. This finding is consistent with Crews et al. (2009), and suggests that a large set of features from metabolomic profiles with greater intensities do not exhibit substantial daily variability within a multi-day sampling protocol. It is worth emphasizing that although metabolomic profiles overall may exhibit low between-person variability in healthy persons, certain features do have greater between-person variability. In Crews, et al., features with high integrated intensities ($>10^6$) clustered with CoVs less than 20% across biological samples in plasma. Our secondary analysis shows that some features within the EPA-matched subset have very little variation while other exhibit broad ranges of intensity values. Metabolomic analyses of low intensity features may be liable to greater inter-individual variability. This may be true, for example, in an assessment targeting features indicative of an exposure to an environmental contaminant, which would likely result in relatively low intensity values compared to endogenous metabolism [61].

The moderate correlations ($r_S \approx 0.5$ to 0.6) between EBC and saliva intensities were somewhat unexpected. In 2008, de Laurentis et al. showed very dissimilar spectral profiles between EBC and saliva in samples of healthy subjects and patients with airway disease,

indicating very low overlap in the composition of measurable compounds between the two matrices. Our findings provide some indication that EBC may be reflecting a large portion of the metabolomic features in both saliva and plasma, and that the metabolomic profiles of EBC were more strongly correlated with saliva. We collected unstimulated, passive saliva. This is known to mostly be generated from the submandibular glands, but still comprising a mixture of saliva produced from the parotid and sublingual glands [62]. The marginally greater number of features measured in saliva may reflect unique microbial signatures. Previous literature on the salivary metabolome have reported the presence of both endogenous and microbial signatures within this matrix [63]. This interpretation should be viewed cautiously, however, since we were not able to discern metabolites generated from human or microbial metabolism for lack of specificity in putative identification. While definitive validation methods, such as tandem MS approaches (MS/MS), were beyond the scope of this pilot analysis, future targeted analyses including formal feature validation may help discern specific contribution from microbial as well as other sources.

It is possible that the strength of the saliva-EBC association may be explained, in part, by salivary contamination of EBC. When examining immunological markers, however, previous studies have found a negligible influence of salivary concentrations on EBC concentrations [64]. Despite this, our metabolomic analysis captures a much larger chemical space than the more limited immunological markers examined previously. In two recent studies of oral and lung microbiota, the taxonomic composition of oral washes and broncheoalveolar lavage were reported to be mostly similar, though still different in specific, lower-abundance taxa by sampling location [65, 66]. These samples, at least partially, were collected from similar physiological locations we sampled through our passive saliva and exhaled breath. The microbiota occupying these similar locations may produce shared metabolites contributing to the observed correlations between EBC and saliva, relative to correlations between either of these matrices with plasma.

Another key finding from this analysis was the consistent strength of association of the four sampling time points over the span of three consecutive sampling days. This finding provides indication that the observed associations between the metabolomic profiles in this pilot study were generally not time-varying over this duration. The striking consistency in the strengths of associations across time, also support our conclusion that these results were not spurious and driven by artifacts in the data analysis plan. However, the result should not be interpreted to mean that the profiles themselves are time-invariant. Ang et al. (2012) found both time-invariant and time-variant metabolites in pooled samples. Their study suggests that the selection of metabolites from a total metabolic profile may warrant special considerations depending on their respective temporal characteristics. Our study had repeated samples at nearly the same time of day from healthy individuals, which may explain the robustness of the associations over time [57].

Although correlations were typically strong among a broad suite of metabolites, without conducting intensive validation, it is unclear whether these were primarily abundant metabolites associated with common metabolic processes, which would not be expected to exhibit daily fluctuation or variability. From our semi-quantitative feature annotation, we found that the 20 metabolites identified from the EPA-matched subset were more likely to be features associated with endogenous metabolism, a food chemical, or consumer product exposure. However, six features, detected in all samples and matrices, were exclusively identified using the USEPA mobile source metabolite list. This may indicate an environmental origin and provides some anecdotal evidence that the current metabolomics platform detected features that are potentially associated with exposure to TRP, given that the sampling protocol followed in this study included a commuting exposure. This conclusion should, however, be viewed cautiously as the utility of mass-matching of high-resolution masses is limited by the comprehensive capture of the annotation databases used. It may be possible that these features are truly unrelated to TRP exposures, and that HMDB has yet to identify these with as great reliability as other

compounds. Our semi-quantitative comparative approach for identifying metabolites of interest does not replace formal validation procedures, such as tandem MS; it does provide a relatively quick and inexpensive means of leveraging available information about the identity and sources of several thousand features.

One limitation of the semi-quantitative analysis is that the matches were identified strictly through a search of proton adducts to a parent compound ion. As our samples were diluted in acetonitrile, there is a high likelihood of acetonitrile, sodium, and potassium adducts, which complicate database annotation by providing many more matches to a single mass-to-charge ratio. Until tools are available to utilize data structure, correlations, and expectations of presence in various biological matrices, simple mass matching will continue to be a challenge for environmental metabolomics when environmental data is still poorly represented in available public datasets.

There are numerous caveats in interpreting the findings from this analysis**.** Most notably is the sample size, which was conducted on a non-random convenience sample of four subjects. Similar sample sizes, however, are not unprecedented in metabolomics analyses [67]. Although the mixed model results showed that the strength of the associations did not vary by subject, given the limited number of observations, it is not possible to generalize these findings to a broader population. To offset noise and potential confounding from uncontrolled factors associated with field-based observational designs involving multiple subjects, we elected to enroll four subjects, who were carefully monitored and followed during their participation in the study protocol. This degree of monitoring would not have been possible given a larger number of participants in a pilot study. Additionally, the purpose of the pilot study was to compare the metabolomic profiling of three distinct matrices, which requires, primarily, enough features measured in the participants. The use of apLCMS and xMSanalyzer produced measurements of 6,000 – 8,000 features from HRM, providing more than adequate power to detect differences among the matrices.

To further address the issues of sample size and generalizability to other study populations, we repeated a similar analysis, using an identical sample collection and metabolic analysis protocol, examining linear correlations between plasma and saliva metabolites in a cohort of 54 college-aged students. EBC was not collected as part of this specific protocol. In this analysis (results not shown), we found comparable correlations in the subset of highly-reproducible features (<10% CoV) (r_S = 0.59 vs. 0.64). While our results are still limited in generalizability to the participants in the study, the general concordance of the current pilot results to those shown in this larger cohort provides some external validation that the pilot study findings were not spurious.

Future work should contribute to the capture of spectral data of those compounds in the Master List of Mobile Source Air Toxics to public annotation databases. The degree of curation in resources such as the Human Metabolome Database provide an invaluable resource for metabolomic analysis, but still lack in compounds associated with or direct metabolites from environmental exposures. Nevertheless, the access to monoisotopic masses of the mobile source air toxics list enabled putative matching using a curated list of compounds known to be emitted from mobile sources. The detection of EPA-matched features in all matrices is encouraging for air pollution exposure science, but cannot be determined with computation alone. Only after definitive identification, through targeted MS/MS analysis, of compounds associated with traffic pollution exposure in each matrix can a determination be made on which of plasma, EBC, or saliva is best suited for exposure assessment while minimizing participant burden.

Collectively, we believe this pilot analysis efficiently demonstrated matrix comparability between plasma, saliva, and EBC and the putative detection of several features that may be associated with TRP. Our methodology was successful in retrieving thousands of features and was able to characterize robust, consistent metabolomic profiles across plasma, saliva and exhaled breath condensate. As metabolomic databases become better curated for

environmental health applications, analyses in studies such as this will presumably improve. Today, there is much ambiguity in putative matching by accurate mass with respect to mobile source air toxics. Detection and validation of these compounds in all three matrices will help towards understanding exposure and relevant biological pathways associated with traffic pollution toxicity.

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Tables and Figures

TABLE 1: Participant Characteristics and Metabolite Levels* from Plasma Metabolomics

	Pre-Exp	Comb. Post-Exp	Accardi et al.	HMDB
Female (%)	25	-	-	-
Age (Range)	29 - 46	-	-	-
Health Indicators				
Glucose (mM)	2.6 ± 0.02	2.5 ± 0.06	6.3 ± 3	3.9 - 6.1
Creatinine	76 ± 0.8	76 ± 0.8	85 ± 15	56 - 108.8
Amino Acids				
Phenylalanine	81 ± 0.6	78 ± 0.6	142 ± 22	48 - 189
Methionine	30 ± 0.5	30 ± 0.4	21 ± 10	22 - 46
Exogenous Chemical				
Benzoic acid	18 ± 0.3	19 ± 0.4	4 ± 0.6	8.1 - 145.3

*Metabolite matching from reference standardization. Values are presented as mean ± SD or ranges. All metabolite levels are in μM concentrations, unless noted otherwise.

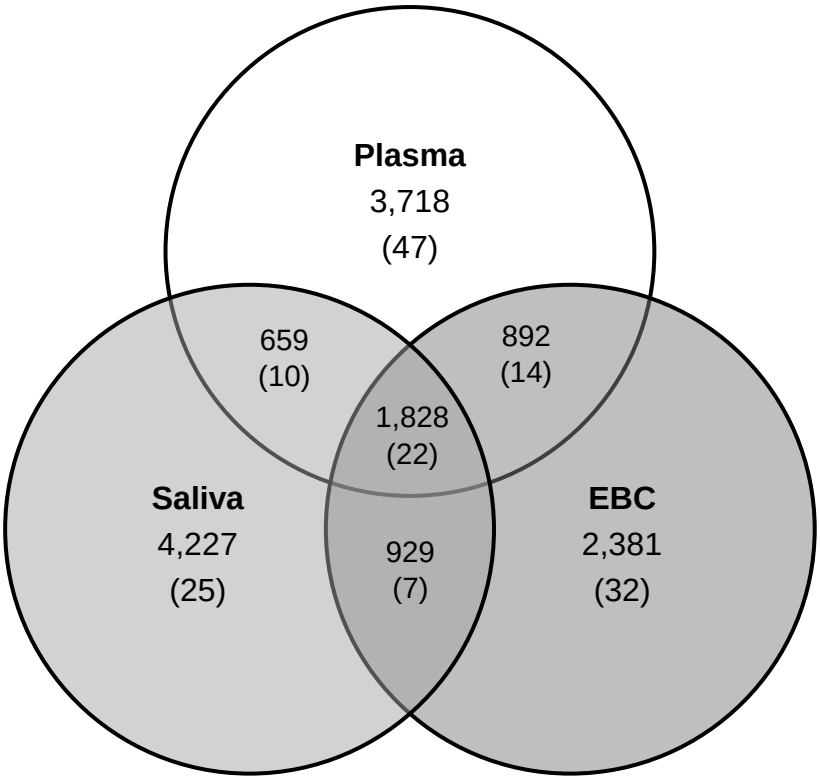

FIGURE 1: Venn Diagram of Feature Counts. Counts are from features detected at any sampling time. Features in one matrix were matched to features in another if they were within 10ppm of their respective m/z and within 30s of retention time. The numbers in the parentheses indicate the counts of features from the USEPA Master List of Mobile Source Air Toxics.

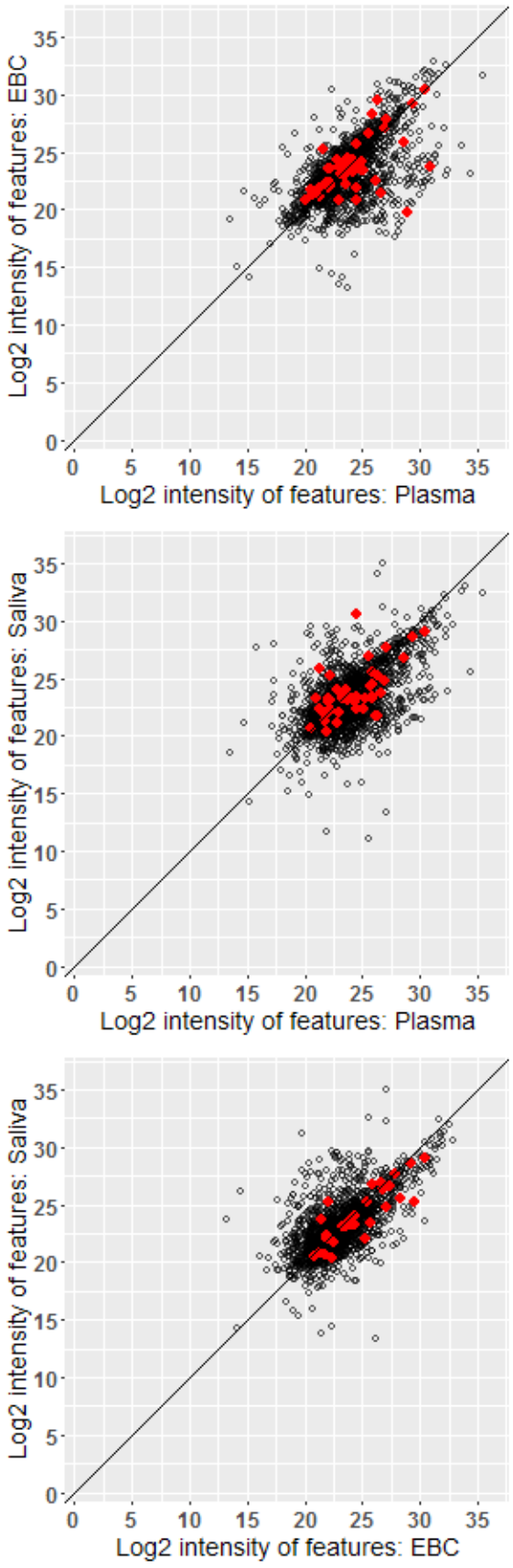

FIGURE 2: Scatterplots of Mean Intensities of Shared Features across Subjects between Matrix Pairs at the Pre-Exposure Sampling Time. Red dots indicate those features that also match the Master List of Mobile Source Air Toxics. Sample sizes (from top to bottom) range from 3,225, 3,085, and 3,255 features for Plasma vs. EBC, Plasma vs. Saliva, and EBC vs. Saliva, respectively.

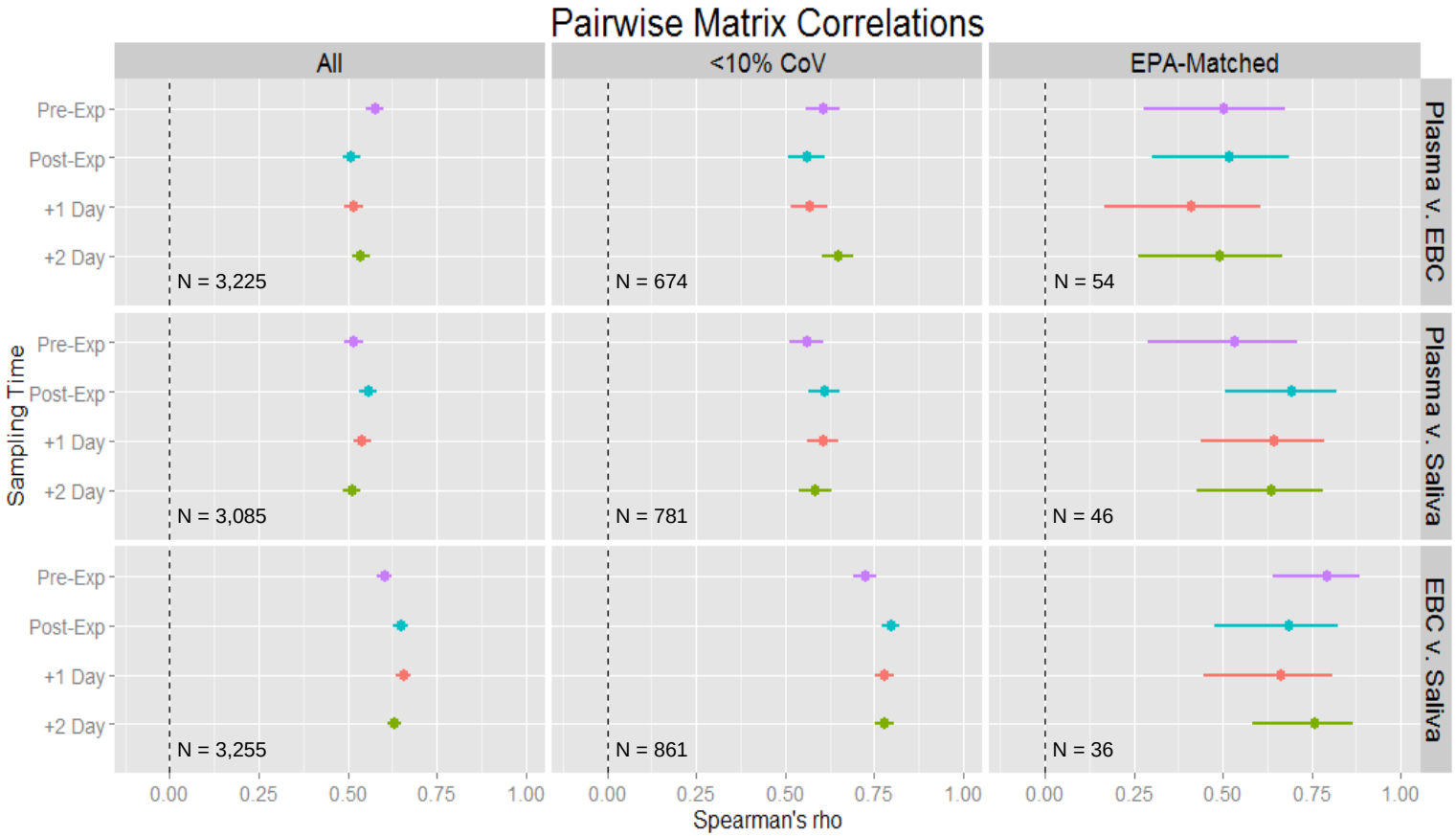

FIGURE 3: Estimates of the Spearman's Rank Correlations between Matrices Stratified by Time. Three sets of the data were considered in turn. The All set (far left) is the complete list of shared, reproducible features. The <10% CoV set (middle) is a subset of All with a very high standard for chemical measurement. The EPA-matched (far right) is the subset of features that match the Master List of Mobile Source Air Toxics. All feature matching here was within m/z of 10ppm and RT of 300s.

TABLE 2: Regression Estimates of Associations between Matrices, Controlling for Subject and Time in the EPA-Matched Subset

Model	*Y*	*X*	*N*	*β*	*SE*	*t-Statistic*	*p value*
1	EBC	Plasma	413	0.60	0.04	16.50	<0.001
2	Saliva	Plasma	318	0.77	0.04	21.42	<0.001
3	Saliva	EBC	240	0.74	0.04	19.57	<0.001

TABLE 3: Putative Annotation of EPA-Matched Features Shared in Plasma, EBC, and Saliva

	Plasma		*EBC*		*Saliva*						
#	m/z	RT	m/z	RT	m/z	RT	# HMDB	# EPA	Overlap	Putative EPA Compound Name/ Chemical Formula	Poss. TRP?
1	101.0714	63.7508	101.0715	63.6356	101.0715	55.4801	2	1	100%	n-nitrosopyrrolidine	N
2	**103.0871**	**40.9973**	**103.0871**	**65.5280**	**103.0871**	**41.0064**	**0**	**1**	**0%**	**n-nitrosodiethylamine**	**Y**
3	111.1174	566.4460	111.1174	567.9528	111.1174	551.2129	5	4	0%	C8H14/C9H20 (i.e., octyne)	N
4	115.0870	64.3534	115.0870	85.3724	115.0870	84.4289	2	1	0%	n-nitrosopiperidine	N
5	**117.0663**	**50.9270**	**117.0662**	**58.1568**	**117.0662**	**59.8724**	**2**	**1**	**0%**	**n-nitrosomorpholine**	**Y**
6	119.0495	92.0831	119.0495	74.8689	119.0495	92.1704	4	1	100%	2,3-benzofuran	N
7	121.0640	44.0034	121.0643	63.3738	121.0644	69.9346	8	7	100%	C8H8O (i.e., methylbenzaldehyde)	N
8	**122.9249**	**80.5810**	**122.9248**	**73.0977**	**122.9248**	**69.6379**	**0**	**1**	**0%**	**tin**	**Y**
9	123.0795	75.2479	123.0794	70.5669	123.0794	70.3230	11	3	66%	C8H10O (i.e., 2,3-dimethylphenol)	N
10	129.1277	490.6521	129.1277	462.2666	129.1277	489.3781	19	1	100%	octanal	N
11	149.1328	32.9848	149.1329	26.4474	149.1331	16.8234	3	12	17%	C11H16 (i.e., pentylbenzene)	N
12	151.1118	525.9616	151.1119	534.2908	151.1119	516.0162	28	1	0%	2,3,5,6-tetramethylphenol	N
13	154.8806	83.0172	154.8806	69.3365	154.8806	66.7111	0	1	0%	carbon tetrachloride	N
14	**185.0414**	**111.3406**	**185.0411**	**113.1992**	**185.0410**	**111.7597**	**0**	**1**	**0%**	**dibenzothiophene**	**Y**
15	201.1855	550.7727	201.1856	543.2826	201.1854	544.6137	13	1	100%	lauric acid	N
16	223.0639	531.9464	223.0640	514.3256	223.0640	526.9002	4	1	0%	hexamethylcyclotrisiloxane	N
17	**235.0572**	**116.1874**	**235.0585**	**101.9014**	**235.0571**	**111.0235**	**0**	**1**	**0%**	**benzonaphthothiophene**	**Y**
18	266.8493	66.2241	266.8495	79.2979	266.8493	69.4841	0	1	0%	pentachlorophenol	N
19	297.0828	561.6014	297.0832	560.7830	297.0827	560.1216	0	1	0%	octamethylcyclotetrasiloxane	N
20	**399.3951**	**33.7569**	**399.3953**	**37.7646**	**399.3954**	**35.3767**	**0**	**3**	**0%**	**C29H50 (i.e., 30-normoretane)**	**Y**

Chapter 4: In-Vehicle, Particulate Metal Exposures Induce Detectable Changes in the Plasma Metabolome in a Commuter Panel Study

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Abstract

Advances in liquid chromatography-mass spectrometry (LC-MS) have enabled high-resolution metabolomics (HRM) to emerge as a sensitive tool for measuring environmental exposures and corresponding biological response. Using measurements collected as part of a large, panel-based study of car commuters, the current analysis examines in-vehicle air pollution concentrations, targeted inflammatory biomarker levels, and metabolomic profiles to trace potential metabolic perturbations associated with on-road traffic exposures.

Methods: A 60-person panel of adults participated in a crossover study, where each participant conducted a highway commute and randomized to either a side-street commute or clinic exposure session. In addition to in-vehicle exposure characterizations, the participants contributed pre- and post-exposure dried blood spots for 2-hr changes in targeted proinflammatory and vascular injury biomarkers and 10-hr changes in the plasma metabolome. Samples were analyzed on a Thermo QExactive MS system in negative electrospray ionization (ESI) mode and resolution of 70,000 with C18 chromatography. Data were processed using apLCMS and xMSanalyzer on the R statistical platform.

Results: HRM detected 10-hr perturbations in 108 features associated with in-vehicle, particulate metal exposures (Al, Pb, and Fe) from a total of 14,341 features measured with a median coefficient of variation 13.5%. These features enriched on arachidonic acid, leukotriene, and tryptophan metabolism. 2-hr changes in proinflammatory biomarkers hs-CRP, IL-6, IL-8, and IL-1β were also associated with 10-hr changes in the plasma metabolome, suggesting diverse amino acid, leukotriene, and antioxidant metabolism effects. A putative match to 20-OH-LTB4 was decreased after in-vehicle exposure to particulate metals, suggesting a subclinical immune response.

Conclusions: HRM is a sensitive, powerful tool for studies of air pollution exposure, and found metabolomic changes associated with exposures to traffic related pollutants.

Introduction

Globally, source apportionment studies attribute 25% of urban ambient particulate matter less than 2.5 microns ($PM_{2.5}$) to traffic sources [1]. The risk of exposure has shown to be higher in car commuters [2]. Nearly ubiquitous, traffic pollution recently demonstrated its pervasive impacts on human health [3, 4] in addition to its well-documented exacerbations of cardiovascular [5, 6] and respiratory diseases [7, 8]. The primary components of traffic-related pollution (TRP) that drive these associations have not been consistent [9].

Mechanisms underlying the health effects of TRP exposures include oxidative stress and inflammation. Chronic cardiovascular diseases and acute respiratory distress for sensitive populations are TRP's most reproducible examples of health effects [10, 11]. The specific constituents of TRP and how they contribute to corresponding biological responses are not well understood. Here, a shift towards multipollutant measurements of exposure has recently demonstrated potential in capturing those aspects of TRP that are responsible for acute asthma distress and congestive heart failure [12] or mortality [13]. This suggests that more sensitive measures of exposure and response may help identify critical components of TRP and their complementary pathways impacting human health.

Metabolomic profiling may bolster detection of exposures to traffic air pollution and provide insight to potential mechanisms through which human biology responds. Rappaport, et. al., explored the potential of a "blood exposome"—the comprehensive quantification of small chemicals in blood. While mining the exposure science literature for food, drug, and endogenous metabolism, the authors captured environmental metals and chemicals at concentrations 1,000 times lower [14]. Metabolomics provides broad-spectrum measurement of human metabolism and has demonstrated utility in measuring environmental chemicals [15]. In air pollution exposures, metabolomic perturbations in urine were associated with polycyclic aromatic hydrocarbon (PAH) exposures [16]. The sensitivity of metabolomics demonstrates

promise to improve exposure assessment of TRP by providing a lower-cost, scalable measure of doses in human blood. High-resolution mass spectrometry, one analytical method for metabolomics, can measure hundreds to hundreds of thousands of signals of chemicals in a sample. Coupled with advanced bioinformatics methods, the method has demonstrated efficiency in measuring the exposome [15, 17].

Panel studies of commuting populations provide an exceptional platform to observe the acute effects of traffic pollution in humans using realistic exposures. This design can harbor the strength of high contrasting exposures [18] while minimizing participant risk to those conditions experienced in the real world [19]. Studies with quasi-experimental exposures have shown usefulness in detecting specific response to TRP [19-21]. By introducing repeated biological sampling, researchers can disentangle short term changes in key biological endpoints, such as inflammation and lung function [22] and potentially reveal new insights on TRP toxicity in humans.

The present analysis couples the strengths of a longitudinal panel of commuters and metabolomic profiling to detect effects of traffic exposure on human metabolism. The Atlanta Commuters Exposure study was a targeted examination of oxidative stress and inflammation for cardiovascular and respiratory outcomes resulting from on-road traffic exposures during morning rush hours in Atlanta, GA. In-vehicle exposures to $PM_{2.5}$, its components, and noise complemented a repeated measures design of biological sampling. This design accommodates the integration of untargeted metabolomic profiling with targeted biomarkers of effect. Here, we examine the associations of: 1) specific TRP to known markers of inflammation and lung function, 2) TRP with perturbations of the metabolome over the course of a day, and 3) markers of inflammation and lung function measured around traffic exposure to perturbations of the metabolome.

Methods

Study Design. The Atlanta Commuters Exposure study was a longitudinal panel of 60 participants conducted in Atlanta, GA from 2011 to 2013. The design, participant characteristics, and exclusion criteria are detailed previously in Golan, et. al., (2015). This analysis focuses on a subset of the population for which plasma samples were available. In brief, the study participants were measured for inflammatory and cardiorespiratory responses before and after distinct exposure scenarios. The three, 2-hr exposure scenarios were highway, side street, and indoor clinic environments during morning rush hours. Every participant was observed on two days, each a week apart. All participants conducted a highway exposure on one day and were randomized to either a side street or clinic exposure for the other day. The order of exposure scenario for an individual was also randomized. The present analysis was conducted on a subset of the study population where venous blood collection was available at both pre- and post-exposure time points for metabolomics analysis. The study was approved by Emory Institutional Review Board.

Exposure Assessment. Exposures were characterized by microenvironmental concentrations. In-vehicle and clinic pollutant sampling was conducted during the exposure period as described previously[23]. Continuous measures, such as $PM_{2.5}$ mass and black carbon concentrations, were captured with high-temporal resolution instrumentation housed in a sampling apparatus located in the passenger seat or clinic room during sampling periods. Fine particulate matter with an aerodynamic diameter less than 2.5 microns ($PM_{2.5}$) and its components were the primary focus of the study. Continuous measurements included $PM_{2.5}$ mass, black carbon (BC), particle number concentration (PNC), particle bound polycyclic aromatic hydrocarbons (pb-PAHs), and noise. The mean 1-minute concentration of each TRP within a sampling period was used to represent exposure. Concentrations of $PM_{2.5}$ components included select elements—Al, Pb, and Fe—and total carbon fractions—organic carbon (OC) and

water-soluble organic carbon (WSOC). These were captured using quartz or Teflon filters and integrated over the exposure period. Elemental concentrations (pg/mL) and carbon fraction concentrations ($\mu g/m^3$) were measured using ICP-MS and TD-GC-MS, respectively. Pollutants were chosen for the present analysis based upon previously demonstrated associations with traffic or mobile source emissions [24].

Biological Sampling. Sampling and targeted biomarker analysis are outlined in detail in Golan, et. al. (2015). In short, a panel of inflammatory and oxidative stress markers was collected at multiple time points before and after each commute. Respiratory markers exhaled nitric oxide (eNO), forced expiratory velocity (FEV_1) and blood-based markers were collected concurrently at both pre-exposure (7AM) and post-exposure (9AM). Dried blood spots were analyzed for high-sensitive C-reactive protein (hs-CRP), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), soluble intercellular adhesion molecule (sICAM) and soluble vascular cell adhesion molecule (sVCAM) using multiplexed inflammatory and vascular injury panels (Luminex). eNO was measured using a NIOX Mino (Circassia) and FEV_1 was measured using a handheld spirometer. The present analysis focuses on the differences between post- (9AM) and pre-exposure (7AM) measurements of these respiratory and inflammatory markers (ΔBiomarkers).

Whole, venous blood was collected pre-commute (7AM) and post-commute (6PM). Blood was collected from the arm, usually the cubital vein, into purple topped containers (containing ethylenediaminetetraacetic acid). Samples were spun and plasma supernatants were aliquoted immediately after collection. All plasma was stored at -80°C.

High-Resolution Metabolomics. Metabolomics was completed using established methods[25]. Briefly, plasma samples were diluted two-fold with acetonitrile and analyzed in triplicate using a dual-chromatography, high-resolution mass spectrometry system (Dionex Ultimate 3000; ThermoScientific QExactive). Analyte separation was accomplished using reverse-phase C_{18} liquid chromatography (Targa C_{18} 2.1mm x 100mm x 2.6µm, Higgins

Analytical) with mass spectral detection completed in positive and negative mode electrospray ionization at 70,000 (FHWM) resolution over a mass-to-charge ratio (m/z) range of 85 to 1250. For quality control, all sample batches included two replicates of a pooled reference material. NIST SRM 1950 was also included at the beginning and end of all study samples. Concentrations of select metabolites were determined by reference standardization using the positive mode data only [26].

Raw data files were processed for feature extraction and quality control in R using the hybrid mode of adaptive processing of liquid chromatography mass spectrometry ('apLCMS') and subsequently modified by 'xMSanalyzer' to generate the final feature tables used for analysis. A feature is a unique ion identified by its m/Z, retention time (RT) and intensity. Feature extraction followed a modified protocol described previously in Uppal, et. al. (2013) and in Chapter 2 with a modification of the known feature table used for 'apLCMS' hybrid mode. The known feature table provided to apLCMS included only those metabolites detected in blood from the Human Metabolome Database v. 3.6 and a list of 1,209 compounds from the Environmental Protection Agency's Mobile Source Air Toxics Inventory (HMDB and EPA). This algorithm was run separately for each of the positive and negative mode raw spectrograms with respective lists of ion adducts optimized for the metabolomics platform. Features included in the final tables for downstream analysis included only those features that were reproduced over two distinct iterations of apLCMS and with median coefficients of variation across technical triplicates less than 30% and prevalence in at least 10% of the biological samples.

Statistical Analysis. Overall, the strategy was two-pronged—focusing first on environmental associations with changes in 1) targeted biomarkers and 2) plasma metabolome, and then on 3) targeted biomarker associations with the plasma metabolome. The approach comprised feature selection by metabolome wide association (MWAS) and human metabolic pathway enrichment of significant features. Metabolome-wide associations were examined, in turn, for each exposure ($Exposure_{ij}$) or change in targeted biomarker ($\Delta Biomarker_{ij}$) using linear

mixed models. The rationale for the two-pronged approach arose from the breadth of information available to the researchers from the ACE population and a need in the field to compare hypothesis-driven, targeted measures of response to hypothesis-free, untargeted measures of response.

All statistical analysis was conducted on the R Statistical Platform (v. 3.3.1) using the packages 'xMSanalyzer,' 'apLCMS,' and 'limma' [27-29]. Associations between TRP and targeted biomarkers followed Golan, et. al., 2017, using mixed models to allow for subject variability and control for baseline targeted biomarker levels. The feature tables generated from metabolomics preprocessing, with log2-transformed intensities, were analyzed using the R statistical package 'limma.' The structural similarity of metabolomics data to microarray data from RNA sequencing analyses permitted ready translation of data input and functions for this analysis plan. 'Limma' was chosen for its ability to run simple mixed effects models with computational efficiency, adoption and acceptance in microarray analysis, and moderation of t-statistics using a simple empirical Bayes method [27].

The analytical strategy was sensitive to the repeated measures study design. Linear mixed models were used to explore these associations separately for each exposure or biomarker of interest within each ion mode. Categorical variables controlled for effects of asthma diagnosis, age, sex, body mass index (BMI), and race. The first model focused on associations between changes from pre- to post-exposure metabolite intensities ($\Delta Feature_{ij}$) and exposure measures ($Exposure_{ij}$) (Model 1). Directly measured pollutants, $PM_{2.5}$, OC, BC, WSOC, pb-PAHs, PNC, noise, Al, Fe, and Pb, were examined, in turn, as continuous variables.

(1) $$\Delta Feature_{ij} = \mu + \theta_i + \beta_1 Exposure_{ij} + \beta_2 Asthma_i + \beta_3 Age_i + \beta_4 Sex_i + \beta_5 BMI_i + \beta_6 Race_i + \varepsilon_{ij},$$

where *j* indexes commute day, *i* indexes subject, $\theta_i \sim N(0,\pi), \varepsilon_{ij} \sim N(0,\sigma^2), and\ \pi = \left(\frac{\sum_1^k \tau^2}{k}\right)_{15\%}$ – the trimmed mean of intra-individual variability.

The second model focused on the association between changes in pre- and post-exposure feature intensities and pre- and post-exposure changes in selected, targeted biomarkers of inflammation and vascular injury (Model 2). The pre- to post-exposure change, ΔBiomarker, was calculated for eNO, FEV_1, hs-CRP, TNFα, IL-1β, IL-6, IL-8, sICAM, and sVCAM.

(2) $\Delta Feature_{ij} = \mu + \theta_i + \beta_1 \Delta Biomarker_{ij} + \beta_2 Asthma_i + \beta_3 Age_i + \beta_4 Sex_i + \beta_5 BMI_i + \beta_6 Race_i + \varepsilon_{ij},$

where indexing of variables and errors are as described for Model 1.

Both models were run in a metabolome-wide association study, generating a *p*-value and moderated t-statistic for each change in intensity ($\Delta Feature_{ij}$). The *p*-values were adjusted for multiple comparisons using the Benjamini-Hotchberg false discovery rate ($FDR_{B\text{-}H}$) with significance met at $FDR_{B\text{-}H} < 0.05$ [30]. These were visualized using Manhattan plots of the $-\log_{10}(p)$ over feature retention time with the average direction of change between post- and pre-exposure metabolite profiles indicated by color.

Human Metabolic Pathway Enrichment and Putative Feature Identification. Pathway analysis was conducted using mummichog (v. 1.0.7) separately for each set of features from the linear mixed models and for the negative ionization mode [31]. The present study focused on the negative ion mode. Lists of significant features from MWAS were identified with an $FDR_{B\text{-}H} < 0.05$ and further restricted for peak quality by extracted ion chromatograph (EIC) filtering. The human reference pathway for mapping came from MetaFishNet, a compilation of KEGG, Edinburgh Human Metabolism Network, UCSD BiGG, and BioCyC network metabolism models.

Matches to the pathways were made within 10 ppm to measured feature m/Z ratios. Pathways were considered strong candidates if at least 3 nodes from the experimental data overlapped with pathway nodes and the mummichog permutation-based enrichment score, s, was less than 0.10. These conditions are more conservative but comparable to previously published studies using these tools [32]. Human metabolic pathway enrichment results were compared within and across exposure and biomarker set lists for overlapping pathway enrichments.

Results

Of 60 total participants in the ACE study, 49 provided venous blood over 73 different sampling days. Sixty-nine of those 73 sampling days were complete samplings, meaning blood was collected both pre- and post-exposure for the individual on a given sampling day. This subpopulation formed the final analytical subset, for which matching respiratory, inflammatory, and oxidative stress markers were available. Population characteristics and exposures are summarized in tables (Table1 and Table 2, respectively). With TRP exposures, highway and non-highway commutes differed significantly in several particulate pollutants and noise, but not particulate metals or WSOC.

Plasma metabolomics yielded 14,341 negative features with median coefficients of variation (CoV) across triplicates < 30% and detected in at least 10% of the samples. The median CoV was 13.5%. Many targeted positive features were annotated, using reference standardization, and had concentrations within reported ranges at the 7AM baseline (Table 3). Some amino acids were above or below reported ranges in HMDB. The study population was lower in asparagine, citrulline, glutamine, leucine/isoleucine, and tryptophan while glutamate and proline were higher. Plasma concentrations of 8 amino acids and carnitine differed between asthmatics and non-asthmatics.

Traffic-related pollutants have few, negative associations with changes in inflammatory cytokines. Associations between TRP and ΔBiomarkers were largely null (Table 4). Pb was

associated with Δhs-CRP (β = -20.1%; $p < 0.005$) and ΔsICAM (β = -24.2%; $p < 0.001$) indicating on average 22% reductions in the acute phase protein and intercellular adhesion molecule-1 per 1 ng/m^3 increase in exposure. Much smaller, but significant, reductions in ΔIL-8 were observed with increases in pb-PAH, PNC, and noise. ΔIL-6 had no significant associations with any exposure measure used, but was poorly measured with much of the distribution below detection (n = 32).

MWAS of environmental exposures highlight associations with certain metal species. Measures of TRP were generally not associated with $\Delta Feature_{ij}$. Of these, only the concentrations of elemental metals Al, Fe, and Pb were associated ($FDR_{B\text{-}H} < 0.05$) with changes in the metabolomes of the study population (Figure 1).

The MWAS for elemental metal exposures identified 11, 6, and 91 features significantly associated ($FDR_{B\text{-}H} < 0.05$) with Al, Fe, and Pb, respectively (Figure 1). Overall, the average change in concentrations were decreasing from baseline for all metal-associated features. Moving from a feature space signified by measured m/Z to annotated compounds by mass-matching, a putative pathway analysis was possible (Figure 2). Runs of mummichog succeeded only for Al-associated and Pb-associated features, likely due to m/Z not matching calculated masses within 10ppm on the metabolic model. Al-associated features enriched for arachidonic acid and leukotriene biosynthesis. 20-OH-leukotriene B4 (molecular weight: 352.225 Da) was a top predicted metabolite and was, on average, decreasing in intensity in the population over time,

MWAS of targeted biomarkers support associations with proinflammatory cytokines and hs-CRP. The targeted biomarkers in this study included lung oxidative stress, systemic inflammation, and vascular injury markers. Prior to the pathway analysis, ΔBiomarkers were examined for correlations with significant features from the combined set of exposure-associated features from MWAS. No significant correlations were observed after multiple hypothesis test correction. Changes in proinflammatory cytokines and acute-phase hs-CRP, but

not respiratory or lung oxidative stress markers, were associated with changes in feature intensities before and after exposure periods (Supplementary Figure 3). Interleukin-6 and hs-CRP had 60 and 47 significant features across both ionization modes, respectively. Metabolome-wide associations with Interleukin-1β and IL-8 revealed 47 and 53 significant features, respectively. For both pairs, IL-6/hs-CRP and IL-1β/IL-8, there was substantial overlap of features within pairs (Supplementary Figure 2). Pathway analysis revealed broader enrichment of features onto pathways of amino acid metabolism, leukotriene metabolism, and ubiquinone biosynthesis.

Overall, significant results are summarized schematically (Figure 3). Overlap between Exposure-associated and ΔBiomarkers-associated MWAS was separated by feature and pathway comparisons, showing very few shared features and only one shared, enriched pathway.

Discussion

Ascertaining the primary components of TRP that induce the milieu of health response has been challenging without tools to capture both exposure and response cost-effectively in population studies. High-resolution metabolomics of blood has demonstrated a broad capture of human metabolism and exposure [17], and may detect specific response in observational human studies [33]. In the present study, we augmented exposure characterization of an in-vehicle environment and targeted biomarkers of oxidative stress and inflammation in road commuters with HRM of plasma. Particulate metal exposures, especially lead, showed 2-hr associations with inflammatory markers (hs-CRP and sICAM) and 10-hr associations with metabolic perturbations. To our knowledge, this is the first indication of a directly measured environmental traffic pollutant associated with changes in human metabolomic profiles. Collectively, across tested exposures and targeted biomarkers, perturbations of human metabolism mapped onto inflammatory responses [34], antioxidant metabolism [35], and amino

acid metabolism [36]. As a proof-of-concept, our data demonstrate HRM to be sensitive to changes in metabolism resulting from traffic-related pollution exposure.

Our study differed from existing air pollution metabolomics studies by observing changes over time to realistic exposures in a human population. The primary aims of the ACE study were to assess in-vehicle particulate matter exposures and examine associations of exposure with select biomarkers indicative of oxidative stress, inflammation, and cardiorespiratory health [23]. The ACE study was designed to minimize the impacts of exposure measurement error from population based studies and capture time-trends in biomarkers. Repeated measures and contrasting exposures within study participants was a strength where changes in measurements over time can be explored with partitioning of inter- and intra-individual variability [37]. Our longitudinal data structure was an improvement over cross-sectional analyses for causal inference [16] and provided high-resolution, speciated microenvironmental exposure assessment.

Nevertheless, associations between TRP exposure and changes in targeted biomarkers (ΔBiomarkers) were sparse and not consistent between the entire ACE population and the subset for which metabolomics data were available (results not shown). In the total ACE population, OC was the only pollutant associated with biomarkers in dried blood spots (Δhs-CRP and ΔsVCAM). This association was not reproduced in the metabolomics subset. Other pollutants (pb-PAH, PNC, noise, and lead) were associated with ΔIL-8, ΔsICAM, Δhs-CRP, and ΔTNFα in the metabolomics subset and not in the total ACE population. This discrepancy is indicative of the challenges faced in air pollution studies focusing on hypothesized, targeted biomarkers of effect on inflammatory and oxidative stress pathways [9, 38]. Despite these differences within our data, the addition of HRM with the use of mixed models enhanced potential insights by measuring changes in the human plasma metabolome (ΔFeature) at relevant time scales and capturing markers used in air pollution studies.

Overall, our data suggested that HRM was sensitive to changes in metabolomes with particulate metal TRP exposures and changes in specific inflammatory biomarkers. We also showed that, while few features associated with exposures overlap with ΔBiomarkers-associated features, the potential metabolic pathways these features enrich for are either shared or complementary along inflammatory and redox reactions (Figure 3). Three key insights arose from these data: 1) either metal-induced or sources implied by particulate metal exposures from a morning commute are associated with 10-hr changes in the plasma metabolome suggesting a role for oxidative potential; 2) functional analysis of significant features shows, overall, 2-hr changes of inflammatory cytokines and acute-phase protein are associated with features enriching on antioxidant pathways; and 3) that HRM was a useful tool in capturing the effects of traffic exposure provided multiple biological measures over time and fine measures of exposure.

The repeated associations across particulate metal exposures from MWAS suggest covariance between these metals in the traffic-related exposures. It is plausible that these are tracers for a traffic-related dust source, such as resuspended soil, road dust, or brake wear. A previous commuter study by our group in Atlanta proposed three potential source factors of in-vehicle PM exposure through a factor analysis: resuspended road dust with contribution of fuel combustion, incomplete gasoline fuel combustion, and brake pad and tire wear [39]. Source apportionment of fine particulate matter in Atlanta, GA reported enrichment of these metals in road dust factors, including Al and Fe [40, 41]. While source apportionment of the in-vehicle environments of the ACE commuting population is pending, Fang and authors examined the water-soluble components of PM filters collected concurrently in Atlanta for their oxidative potential [42]. Fe correlated ($r > 0.65$) consistently with oxidative potential as measured by dithiothreitol activity over a year-long sampling. Together, these support both the presence of elemental metals in a roadway commuting environment and the potential to elicit biological response through oxidative stress.

Particulate metals have been identified as contributors to cardiovascular disease morbidity [43] and mortality [44]. Transition metals, such as Fe, are redox-active—where the metal ion can serve as both electron acceptor and donor in reactions to generate radical ions. Metals, both redox-active and redox-inactive, can promote oxidative stress through redox cycling or quenching antioxidant capacity [45]. These toxic processes driven by metal exposures are found to be induced by near roadway PM. Pardo, et. al., demonstrated that PM extracts from a roadside monitor in London and a simulated metal solution, including Al, Fe, and Pb, induced increases in IL-6 and TNFα in murine bronchoalveolar fluid over a 24-hour period [46]. In this model, inflammatory biomarker levels returned to baseline over a 48-hr period. In contrast, ΔTNFα and ΔIL-6 were not associated with any metal exposures in our commuting population. However, Pb was associated with Δhs-CRP and ΔsICAM. For each unit increase in Pb exposure, the percent change in these biomarkers averaged a 22% decrease over a 2-hr period. A decrease in proinflammatory markers from baseline after Pb exposure is contrary to expectation [47, 48]. However, there is the potential confluence of circadian patterns inflammatory markers and rapid resolution of inflammatory signaling [49] that may result in our observed depression. In an experimental mouse model, acute instillation of urban air particles (at 1mg/kg) resulted in 3-hr increases in TNF-α and IL-6 that resolved before the subsequent 24-hr measurement. Our findings may be an initial indication of higher concentrations of metal exposures, specifically Pb, driving proinflammatory responses to resolution at a 2-hr scale as measured by dried blood spots, which was echoed in the results of our metabolomics analyses.

The MWAS and pathway enrichments combined suggest that a morning exposure to particulate metals result in detectable perturbations in leukotriene, arachidonic acid, and tryptophan metabolism over a 10-hr period. Enrichment of features on tryptophan metabolism for Pb exposures is consistent with the evidence of lead, although redox-inactive, participating in the depletion of antioxidants [45, 50]. While Al is redox-inactive, it does demonstrate the ability to shift biological systems into oxidative stress [51]. Of Al-associated features, two (m/Z:

350.2105 Da and 350.2102 Da) drive the enrichment of leukotriene and arachidonic acid pathways and suggest an average decrease of these features across the study population after controlling for confounders and asthma status over the 10-hr period. These two features match a metabolite of proinflammatory chemoattractant leukotriene B4 (LTB4): 20-OH-LTB4. IL-6-associated features also enriched for leukotriene pathways, providing an initial concordance with the exposure-based enrichments. Metabolites of LTB4, 20-OH-LTB4 and 12-oxo-10,11-dihydro-20-COOH-LTB4, were putatively identified in mummichog.

Leukotrienes are important lipid mediators in inflammation response and are specific targets for the management of asthma (Figure 4) [52, 53]. Briefly, cleaved phospholipids from cellular membranes are metabolized into arachidonic acid, a versatile molecule that participates in prostaglandin, leukotriene, and other phospholipid biosynthesis. Leukotriene B4, created through reactions catalyzed by 5-lipoxygenase (ALOX5) and leukotriene A4 hydrolase, is preferentially created in neutrophils and alveolar macrophages leading to enhancement of endothelial attachment and chemotaxis of additional cells. Inactivation of LTB4 begins with its hydroxylation by cytochrome P450 enzymes: CYP4F2, CYP4F3, and CYP4F12. Putatively identified 20-OH-LTB4 was associated with both Al exposures and ΔIL-6 in our metabolomics population. The hydroxylated LTB4 is further oxidized for an irreversible inactivation of the leukotriene which is subject to further beta-oxidation. Δhs-CRP-associated features had substantial overlap with ΔIL-6-associated features (Supplementary Figure 2), and enriched for leukotriene metabolism, but not with at least 3 features mapping onto the pathway. Together, these results implicate inactivated leukotrienes in various forms as being responsive to TRP exposure.

Leukotriene B4 in breath has been associated with the inflammatory response to traffic exposures [34]. The authors showed long-term traffic exposures, assessed by land use regression models estimating exposure to $PM_{2.5}$, were associated with increased LTB4 measured in induced sputum (2μg/m^3 $PM_{2.5}$: ~23% $CI_{95\%}$(4%-42%) LTB4). Some attention has

grown for leukotrienes in response to TRP exposures. Rabinovitch, et al., (2016) recently reported increases (24% $CI_{95\%}$(1.5%,51.5%)) in urinary cysteinyl leukotriene (LTE4) after very short term exposures to $PM_{2.5} \geq 5\mu g/m^3$ in asthmatic children. The cysteinyl leukotrienes, LTC4, LTD4, and LTE4, are created from the enzymatic reactions of LTA4 and glutathione (Figure 4). These leukotrienes operate extracellularly, binding to receptors of neighboring cells and promote vascular permeability [54].

The strengths and limitations of our study and analyses provide the opportunity to discuss lessons learned. Metabolic profiles are subject to polymorphisms across several known dimensions. Fortunately, a thorough review of the current science in understanding the impacts of diurnal variation, sex, age, disease, diet, and geography exists [15]. We controlled, at least in part, for diurnal variation, sex, age, asthma status, obesity, and Metro Atlanta residency. The clustered design of the ACE study allowed for using linear mixed models in an exposure assessment setting [55] to control for the effects of many determinants of metabolic phenotypes at the stage of feature selection or MWAS. Thus, subsequent pathway analyses have a higher confidence of being associated with the primary exposures of interest at the expense of external validity. The ACE study, while capturing self-reported food intake over the study days, did not explicitly control diet (except for the consumption of nitrate rich foods and leafy greens). Diet quality, in small part, may be influenced by race. Hiza et al. (2013) reported Hispanics chose healthier diets than whites or blacks from the 2003-2004 National Health and Nutrition Examination Survey. Our study population had a negligible number of Hispanic persons, but a large Asian population (20%). Including race in our models with BMI may have captured some of the variance attributable dietary choices. A future study may consider including targeted measures or including standards of common foods that are known to promote proinflammatory responses in humans [56].

We chose to be especially stringent in our standards for statistical significance in the present analysis compared to typical exploratory metabolomics analyses. As a first step to using

HRM in air pollution panel studies, our motivation was to find the strongest evidence of an environmental exposure predicting changes in metabolomes. At the MWAS stage, features were significant if the FDR < 0.05 and the EICs of peak were of reasonable quality. For pathway enrichment, we again restricted our examination to pathways with enrichment scores, s, < 0.10. Our approach quickly reduced complexity and identified only the strongest associations. It likely also removed consideration of the greater breadth of human response to TRP exposure. Relaxing FDR to < 0.20 would not have changed the results of our MWAS findings, except for the addition of 2 single features across BC and WSOC exposures. However, relaxing the FDR of ΔBiomarkers models would have greatly expanded pathways to consider, at the expense of specificity to traffic exposure. Only three variables representing particulate metal exposures were associated with metabolic perturbations. Other TRP parameters that are widely used, such as $PM_{2.5}$ mass and PNC, demonstrated no associations. We also explored categorical exposures using the contrasts in our study design (data not shown), and found no significant associations even up to FDR < 0.20. The availability of rich exposure characterization, that included metal content, at the microenvironmental level aided in our discovery, but also tells of the sampling and characterization involvement required in a human observation study.

Finally, we would remind investigators of the interpretive challenges in an untargeted, HRM analysis from matching features m/Z onto online databases of metabolites. Measured features are typically matched by m/Z alone to annotation and network databases. This results in 'many-to-many' matches of features to compounds in the databases. For example, 2,716 features, less than 20% of the total supplied to mummichog (n = 14,282) for Pb-based enrichment, matched 7,309 compounds on the human metabolic network. This expansion is then reduced by network analysis, vastly improving interpretability [31]. Nevertheless, there remains a large proportion of unknown metabolites that are changing in response to Pb exposure. Our data, like others at the stage of pathway enrichment, provide strong suggestions of pathway level perturbations, but require further study. In the absence of definitive feature

identification, correlation analysis may provide additional insight to the identification of important compounds [32]. We explored the correlations of the putative 20-OH-LTB4 features with the remainder of the measured metabolome and clinical changes in neutrophils or eosinophils from complete blood counts in our population, and found no significant associations (results not shown). Either definitive identification of specific features using MS/MS or reliable reference standards can improve certainty of our results. Thus, the interpretation of pathway enrichments on metabolomic data alone is not sufficient to implicate altered metabolism, but is necessary to direct further study.

While there is support in the air pollution literature of a metal-associated response in humans, its detection using metabolomic profiling around traffic related exposures needs to be replicated. The general inconsistencies of inflammatory markers and their associations with certain $PM_{2.5}$ components require studies to be reproduced to provide robust assessments of TRP toxicity in humans. We demonstrated that HRM was a sensitive tool when used within a semi-experimental study of human exposure. Also, our environmental measures go beyond standard air pollution sampling practices used in large population studies. These advantages in study design and data capture enhanced our ability to capture changes in human metabolism with respect to TRP exposure. For the purposes of bioeffect screening, HRM of plasma was useful in providing interpretable results. Further study should improve upon repeated sampling of plasma with shorter windows and multi-day sampling after single exposures in a human population. If our metabolomic sampling of plasma echoed the sampling of dried blood spots—used for targeted biomarkers in this study—then perhaps pro- and anti-inflammatory processes associated with TRP can be tracked over time. Finally, while much of air pollution toxicology research focuses on proinflammatory markers of response, we show cause to study pro-resolving mediators as well. For example, targeting eicosanoids, specifically LTB4, in a future iteration of a traffic exposure metabolomics study may improve our understanding of the interplay of systemic inflammation in acute response.

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Tables and Figures

Table 1: Population Health Characteristics

Participant Characteristics	
N	49
Age in years	26 (5)
Female	47%
Race	
White	64%
Asian	20%
Other	16%
Health Status	
BMI ($kg \cdot m^2$)	23.15 (3.53)
Asthma Diagnosed	53%

Values are Mean (SD), unless noted otherwise

* indicates significant difference between means with $p < 0.05$

Table 2: Mean In-Vehicle Exposures by Commute Type

Exposure Characteristics	
Commute Type (N)	
Highway	36
Non-Highway	37
$PM_{2.5}$ ($\mu g \cdot m^{-3}$) *	
Highway	17.14 (6.18)
Non-Highway	11.18 (8.58)
BC ($\mu g \cdot m^{-3}$) *	
Highway	5.33 (2.23)
Non-Highway	1.58 (1.43)
OC ($\mu g \cdot m^{-3}$) *	
Highway	7.66 (1.98)
Non-Highway	6.07 (1.70)
WSOC ($\mu g \cdot m^{-3}$)	
Highway	8.48 (3.75)
Non-Highway	7.95 (3.45)
PNC ($\# \cdot m^{-3}$) *	
Highway	34,808 (12,918)
Non-Highway	10,649 (8,147)
pb-PAH ($\mu g \cdot m^{-3}$) *	
Highway	113.93 (30.51)
Non-Highway	65.21 (38.94)
Noise (dBA) *	
Highway	68.59 (2.73)
Non-Highway	58.40 (11.23)
Aluminum (Al) ($ng \cdot m^{-3}$)	
Highway	29.36 (28.67)
Non-Highway	24.37 (19.71)
Iron (Fe) ($ng \cdot m^{-3}$)	
Highway	176.33 (171.19)
Non-Highway	121.55 (115.99)
Lead (Pb) ($ng \cdot m^{-3}$)	
Highway	0.45 (0.45)
Non-Highway	0.92 (1.64)

Mean (SD), unless noted otherwise; * denotes $p < 0.05$ for Student's *t* test

Table 3: Select Metabolite Levels at Baseline for Commutes

	Non-Asthmatics			Asthmatics		
Metabolite	*N*	*Mean*	*SD*	*N*	*Mean*	*SD*
Amino Acid						
Alanine *	34	446.49	(7.19)	39	441.97	(7.58)
Arginine *	34	87.31	(1.67)	39	85.44	(1.91)
Asparagine *	34	7.13	(0.11)	39	7.06	(0.12)
Citrulline	34	10.41	(0.17)	39	10.32	(0.22)
Glutamate	34	157.91	(4.80)	39	155.89	(7.26)
Glutamine	34	61.70	(0.59)	39	61.36	(0.73)
Histidine *	34	124.82	(1.37)	39	123.72	(1.22)
Isoleucine/Leucine	34	122.44	(1.62)	39	121.53	(1.82)
Lysine *	34	348.41	(4.60)	39	344.13	(5.41)
Methionine *	34	29.79	(0.44)	39	29.50	(0.47)
Phenylalanine *	34	79.83	(1.57)	39	78.24	(2.85)
Proline	34	278.95	(5.95)	39	275.61	(7.67)
Serine	34	93.03	(1.41)	39	92.64	(1.27)
Threonine	34	123.40	(2.12)	39	122.89	(2.27)
Tryptophan	34	33.62	(0.42)	39	33.34	(0.58)
Tyrosine *	34	61.80	(0.94)	39	61.12	(1.07)
Amino Acid Metabolites						
Kynurenine	34	2.01	(0.03)	39	2.00	(0.04)
Oxoproline	34	35.48	(0.47)	39	35.19	(0.80)
Exogenous Chemical						
Caffeine	34	74.26	(3.49)	39	73.45	(3.54)
FA Metabolism						
a-Linolenic acid	34	106.98	(2.21)	39	107.11	(2.75)
Acetyl-carnitine	34	1.78	(0.08)	36	1.77	(0.11)
Carnitine *	34	27.98	(0.70)	39	27.12	(1.35)
Linoleic acid	34	1137.79	(12.23)	39	1138.04	(14.74)
Health Indicators						
Cholesterol	3	1546.00	(104.16)	2	1432.60	(41.03)
Creatine	33	37.06	(1.89)	38	36.52	(1.20)
Creatinine	34	72.86	(0.95)	39	72.98	(0.77)
Lipid Metabolism						
Choline	34	1.26	(0.01)	39	1.27	(0.02)
Sphinganine	13	0.03	(0.00)	20	0.03	(0.00)

Values are Mean (SD) in µM; Sample sizes (N) represent samplings, not individual participants; * denotes statistical significance with FDR < 0.05 between Asthmatics and Non-Asthmatics; FA = Fatty Acid

Table 4: Percent Change in Targeted Biomarker per Unit of Exposure

	Δhs-CRP	**ΔTNFα**	**ΔIL1β**	**ΔIL6**	**ΔIL8**	**ΔsICAM**	**ΔsVCAM**	**ΔeNO**	**ΔFEV1**
BC	0.87	-2.09	-1.72	-2.18	-2.10	-0.08	0.50	-0.32	-0.32
OC	4.97	-1.53	-8.62	-6.35	*-3.21*	9.50	9.98	-2.02	-0.47
WSOC	1.88	2.29	-0.57	--	0.05	1.36	2.64	-0.27	0.12
pb-PAH	-0.10	-0.04	-0.16	-0.46	**-0.23***	-0.03	0.07	-0.06	-0.02
PNC	0.50	-0.08	<0.01	-0. 78	**-0.55***	0.58	0.61	-0.20	**<-0.01***
Noise	-0.63	**-0.77***	-1.07	1.21	**-0.80***	-0.85	-0.54	-0.14	-0.02
Al	-0.05	-0.13	0.42	0.37	0.07	-0.30	-0.23	0.05	-0.02
Fe	-0.03	-0.04	0.01	-0.09	-0.01	-0.04	-0.02	<-0.01	-0.01
Pb	**-20.14***	0.84	-7.13	-5.15	0.81	**-24.21***	-31.09	-0.16	0.07

* Indicates p-value significant at $\alpha < 0.05$; -- indicates a model that did not converge;
PNC % change reflect change in concentration in thousands (1,000s) of particles

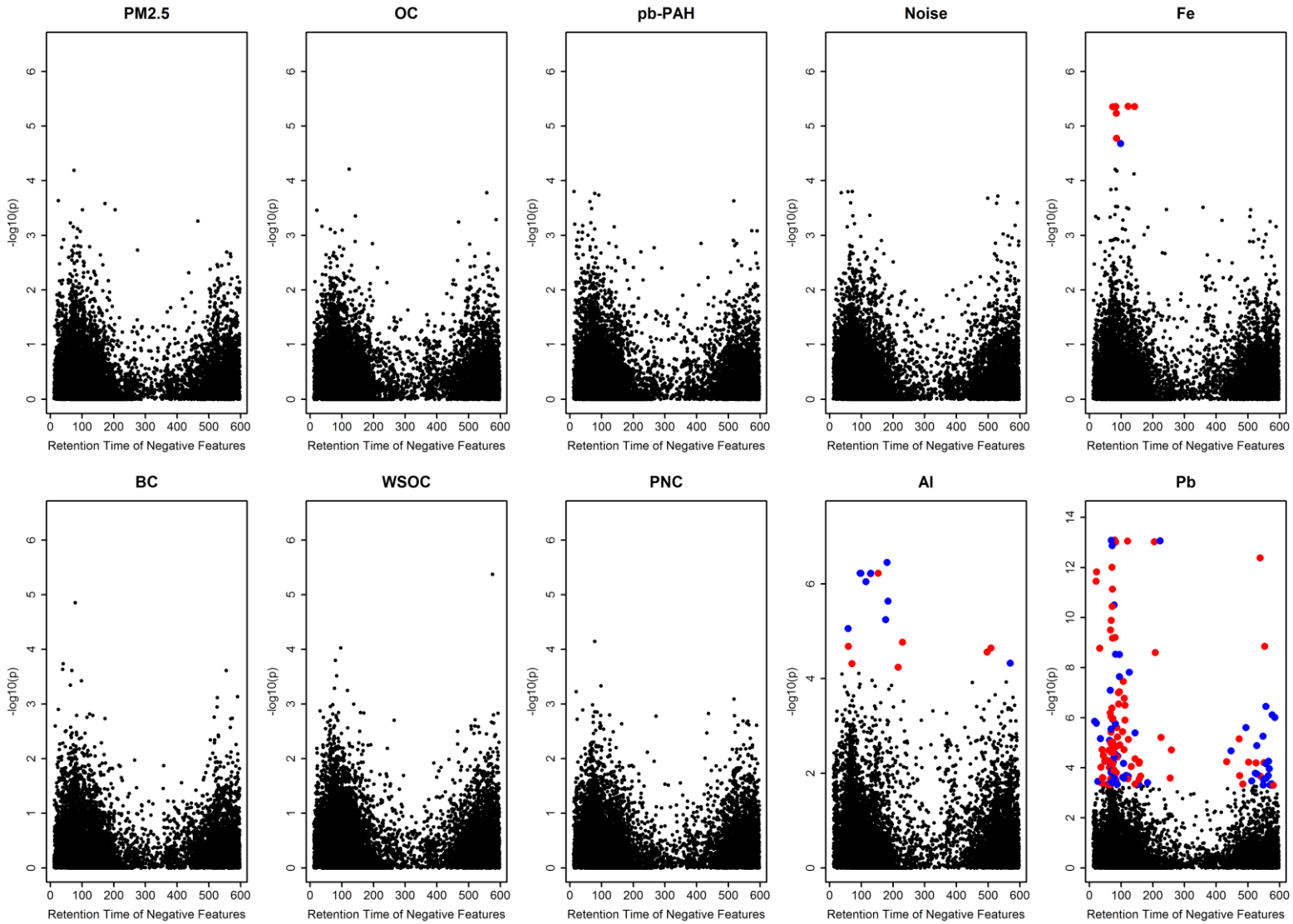

FIGURE 1: Manhattan plots of associations between changes in feature intensities with in-vehicle, traffic-related pollutants. Colored points are significant at $FDR_{B\text{-}H} < 0.05$ and indicate average increase (red) or decrease (blue) in feature intensity.

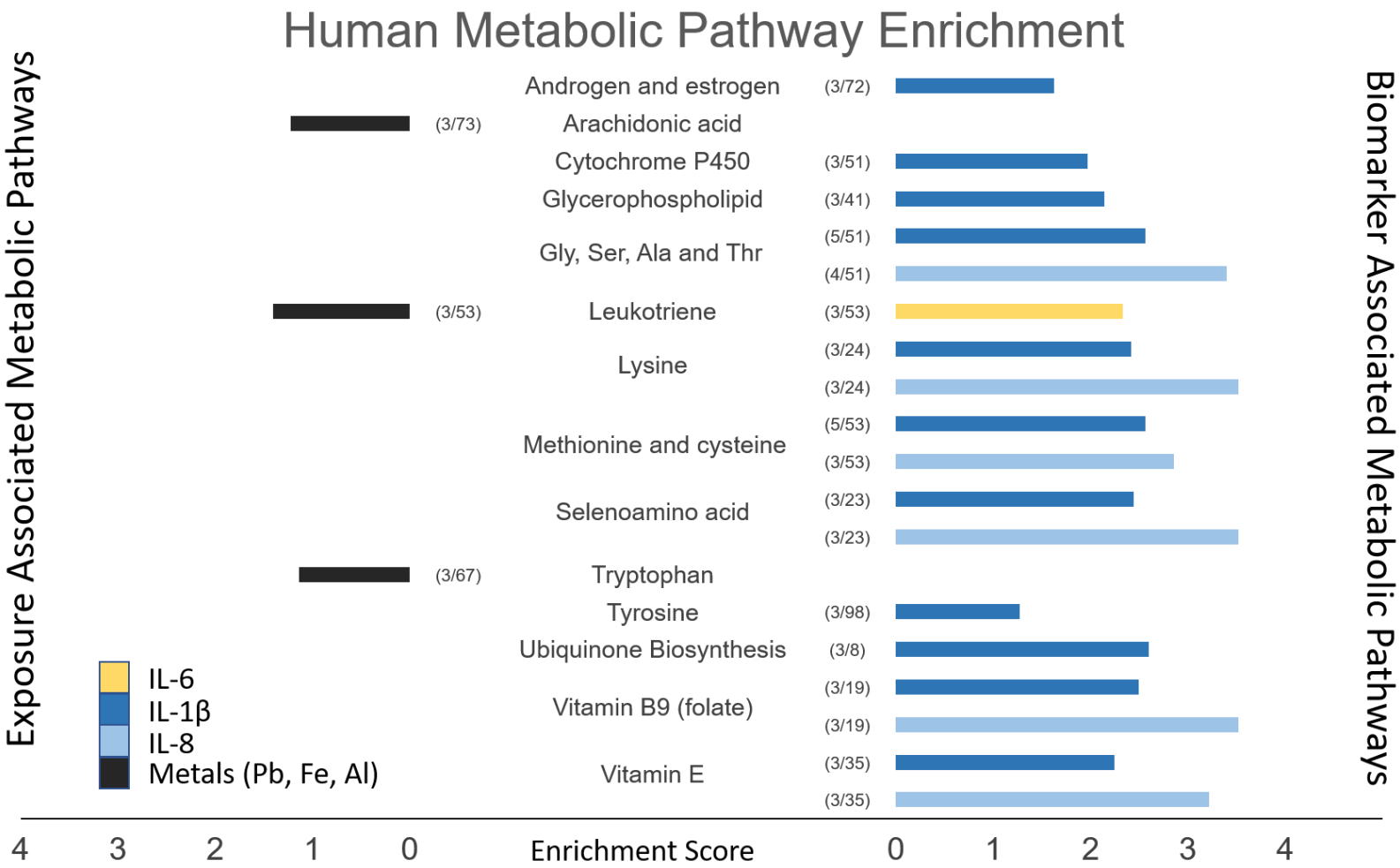

FIGURE 2: Pathway enrichment of exposure-based and biomarker-based significant features. Colored bars indicate the $-\log_{10}(s)$ of enrichment scores from mummichog. Numbers in parentheses indicate the ratio of matching features onto a human reference pathway.

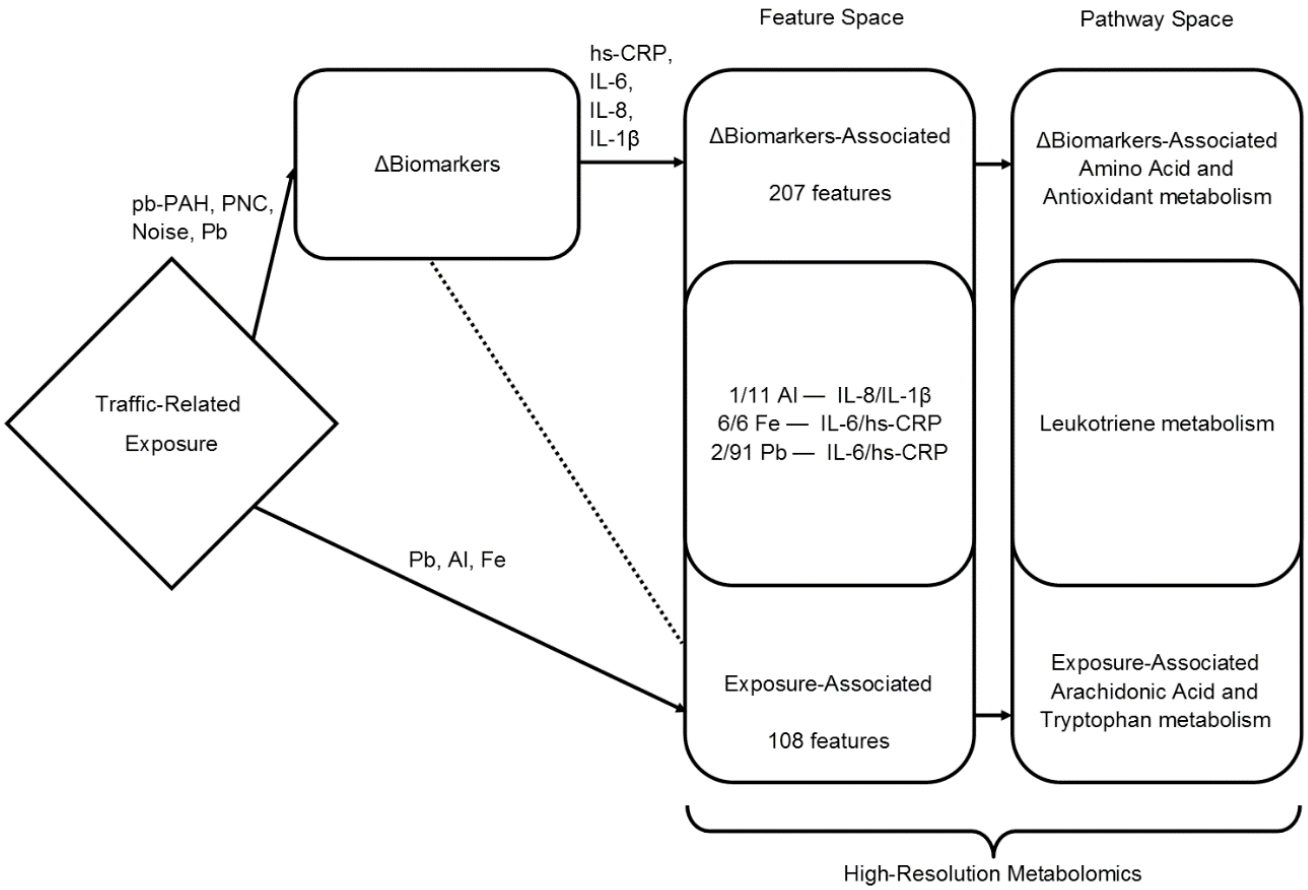

FIGURE 3: Representation of combined results of MWAS and pathway enrichment of both Exposure and ΔBiomarkers. Significant predictors or enriched pathways ($p < 0.05$ or $s < 0.10$) are explicitly named. Particulate metal exposures were the exclusive in-vehicle traffic related pollutants associated with changes in 108 features of the plasma metabolome. Few significant features overlapped between Exposure-associated and ΔBiomarkers-associated features. Leukotriene metabolism was enriched from Al-associated features and ΔIL-6-associated features.

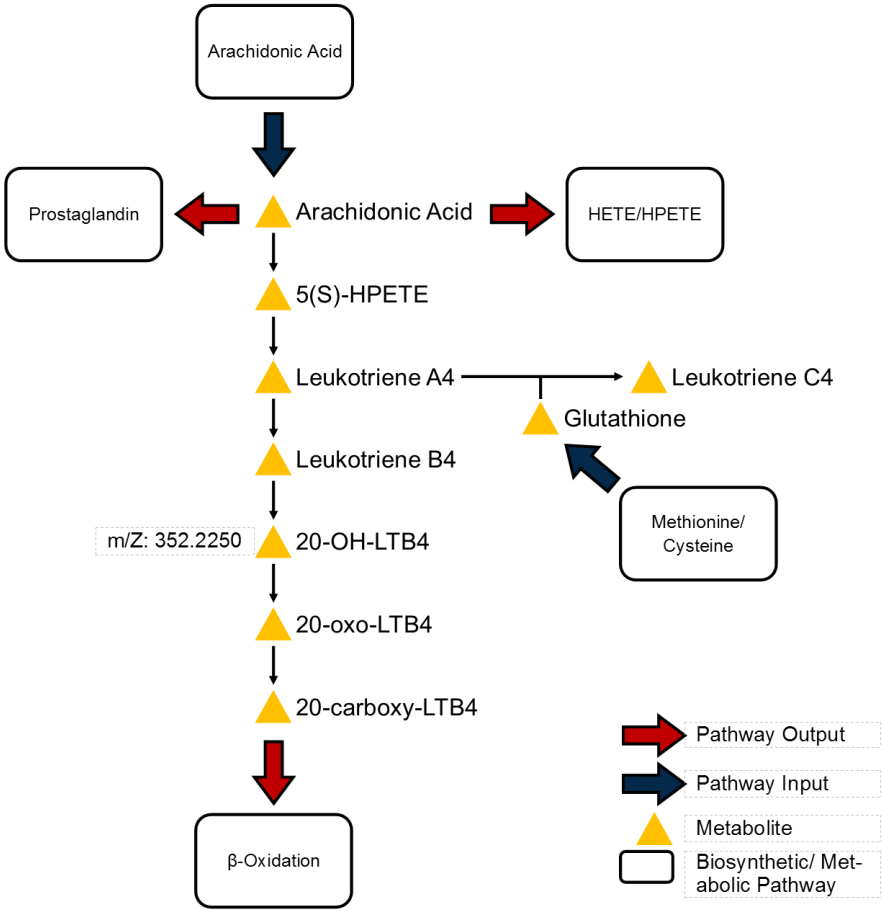

FIGURE 4: Pathway of leukotriene biosynthesis and catabolism in humans. Leukotriene metabolism was the only pathway to be enriched for in both exposure-based and biomarker-based models using mummichog (overlap ≥ 3 and s ≤ 0.10). The features selected with Al, Pb, and ΔIl-6 models have overlapping matches on this pathway with 20-OH-LTB4. The metabolite putatively detected is a biologically inactive form of leukotriene B4. Arachidonic acid metabolism and glutathione synthesis feed into the pathway to generate the variety of signaling molecules on this pathway. Adapted from MetaCore by Thomson Reuters.

Chapter 5: Conclusions

The reality of human exposure to environmental pollutants is dynamic and complex. As exposure changes, it interacts with our biology in equally intricate ways. Systems are interacting with one another, in real-time, seeking a balance which may impact human health. One example, which has been the focus of this dissertation, is that of traffic pollution exposures. Traffic exposures encompass complexity over physical, chemical, temporal, and spatial domains. In many urban locales, these mixtures, both in exposure and effect, contribute substantially to total daily exposure, potentially resulting in numerous adverse health responses. The requisite travel from home to place-of-work may constitute a minor portion of time spent during an average day, but may disproportionately lead to the bulk of harmful exposure to air pollution and stress, creating lasting effects in human biology. To derive insight from the complexity, this dissertation focused on simple, fundamental questions that form the foundation of further study of environmental mixtures.

This dissertation took to understanding key relationships between components of environmental and biological mixtures and how they may correspond to one another. It began with the examination of associations between particulate matter and noise exposures in different microenvironments that humans are likely exposed. Then, the dissertation turned inward to explore the comparability of biological mixtures in metabolomic profiles across different biological matrices used in exposure assessment studies. Finally, it closed with a study exploring the link between exposures to PM components and noise and changes in human metabolism.

In Chapter 2, the relationship between noise and particulate matter was shown to be different between pollutants, microenvironments, and time. We showed that the in-vehicle environment is distinct from the near roadway environment in the association between noise and each of $PM_{2.5}$ mass, black carbon, and pb-PAHs, but not PNC. For long-term exposures, some studies have shown little to no effect of confounding of air pollution associations by noise for certain health effects. [1, 2]. The finding from the present work is important in addressing the

potential for confounding of noise and air pollution and their respective associations with human disease in the acute setting.

Irrespective of the three microenvironments sampled, PNC and noise had stable, positive associations indicating a shared variance in both space and time. Ultrafine particles, indicated by PNC in this study, have shown to disrupt the balance of pro-inflammatory and anti-inflammatory lipid signaling [3, 4]. Many studies examined the role of ultrafine particle exposures and health response; however, we show that those associations have the potential to be modified by concomitant noise exposures in acute health response settings.

Chapter 3 pushed examinations of different microenvironments into different biological matrices within the human body using high-resolution metabolomics. It hypothesized that biofluids typically sampled for air pollution exposure assessment may be comparable within a person with respect to their metabolomic profiles. To date, this is the first examination of saliva, breath, and plasma simultaneously in a population using high-resolution metabolomics. Indeed, for more abundant features in each matrix, the correlations were moderate (r_S: 0.41 - 0.80) between shared features. Furthermore, 6 features measured and found in all three matrices also matched, by mass alone, to known mobile source air toxics. Further chemical analysis is necessary to confirm the identity of these features, but the analysis reduced the chemical space of interest for air pollution exposure assessment to a candidate list of compounds.

Finally, Chapter 4 capitalized on a repeated measures study of traffic exposures and cardiorespiratory responses in a commuting population to explore the ability of high-resolution metabolomics to detect short-term metabolomic perturbations. Traditional exposure measures of air pollution, such as $PM_{2.5}$ mass and respective carbon fractions, measured in microenvironments where participants were exposed did not predict 10-hr changes in metabolomic profiles. However, some particulate metals did demonstrate associations with metabolomic changes at that time scale. This suggested that the oxidizing potential of these metals, Fe, Al, and Pb, or aspects of the particle sources from which they arise, correspond to

changes in hundreds of metabolic features. Importantly, through analytical platforms used, some of this 10-hr perturbation may include the resolution of inflammatory response through reductions in leukotriene metabolites.

The strengths of Chapter 4 lie in the lessons learned in the integration of an important epidemiological tool—the panel study—with cutting-edge technology in chemical measurement and bioinformatics. The “meet-in-the-middle” approach to garnering causal insight for -omics data was introduced by Chadeau-Hyam, et. al. (2010) and inspired the integration of metabolomics with the Atlanta Commuter Exposures study [5]. This study demonstrated that a variety of scientific tools can be used together in novel settings to understand the exposome and human health, especially combining hypothesis-driven and hypothesis-generating approaches in quasi-experimental observational studies.

Overall, the presented dissertation is a collection of insights into the complexity of traffic exposure mixtures and their resulting, acute impacts on human biology. In the short term, future research should include characterizing and identifying the in-vehicle sources that include particulate metal exposures found to associate with metabolomic changes. While there is a vast body of source apportionment literature, an analysis of source factors and how they were measured will aid in attributing appropriate, spatiotemporally relevant mixtures to biological responses. Another immediate research question includes the definitive identification of compounds associated with traffic exposures. From tens of thousands of metabolic features, this dissertation whittled down the chemical space of interest to around 100 features. Even still, 6 features found in three different biofluids, are potentially emitted directly from mobile sources. Identification of this relatively short list of compounds will lead to further research questions of both exposure and the breadth of acute human response to traffic pollution. In the near future, these results should be replicated and included into curated databases of exposures and responses to aid future investigations into aspects of human exposure to traffic pollution and its acute biological impacts.

References

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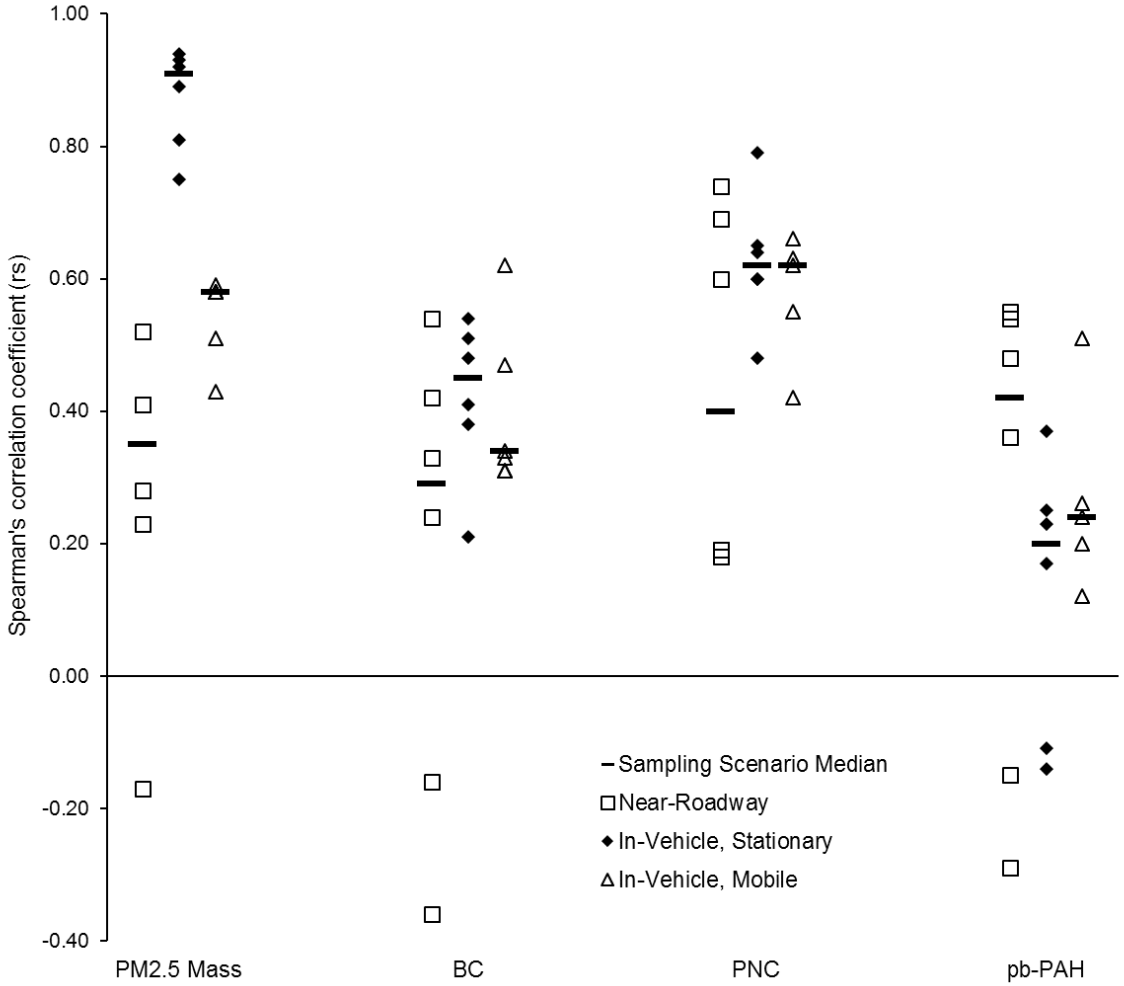
3. Beck-Speier, I., et al., *Ultrafine particles affect the balance of endogenous pro- and anti-inflammatory lipid mediators in the lung: in-vitro and in-vivo studies.* Particle and Fibre Toxicology, 2012. **9**.
4. Li, R.S., et al., *Ambient ultrafine particles alter lipid metabolism and HDL anti-oxidant capacity in LDLR-null mice.* Journal of Lipid Research, 2013. **54**(6): p. 1608-1615.
5. Chadeau-Hyam, M., et al., *Meeting-in-the-middle using metabolic profiling - a strategy for the identification of intermediate biomarkers in cohort studies.* Biomarkers, 2011. **16**(1): p. 83-88.

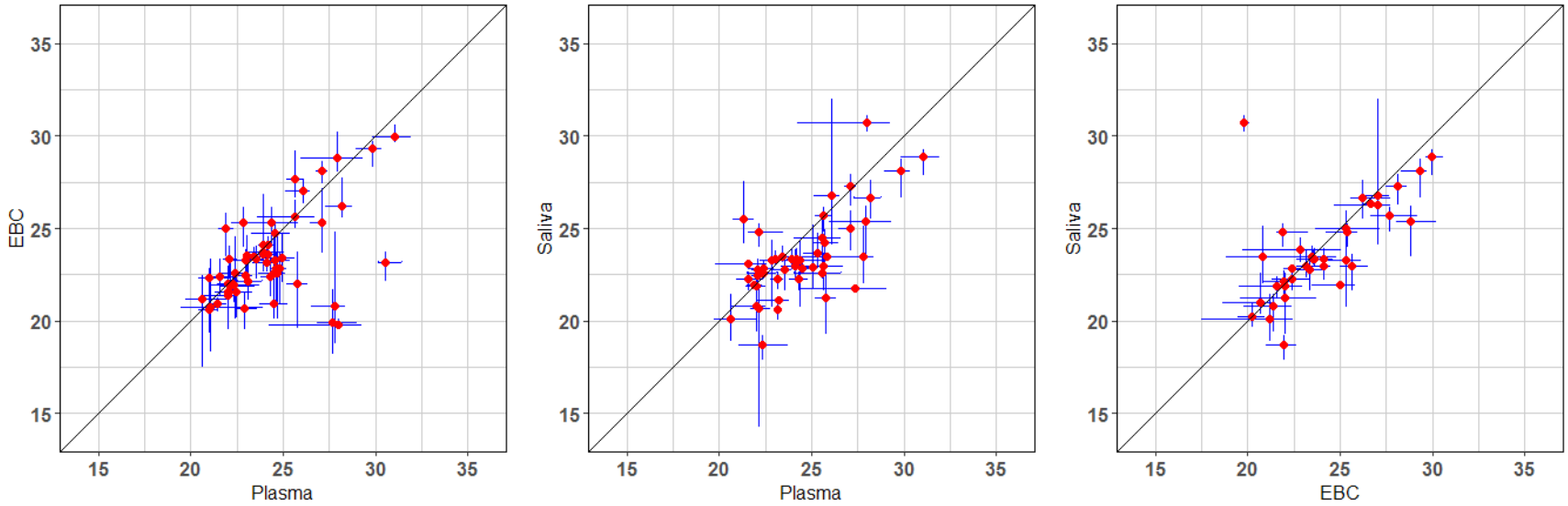
Appendices

Chapter 2 Supplementary Figures



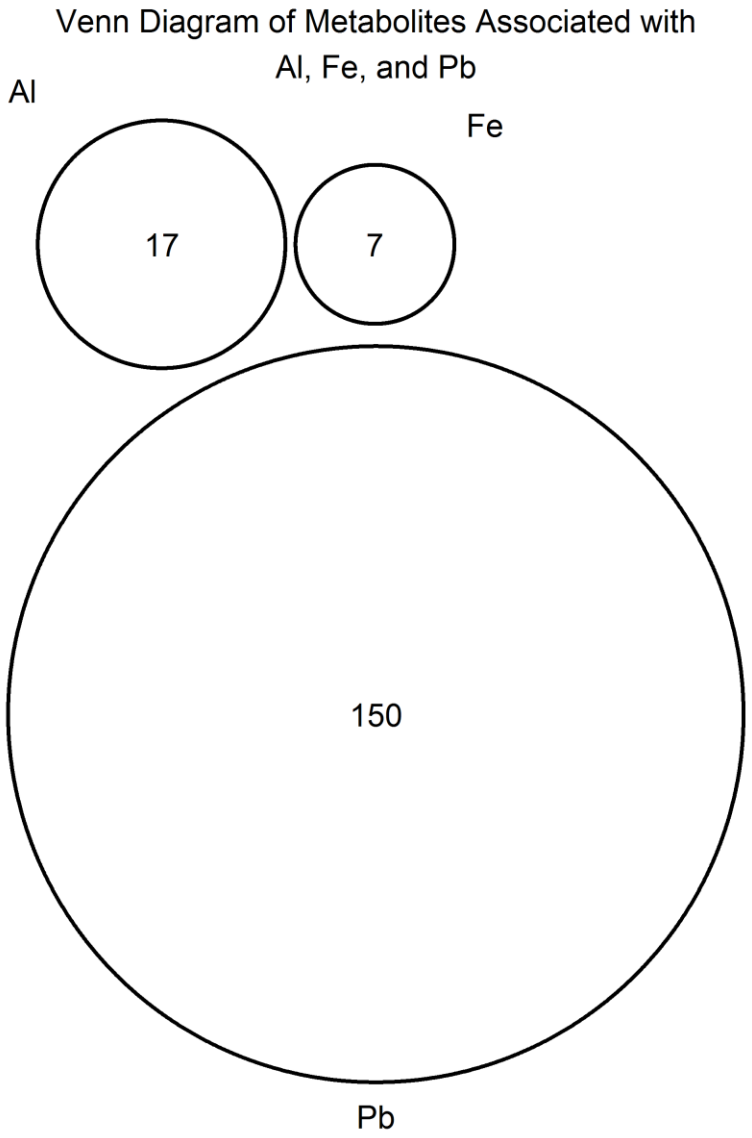
Supp. FIGURE 1: Sampling session-specific and sampling scenario median Spearman's rank correlations between noise levels and traffic-related pollutant concentrations for PM2.5 mass, BC, PNC, and pb-PAHs at 1-min resolution. Filled diamonds (♦), open triangles (▵) and open squares (□) are the IVS, IVM, and NR scenarios, respectively.

Chapter 3 Supplementary Figures

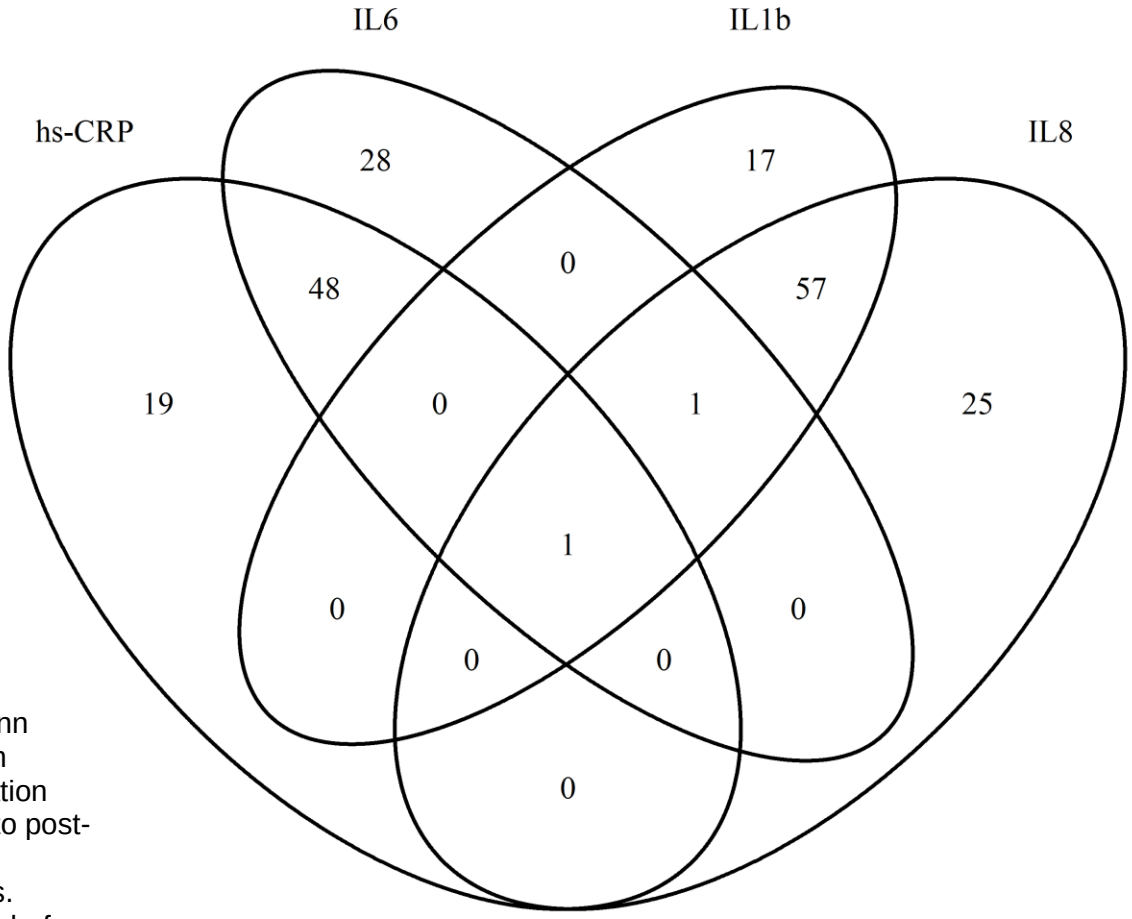


Supp. FIGURE 1: Mean Log-Transformed Intensities of Shared Features across Subjects and Samplings between Matrix Pairs in the EPA-matched Subset. Red dots indicate those features that match the Master List of Mobile Source Air Toxics. Blue bands represent the max-min range of log-transformed feature intensities across all subjects and sampling times. Sample sizes (from top to bottom) range from 54, 46, and 36 features for Plasma vs. EBC, Plasma vs. Saliva, and EBC vs. Saliva, respectively.

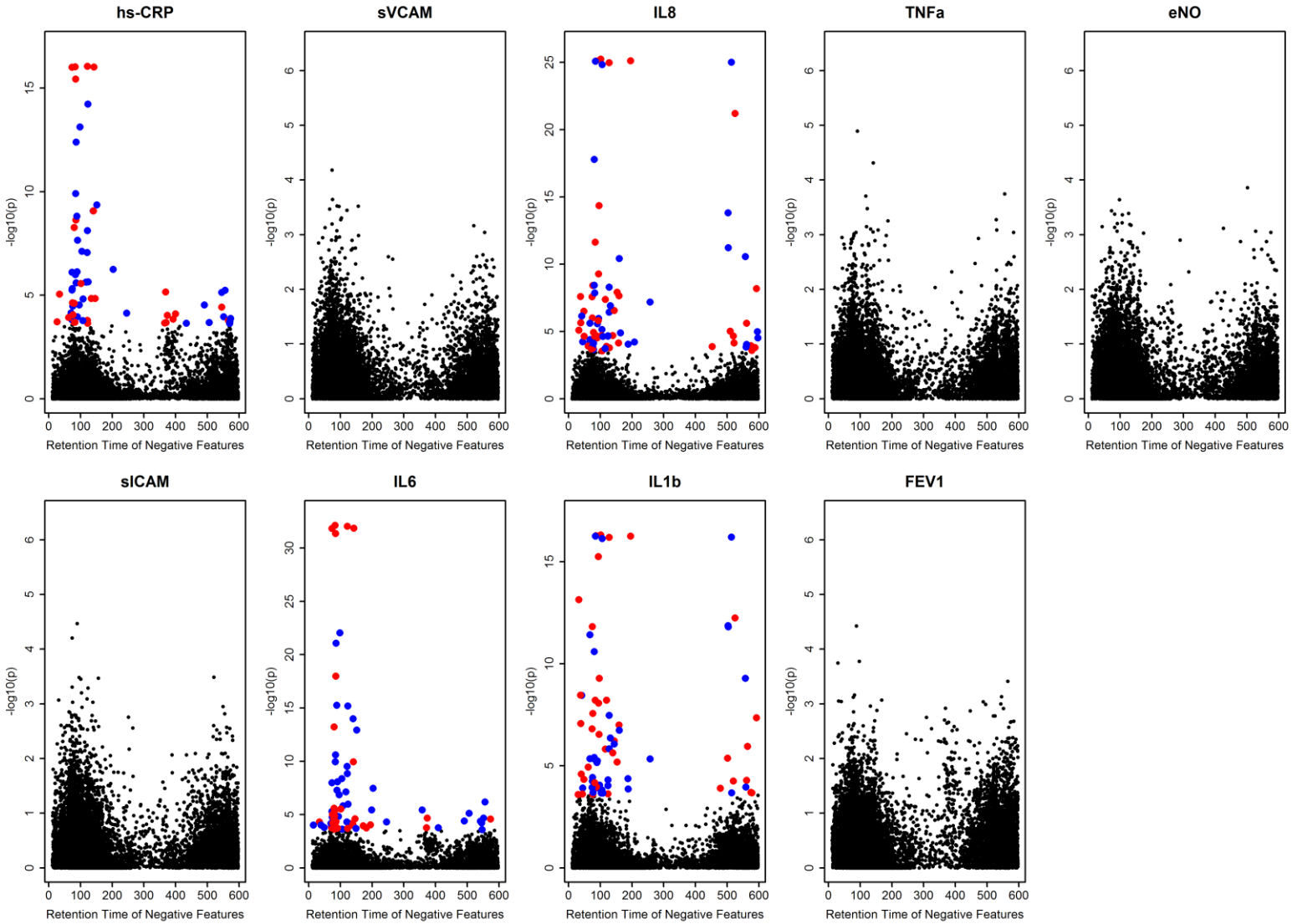
Chapter 4 Supplementary Figures



Supp. FIGURE 1: Venn diagram of features in negative mode ionization associated with variability in in-vehicle Al, Fe, and Pb exposures. Features counted are before EIC filtering.



Supp. FIGURE 2: Venn diagram of features in negative mode ionization associated with pre- to post-exposure changes in inflammatory markers. Features counted are before EIC filtering.



Supp. FIGURE 3: Manhattan plots of associations between changes in feature intensities with changes in targeted biomarkers (ΔBiomarkers). Colored points are significant at $FDR_{B\text{-}H} < 0.05$ and indicate average increase (red) or decrease (blue) in feature intensity.