Spring 5-13-2016

An Examination of the Effects of Air Pollution and Physical Activity on Markers of Acute Airway Oxidative Stress and Inflammation in Adolescents

Emilia Pasalic

ABSTRACT

AN EXAMINATION OF THE EFFECTS OF AIR POLLUTION AND PHYSICAL ACTIVITY ON MARKERS OF ACUTE AIRWAY OXIDATIVE STRESS AND INFLAMMATION IN ADOLESCENTS

By

EMILIA PASALIC

May 13, 2016

INTRODUCTION: Airway inflammatory response is widely believed to be a central mechanism in the development of adverse health effects related to air pollution exposure. Increased ventilation and inspiratory flow rates due to physical activity in the presence of air pollution will increase the inhaled dose of air pollutants. However, physical activity can also affect lung function and may moderate the relationship between air pollution and lung function. The mechanisms that underpin the complex interplay between air pollution, physical activity, and lung function may be more sensitive to the inhaled dose of air pollution than to ambient air pollution exposure alone. Despite this, the majority of literature on the topic measures only the ambient concentration of air pollution.
AIM: This study aims to characterize the relationship between inhaled air pollution dose, physical activity, and respiratory response markers of lung function, oxidative stress and inflammation among healthy adolescents. Respiratory response measures include exhaled nitric oxide (eNO), percent oxidized exhaled breath condensate glutathione (%GSSG), percent oxidized exhaled breath condensate cysteine (%CYSS), the percentage of total oxidized compounds (%Oxidized), and changes in pulmonary function, namely, forced vital capacity (FVC), forced expiratory volume (FEV_1), and forced expiratory flow (FEF_{25-75}). Air pollution measures include cumulative inhaled doses of fine particulate matter ($PM_{2.5}$), ozone (O_3), black carbon (BC), and particle number total (PNT).
METHODS: Using a non-probability sample of high school athletes, outcomes were measured prior to and after participation in extracurricular sports practice. The inhaled dose of air pollutants during the sports practice was estimated for each participant using a novel method developed by Dr. Roby Greenwald. This observational study estimates the association between air pollution dose and outcome measures using general linear mixed models with an unstructured covariance structure and a random intercept for subject to account for repeated measures within subjects. All data analysis was completed using SAS.
RESULTS: A one IQR (i.e. 345.64 µg) increase in O_3 inhaled dose is associated with a 29.16% average decrease from baseline in %Oxidized. A one IQR (i.e. 2.368E+10 particle) increase in PNT inhaled dose is associated with an average decrease in FEF_{25-75} of 0.168 L/second from baseline. The relationship between PNT inhaled dose and eNO is

moderated by activity level, with increasing activity levels attenuating the relationship. Similarly, the relationship between O_3 inhaled dose and %CYSS is attenuated by activity level, with increasing activity levels corresponding to smaller changes from baseline for a constant O_3 inhaled dose.
DISCUSSION: Someone who inhales a high cumulative dose despite a low activity level is likely breathing in a higher concentration of air pollution in a shorter period of time than a person who receives the same dose with a high activity level. The moderating effects of activity level suggest that peaks of high concentration doses of air pollution may overwhelm cells' endogenous redox balance resulting in increased airway inflammation. Further research that examines the relationships between dose peaks over time and inflammation could help to determine whether a high concentration dose over a short period of time has a different effect than a lower concentration dose over a longer period of time.

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B.A., GEORGIA STATE UNIVERSITY

A Thesis Submitted to the Graduate Faculty
of Georgia State University in Partial Fulfillment
of the
Requirements for the Degree

MASTER OF PUBLIC HEALTH

ATLANTA, GEORGIA
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APPROVAL PAGE

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Acknowledgments

A big thank you goes out to all of you who have helped me write this thesis. Dr. Roby Greenwald provided mentorship, expertise, enthusiasm, and data from the Study of Air Pollution and Physical Activity. Dr. Matt Hayat has given numerous patient explanations, encouragement, and inspiration throughout my time at the School of Public Health, but especially through the process of writing this thesis. My colleagues Maithili Bhat, Gala Chapman, Aleta Christensen, Nick Johnson, Tessa Lee Johnson, and Johnna Szegda have all offered good advice and helped me understand important things. My partner Emir is a programming super wizard and an excellent cook; without his help I would probably be very hungry somewhere, still estimating inhaled doses for each participant, one by one, with a pocket calculator. And coffee. Thank you, coffee.

Author's Statement Page

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Chapter 1. Introduction

The benefits of physical activity are well documented and include reduced mortality and morbidity as well as increased mental and physical wellbeing [1]–[3]. Adolescents who engage in regular physical activity establish habits that will improve their health over the course of their lifetimes [4]. However, increased ventilation and inspiratory flow rates due to physical activity in the presence of air pollution will increase the inhaled dose of air pollutants [5]–[10]. Numerous studies have shown air pollution to be related to increased mortality and morbidity, including respiratory and cardiovascular ailments [11]–[15]. Adolescents are uniquely susceptible to adverse health effects related to air pollution exposure, respiratory inflammation, and decreased lung function [16]–[18].

We aim to characterize the relationship between inhaled air pollution dose, physical activity, and respiratory response among adolescents. Respiratory response measures include exhaled nitric oxide (eNO), percent oxidized exhaled breath condensate glutathione (%GSSG), percent oxidized exhaled breath condensate cystine (%CYSS), the percentage of total oxidized compounds (%Oxidized), and changes in pulmonary function, namely, forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), and forced expiratory flow during the middle half of FVC maneuver (FEF_{25-75}). Air pollution measures include the inhaled dose of fine particulate matter ($PM_{2.5}$), ozone (O_3), and black carbon (BC), as well as the total particle number count in the inhaled dose (PNT).

Chapter 2. Review of the Literature

Airway inflammatory response is widely believed to be a central mechanism in the development of adverse health effects related to air pollution exposure [19]–[21]. Reactive oxygen species and oxidative stress play an important role in airway inflammatory response during exposure to airborne particles [19]. Airborne particles are believed to trigger oxidative stress resulting in a systemic and pulmonary inflammatory response [22]–[24]. A long held theory suggests that airborne particles, which can consist of oxidants, trigger a cellular inflammatory response through the direct formation of reactive oxygen species outside of the cell wall, resulting in oxidative stress [19]. However, emerging evidence suggests that the production of cellular inflammatory response may be part of the cell's endogenous redox process, such that airborne particles, whether or not they contain oxidants, can trigger reactive oxygen species generation and oxidative stress within the cell walls, further inducing toxicity [19].

Exhaled nitric oxide (eNO), which is expressed through the respiratory epithelium during a process of inducible NO synthase, signals inflammatory mechanisms in the bronchial mucosa [25]. eNO is used widely as a marker of airway inflammation and oxidative stress [26]–[28]. Air pollution, particularly $PM_{2.5}$ and O_3, is associated with increases in eNO [25], [29]. Studies examining physical activity and eNO have produced varying results, however, physical activity is generally associated with an acute reduction in the concentration of exhaled nitric oxide in healthy subjects [26], [30]–[32]. Sachs-Olsen et al. found that vigorous physical activity was significantly associated with an increase in eNO among non-asthmatic adolescents, however, the study did not take into account the presence of air pollution, the effects of which may have been intensified by increased breathing rates [28].

Glutathione, an antioxidant which plays a protective role against oxidative stress in the airway, is part of the cell's endogenous redox process [33]. Exhaled breath condensate glutathione can be measured in its reduced (GSH) and oxidized (glutathione disulfide, or GSSG) forms [34]. Changes in the redox balance, i.e. reductions in the ratio of GSH to GSSG (GSH/GSSG), may be a key factor in airway inflammation and oxidative stress [33], [35]. In healthy individuals, an acute increase in GSH in response to low $PM_{2.5}$ exposure serves as an adaptive defense against oxidative stress [22], [36], [37]. However, studies suggest that higher doses of pollutants can overwhelm the body's endogenous protective antioxidant response leading to airway inflammation in response to oxidative stress marked by a dose-dependent decrease in GSH/GSSG [36], [38]. Another way to measure oxidative stress is to calculate %GSSG, the percentage of the oxidized form, GSSG, out of the total glutathione, GSSG + GSH [39]. As %GSSG increases, the ratio of GSH/GSSG decreases proportionally.

Glutathione is a tripeptide made of glutamine, glycine, and cysteine. Similarly to GSH/GSSG, the redox balance of the antioxidant cysteine (CYS) to its oxidized form cystine (CYSS) serves as a marker of oxidative stress [39]. CYS is a precursor to the formation of GHS, however the ratio of CYS/CYSS is independent of the ratio of GSH/GSSG as these redox pairs are regulated in different sub-cellular compartments, each indicating the presence of diverse oxidative stress responses [39], [40]. Just as

GSH/GSSG and %GSSG share a curvilinear, inverse relationship, so do CYS/CYSS and %CYSS, the percentage of the oxidized form, CYSS, out of the total, CYSS+CYS. Researchers have shown that among mice, diesel exhaust exposure in combination with house dust mite exposure is associated with significant increases in %CYSS when compared to diesel exhaust or house dust mite exposure alone [39].

FVC is the maximal volume of air exhaled as forcefully and completely as possible, after inhaling to the maximum capacity of the lungs, while FEV_1 is the volume of air exhaled during the first second of the FVC maneuver, and FEF_{25-75} is the volume of exhaled air during the middle half of the FVC maneuver divided by the time it took to exhale it [41]. These measures of spirometry are used widely to evaluate general respiratory health [41]. Rice et al. studied short term exposure to air pollution within levels deemed acceptable by the U.S. Environmental Protection Agency (EPA) and found that exposure to higher levels of $PM_{2.5}$, NO_2, and O_3 is associated with a reduction in FEV_1 and FVC [42]. Physical activity is also associated with acute reductions in lung function among children with asthma [43], [44]. However, among people with healthy lung function, physical activity can be expected to cause bronchodilation and slight increases in spirometry measures of lung function [43].

Because both physical activity and air pollution can independently affect lung function and markers of oxidative stress and airway inflammation, understanding the interplay between these two factors is necessary in order to interpret the effects of air pollution on lung function and oxidative stress in the presence of physical activity [45]. Relatively few studies investigate interactions between physical activity and air pollution, or adjust for the effects of physical activity when exploring the relationship between air pollution and lung function or oxidative stress in the airways. Among those that do, the results are conflicting. One study of adult hikers found that, adjusting for smoking status, asthma, hours hiked, and other covariates, for every 50 ppb increase in mean O_3, there was a 2.6% decrease in FEV_1, and a 2.2% decrease in FVC [46]. Rundell et al. showed that exposure to high levels of fine particulate matter during exercise was associated with a decrease in FEV_1 and FEF_{25-75}, and a non-significant decrease in eNO, while lung function did not change after exposure to low levels of fine particulate matter during exercise [47]. Yet another study showed while exposure to high concentrations of fine and ultrafine particulate matter while exercising were associated (non-significantly) with an immediate increase FEV_1 and FVC, 6 hours after the exposure participants showed a non-significant decrease in these same measures [48]. Kubesch et al. employed a crossover design in order to disentangle the effects of physical activity and traffic related air pollution (TRAP) on respiratory and inflammatory response. This study examined each participant in four conditions: either moderate exercise or rest in either low TRAP or high TRAP environments. The researchers concluded that air pollution and physical activity have independent effects; exercise was associated with increases in FEV_1, FVC, FEF_{25-75}, and surprisingly, eNO and systemic inflammation markers, independent of TRAP levels, while increases in course particulate matter were also associated with an increase eNO [45].

One plausible explanation for contradictory results among studies that examine physical activity, air pollution, and respiratory response is that many studies relied on measures of air pollution exposure. Yet, mechanisms between air pollution and pulmonary response

may be more sensitive to the inhaled dose of air pollution than to ambient air pollution exposure alone. The inhaled air pollution dose varies based on ambient air pollution levels, individual physical characteristics, and breathing rate at the time of exposure [5]–[10]. The relationship between physical activity, air pollution, and respiratory response is further complicated in that physical activity increases the ventilation rate, increasing the inhaled dose of air pollutants as well as particle deposition in human lungs [49], [50]. Studying the inhaled dose of air pollution rather than simply the exposure allows the researcher to effectively isolate and investigate any possible interactions between physical activity and air pollution, and can provide better insight into the effects of each of these factors on respiratory response.

Only a handful of studies have examined the in vivo human respiratory response to inhaled doses of air pollution. Buonanno et al. estimated the dose-response relationship between daily alveolar deposited surface area dose of airborne particles and measures of spirometry and eNO among asthmatic children, finding that a daily dose increase of $100mm^2$ was associated with a 4.1 ppb increase in eNO and a 0.8% decrease in FEF_{25-75} [51]. One limitation of this study was that the inhalation rate used in the dose calculation was estimated using U.S. EPA inhalation rate estimates for different daily activities, which were self-reported by the participants over several days. In a randomized controlled cross-over trial, Behndig et al. exposed each group to either diluted diesel exhaust at a steady concentration of 100 $\mu g/m^3$ or filtered air while exercising, in randomized order several weeks apart. The researchers found an increase in GSH as well as an increase in airway inflammation after diesel exposure in the bronchial airway and nasal lavage samples, but not in the alveolar lavage [36]. While this study did not specifically measure the inhaled dose of air pollutants, the researchers fixed the concentration of diesel exposure and the duration and intensity of exercise, and differences in individual ventilation rates and physical characteristics that would affect dose were likely controlled by the randomized crossover design. In another crossover study, Adams et al. (2000) regulated the inhaled dose of O_3 by exposing participants for 6 hours to constant levels of zero or 0.12 ppb of O_3, while varying the exercise minute ventilation ($\dot{V}_E$) to achieve equivalent ventilation rates (EVR = $\dot{V}_E$/body surface area in m^2) between participants. Each participant, serving as their own control, was exposed to 0.12 ppb 0_3 on three separate occasions, at three separate EVR levels, allowing Adams et al. to evaluate the effects of four separate O_3 dose levels on pulmonary function (0, 1187, 1384, and 1573 ppb, respectively). The researchers found that FEV_1 did not change at an O_3 dose level of zero, but decreased significantly after exposure at all three O_3 dose levels above zero. Though a pattern of dose-response was numerically established, the differences in effect size between dose levels were not significant [52]. The small sample size, and relatively small variation between dose levels in this study may have been limiting factors. While Rundell et al. and Kubesch et al. did not calculate an inhaled dose of air pollutants, both studies compared respiratory response after exercise during exposure to low and high TRAP environments, and demonstrated dose-response relationships between air pollution and respiratory response [45], [47].

The present analysis examines the acute respiratory effects of physical activity and participant-specific inhaled doses of $PM_{2.5}$, O_3, and BC, and the total number of inhaled particles (PNT) in healthy, active adolescents. We hypothesized that interactions exist

between physical activity and air pollution, and that when controlling for physical activity, increased inhaled doses of air pollutants would be associated with a decrease in measures of lung function, an increase in eNO, and an increase in the %GSSG, %CYSS, and %Oxidized as GSH and CYS are oxidized during the course of exposure.

Chapter 3. Methods and Procedures

3.1 Study Design

Data for this analysis were provided by the Study of Air Pollution and Physical Activity (SAPPA). Data collection for this observational study was conducted at two high schools in Atlanta, GA. One high school was set in a wooded, suburban area, while the other was set in an urban area close to major roadways. Recruitment took place between October, 2012 and July, 2014 and data were collected from December, 2012 to July, 2014. Approval for this study was provided by the Emory University Institutional Review Board and the Georgia State University Institutional Review Board.

A convenience sample of 126 students was recruited from the two high schools. All participants were healthy and engaged in one or more extracurricular sports including marching band, track and field, football, soccer, basketball, and cheerleading. Participants over the age of 18 provided written consent. Participants under the age of 18 provided written assent as well as written parental consent.

Prior to beginning sports practice and for the duration of the practice session, participants were fitted with a chest strap that records continuous measurements of heartrate (HR), breathing rate (F_B) and motion. Spirometry was conducted prior to and after practice. Spirometry measures taken were FVC, FEV_1, and FEF_{25-75}. Baseline and post-exposure measurements of eNO, GSH, GSSG, CYS, CYSS, and mixed disulfides (MD) were also taken. Ambient levels of $PM_{2.5}$, O_3, BC and particle number concentration (PNC) were monitored on site throughout the practice session. The cumulative inhaled dose of each air pollutant was calculated by multiplying ambient levels of the air pollutant at each minute of participation by the participant's minute ventilation ($\dot{V}_E$) normalized to FVC, and summing the estimated dose for each minute. The method used for air pollution dose estimation is described in more detail below.

3.2 Data Collection

3.2.1 Predictor Measurement Ambient air pollution levels, including $PM_{2.5}$, O_3, BC, and PNC, were measured on site. All air pollution measures were converted to concentration/L taken in one minute intervals. Ambient PNC was measured using the Hand-held Condensation Particle Counter Model 3007 (TSI Inc., Shoreview, MN). Model 3007 is an isopropyl alcohol based condensation particle counter that uses a continuous laminar flow method to condense alcohol onto particles in the sample stream and an optical detector to count particles. Model 3007 can detect particles larger than 10 nM. PNC was converted to the number of particles/L. Ambient $PM_{2.5}$ was measured using the Portable Laser Aerosolspectrometer and Dust Monitor, model 1.109 (Grimm Aerosol, Ainring, Germany). $PM_{2.5}$ was measured in $\mu g/m^3$, and converted to µg/L. Ambient O_3 was measured using the Model 49i Ozone Analyzer (Thermo scientific, Waltham, MA). The Ozone Analyzer uses a dual cell photometer and employs temperature and pressure correction. The instrument can detect ozone concentration from 0.05 ppb to 200 ppm. O_3 was measured in parts per billion and converted to µg/L. In the event that on-site ambient pollution measurements failed, one minute ambient levels of $PM_{2.5}$ and O_3 were collected from the Ambient Air Monitoring Network site closest to

each school that engaged in continuous sampling of $PM_{2.5}$ and O_3. These two monitoring stations, operated by the Georgia Environmental Protection Division, were located approximately 2 and 10 miles from the respective schools. Ambient BC was measured using the microAeth Model AE51 Aethalometer (AethLabs, San Francisco, CA). The aethalometer captures particles on a T60 Teflon-coated borosilicate glass fiber filter and uses a photo diode detector to track the rate of change of absorption of light from an 880nm LED, relative to a reference portion of the filter. BC was measured in ng/m3 and converted to ng/L.

Continuous measurements of HR (beats per minute), F_B (breaths per minute), and activity level ("the vector addition of three dimensional acceleration expressed as a fraction of standard gravity" [6]) were taken in one second intervals using a chest strap with a physiological monitoring module, BioHarness™ 3 (Zephyr Technology Corporation, Annapolis, MD). These data were collected in real time using laptops on site. The chest strap houses two leads which measure the electrical activity of the heart, a chest expansion sensor that measures F_B, an accelerometer, and a Bluetooth® transmitter. For use as a predictor, a cumulative activity level was estimated by averaging one-second intervals of activity level over the course of one minute, and summing the activity level for all minutes.

3.2.2 Dose Estimation: Minute ventilation in liters ($\dot{V}_E$) was estimated using a novel method developed by Greenwald et al. [6]. Greenwald describes several models for estimating $\dot{V}_E$ normalized to FVC using easily collected data [6]. This study employs Greenwald's two predictor model using HR and BR averaged over 30 second intervals to estimate a 30 second interval of $\dot{V}_E$ normalized to the participant's highest overall measurement of FVC:

$$\frac{\dot{V}_E}{FVC} = -4.2469 + (0.0595HR) + (0.2255BR)$$

30 second intervals of $\dot{V}_E$ normalized to FVC are then are then multiplied by the participant's highest overall measurement of FVC to produce a unique estimate of $\dot{V}_E$ for that 30 second interval. The 30 second intervals of $\dot{V}_E$ were averaged over one minute and multiplied by the ambient level of air pollution concentration per liter measured at that minute. An inhaled dose of air pollution was estimated for each minute a participant was engaged in sports practice. Minute pollution doses over the entire period were then totaled for each participant to produce a measure of the cumulative total air pollution dose (rather than the concentration) for each pollutant to test as predictors of respiratory response. In order to prevent an error caused by numerical overflow during statistical analysis, PNT was divided by 10,000,000, thus converted to tens of millions of particles.

3.2.3 Outcome Measurements: Participants provided non-invasive samples of breath condensate which were tested for MD, GSH, GSSG, CYS and CYSS using a high performance liquid chromatography (HPLC) method for exhaled breath condensate as described by Yeh et al., and originally developed for plasma samples by Jones et al. [34], [35]. Samples were collected by trained study staff using an R-tube which consists of a sterile polypropylene tube with a saliva trap and mouthpiece (Respiratory Research, Charlottesville, NC). The tubes were kept chilled at -70°C using an outer aluminum

sleeve during collection. Participants were required to engage in tidal breathing for 10 minutes during sample collection and were instructed to swallow saliva to avoid salivary contamination in the collection tube. Breath condensate samples of 300 µL were immediately preserved with a solution of chloric acid (5% final), iodoacetic acid (13.4 mM final) boric acid (0.1 M final), and an internal standard gamma-Glu-Glu (5nM final) and stored at -70°C. The percentage of oxidized glutathione was calculated as %GSSG = [GSSG / (GSSH + GSH)] x 100. Similarly, the percentage of cystine was calculated as %CYSS = [CYSS / (CYSS + CYS)] x 100. The percentage of total oxidized compounds was calculated as %Oxidized = [(GSSG + CYSS + MD) / (GSSG + CYSS + MD +GSH + CYS)] x 100.

Prior to the performance of spirometry maneuvers, trained study staff measured eNO using a hand-held instrument, the NIOX MINO (Aerocrine, Morrisville, NC). The NIOX MINO measures nitric oxide using an electrochemical analysis method, adapting guidelines established by the American Thoracic Society to this method. The NIOX MINO does not analyze the first part of the exhalation in order to avoid sample contamination from the mouth. Study staff instructed participants to exhale fully before inhaling to total lung capacity through the NIOX MINO filter and exhaling slowly again through the filter. Using the NIOX MINO, only one valid measurement is necessary. For outdoor sessions, if weather conditions fell outside of the specified operating range for the instrument (16 to 30°C, and 20-60% relative humidity), eNO measurements were conducted indoors. The NIOX MINO has been validated in numerous studies [53]–[56].

Study staff were trained in spirometry test procedures according to guidelines from the American Thoracic Society. Staff guided participants as they performed 3 FVC maneuvers both before and after each sports practice session using the EasyOne Plus handheld spirometer (ndd Medical Technologies Inc., Andover, MA). For each maneuver, study staff recorded FVC, FEV_1, and FEF_{25-75}. For analysis, data from the maneuver with the highest value of FVC out of the three maneuvers were used. FVC and FEV_1 are expressed in L, while FEF_{25-75} is expressed in L/sec.

3.3 Statistical analysis

SAS 9.4 (SAS Institute Inc., Cary, NC) was used for all data analysis. The α level was set a priori to 0.05. Normality of outcome variables was checked visually. In the event that outcome variables did not approximate a normal distribution, natural log transformations were taken to more closely approximate normality. Multicollinearity between predictors was tested and ruled out first by examining bivariate correlations using Pearson's correlation coefficient and scatter plots, and second by regressing each predictor on all the others and examining tolerance and variance inflation factors as well as condition indices. Observations with missing data were assumed to be missing completely at random and excluded from the analysis. For the outcome eNO, all values below five were outside the detectable range of the instrument. A sensitivity analysis was performed to assess the sensitivity of the multi-pollutant model to different imputed values: 0.00001, 2.5, and 5, and "missing". The three numerical values were selected to represent the range of possible values for these observations. For each model in the sensitivity analysis, a natural log transformation of eNO was taken after the single imputation at the specified

level. For our final analysis, the nine values of eNO which were below the detectable limit were imputed with the value 2.5.

Data were analyzed using a general linear mixed model with an unstructured covariance matrix. In order to select the covariance structure, multi-pollutant models for two outcomes (log of eNO and log of %GSSG) were run with unstructured, compound symmetry and variance component covariance matrices. Covariance structures were compared using the Akaike Information Criterion (AIC). The final models include a random intercept for subject to account for repeated measurements taken on each individual. Random slopes for the effect of time and time*occurrence to account for repeated measurements taken on each individual as well as the repeated participation of subjects during multiple practice sessions were tested and left out of the model due to estimability problems, which are described in more detail in the results section. Separate models were constructed for each outcome. All models included fixed effects for each air pollutant dose*time and activity*time to evaluate the change between pre and post measurements. All models controlled for BMI, sex, and age. The basic multi-pollutant model for each outcome contained terms for $PM_{2.5}$, O_3, and PNT, but not BC. The basic multi-pollutant model was as follows:

$$Y_i = \beta_0 + \beta_1(PM_{2.5}dose)_i + \beta_2(PNTdose)_i + \beta_3(O_3dose)_i + \beta_4(activity)_i + \beta_5(time)_i + \beta_6(PM_{2.5}dose * time)_i + \beta_7(PNTdose * time)_i + \beta_8(O_3dose * time)_i + \beta_9(activity * time)_i + \beta_{10}(sex)_i + \beta_{11}(age)_i + \beta_{12}(BMI)_i + \gamma_i + \varepsilon_i$$

For each multi-pollutant model, interaction terms between activity level, time, and each type of air pollution were each tested individually in this multivariable model and retained in the model only if the interaction term was significant.

In addition, single pollutant models were constructed for each outcome and compared to multi-pollutant models. Single pollutant models, as follows, were constructed separately for each pollutant, including black carbon:

$$Y_i = \beta_0 + \beta_1(dose)_i + \beta_2(activity)_i + \beta_3(time)_i + \beta_4(dose * time)_i + \beta_5(activity * time)_i + \beta_6(sex)_i + \beta_7(age)_i + \beta_8(BMI)_i + \gamma_i + \varepsilon_i$$

Because a single unit change in air pollution dose is relatively miniscule and the interpretation of a change this small holds little practical value, final results are presented as the change from baseline in outcome measurement per interquartile range increase in inhaled dose or activity level (Δ). For natural log transformed outcomes, estimates are presented as a percent change and were calculated as $\Delta = \left[\left(exp^{\beta time + \beta dose * time \times IQR}\right) - 1\right] \times 100\%$ where βtime is the coefficient estimate for time of outcome measurement (pre or post, coded as 0,1) in the mixed model, βdose*time is the coefficient estimate for the dose by time interaction, and the IQR is the interquartile range of the predictor in question. For non-transformed outcomes, estimates are presented as an absolute change and were calculated as $\Delta = \beta time + \beta dose * time \times IQR$.

Chapter 4. Results

4.1 Descriptive Analysis

Participant characteristics are presented in Table 1. A total of 126 participants were recruited to and included in the study. The average age of all participants was 16 years and 4.5 months (16.38 ±1.34). For males, the average age was 16.49 (±1.37), and for females, 16.16 (±1.28). A total of 85 (67.46%) participants were male, and 41 (32.54%) were female. 122 (96.83%) participants were black while the remaining 4 (3.17%) were Hispanic. The median BMI among all participants was 23.53 (IQR 20.93-25.90). Among females, the median BMI was 22.33 (IQR: 20.27-24.56), by comparison, the 50th percentile BMI for 16 year old females in the U.S. is 20.5 [57]. Among males, the median BMI was 23.54 (IQR: 21.57-26.21), by comparison, the 50th percentile BMI for 16 year old males in the U.S. is around 20.83 [58]. All participants were non-smokers. No participants had a current physician's diagnosis of asthma. A summary of participant air pollution doses and activity levels is presented in Table 2. A summary of outcome characteristics at baseline and follow-up is presented in Table 3.

Table 1.

Participant Characteristics (n=126)

Characteristics	n (%)	Missing n (%)
Sex		
Female	41 (32.54)	0
Male	85 (67.46)	0
Race		
Black	122 (96.83)	0
Hispanic	4 (3.17)	0
School		
Rural	68 (53.97)	0
Urban	58 (46.03)	0
Age; Mean(SD);	16.38 (1.34)	3 (0.023%)
BMI; Median (IQR)	23.53 (20.93,25.90)	1 (0.008%)

Abbreviations: BMI, body mass index; IQR, interquartile range; n, number; SD, standard deviation;

Table 2.

Air Pollution Dose and Activity Level Characteristics (n=247*)

Predictor (unit)	Median (IQR)	Missing n(%)
$PM_{2.5}$ Dose (µg)	34.33 (19.74-50.72)	29 (11.74%)
PNT Dose (1E+7 particles)	1788.04 (1015.74-3384.07)	42 (17%)
O_3 Dose (µg)	249.8 (56.05-401.7)	44 (17.81%)
BC Dose (ng)	1340.8 (883.35-2562.9)	64 (25.91%)
Activity Total	28.474 (20.17-35.16)	29 (11.74%)

*Some participants participated on more than one observation day for a total of 247 observations.

Abbreviations: BC, black carbon; IQR, interquartile range; n, number; O_3, ozone; $PM_{2.5}$, particulate matter 2.5; PNT, particle number total; SD, standard deviation;

Table 3.

Outcome Characteristics (n=247*)

Outcome	Baseline	Missing n(%)	Follow-up	Missing n(%)
eNO ; Median (IQR)	18 (12-33)	1 (0.4%)	18 (11-32)	21 (8.5%)
Log of eNO; Mean (SD)	2.98 (0.83)	1 (0.4%)	2.94 (0.83)	21 (8.5%)
GSSG; Median (IQR)	0.41 (0.13-1.3)	115 (46.6%)	0.66 (0.17-2.28)	129 (52.2%)
%GSSG; Median (IQR)	1.94 (0.93-3.59)	117 (47.4%)	2.34 (1.1-5.16)	129 (52.2%)
Log of %GSSG; Mean (SD)	0.52 (1.11)	117 (47.4%)	0.70 (1.28)	129 (52.2%)
CYSS; Median (IQR)	0.97 (0.62-1.57)	115 (46.6%)	1.15 (0.71-1.79)	129 (52.2%)
%CYSS; Median (IQR)	74.26 (42.09-82.71)	115 (46.6%)	59.87 (27.09-82.97)	129 (52.2%)
Log of %CYSS; Median (IQR)	4.31 (3.74-4.42)	115 (46.6%)	4.09 (3.3-4.42)	129 (52.2%)
%Oxidized; Median (IQR)	9.08 (5.65-13.81)	115 (46.6%)	9.91 (6.01-13.1)	129 (52.2%)
Log of %Oxidized; Mean (SD)	2.2 (0.68)	115 (46.6%)	2.19 (0.62)	129 (52.2%)
FEF_{25-75}; Mean (SD)	3.77 (1.13)	41 (17%)	3.58 (1.09)	50 (20.2%)
FEV_1; Mean (SD)	3.27 (0.66)	44 (17.8%	3.21 (0.63)	55 (22.3%)
FVC; Mean (SD)	3.75 (0.75)	41 (17%)	3.72 (0.73)	49 (19.8%)

*Some participants participated on more than one observation day for a total of 247 observations. Abbreviations: %CYSS, percent oxidized cysteine; %GSSG, percent oxidized glutathione; %Oxidized, total percent oxidized of measured antioxidants; CYSS, cystine; eNO, exhaled nitric oxide; FEF_{25-75}, forced expiratory flow; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; GSSG, glutathione disulfide; IQR, interquartile range; n, number; SD, standard deviation;

4.2 Missing Data

Missing data are reported in Tables 1, 2, and 3. For missing values of air pollution dose measurement, covariate values were not measurable as a result of instrument error. Missing values of %GSSG, %CYSS, and %Oxidized are a result either of the contamination of the sample or because of a failure to collect the minimum amount of exhaled breath condensate necessary for analysis. Missing values of spirometry measures are a result of measurement error. The numbers of observations analyzed in each model are presented in Tables 5 and 6.

4.3 Multicollinearity Testing

The highest bivariate correlation between any two predictors, $PM_{2.5}$ and 0_3, was r=.67. The lowest tolerance level found was 0.35, with a variance inflation factor of 2.85. No condition indices were higher than 5 when adjusting out the intercept using the "collinoint" option in SAS.

4.4 Covariance Structure and Random Effects Selection

The sparseness of frequency counts in the number of repeated occurrences created an estimation problem for all models that included a random effect term for time*occurrence. While some participants participated on up to five separate occurrences, very few participants had more than three occurrences. In addition, large amount of missing data for some predictors and outcomes may have hindered estimability for models with random effect terms for time. In models for both eNO and %GSSG, unstructured and variance component structured matrices were tied for the lowest AIC. The unstructured covariance structure was ultimately selected because it is the most

Table 4.

Covariance structure comparison

Covariance matrix structure	Random effects	AIC
Log of eNO		
Compound Symmetry		
	Intercept*	606.6
	Intercept, Time	699.7
Unstructured		
	Intercept	604.6
	Intercept, Time*	605.8
Variance component		
	Intercept	604.6
	Intercept, Time*	604.6
Log of %GSSG		
Compound Symmetry		
	Intercept*	706.5
	Intercept, Time	707.2
Unstructured		
	Intercept	704.5
	Intercept, Time*	706.2
Variance component		
	Intercept	704.5
	Intercept, Time*	704.5

*Indicates a problem with model estimability. Abbreviations: %GSSG, percent oxidized glutathione; AIC, Akaike information criterion; eNO, exhaled nitric oxide;

flexible of the covariance structures. By comparison, the variance component structure assumes independence of within-subject measurements, an assumption that is not appropriate for our data [59]. Results for covariance structure selection are presented in Table 4.

4.5 Multi-pollutant General Linear Mixed Models

The results of all multi-pollutant models are presented in Table 5. Significant associations are seen between O_3 and %Oxidized, and PNT and FEF_{25-75}. A one IQR (i.e. 345.64 µg) increase in O_3 inhaled dose is associated with a 29.16% average decrease from baseline in the percentage of total oxidized compounds. A one IQR (i.e. 23,683,300,000 particle) increase in PNT inhaled dose is significantly associated with an average decrease in FEF_{25-75} of 0.168 L/second from baseline. A statistically significant association is also seen between PNT and eNO, however, this association is attenuated by activity level. At a total activity level of zero, a one IQR (i.e. 23,683,300,000 particle) increase in PNT inhaled dose is associated with an average increase in eNO of 14.77% above baseline, while at the 25th quartile activity level of 20.17, a one IQR increase in PNT was associated with a smaller, 2.59%, increase in eNO. As activity levels rise, the relationship between PNT and eNO becomes negative. At the median activity level of 28.474, a one IQR increase in PNT is associated with a 2.05% decrease in eNO, and at the 75th quartile of activity level, 35.15, PNT is associated with a decrease of 5.62% in eNO. A graphical depiction of this relationship is found in Figure 1. Similarly, the relationship between O_3 and %CYSS is attenuated by activity level, with increasing activity levels corresponding to smaller changes from baseline for a constant level of 0_3. When activity level is zero, an IQR change of 345.64 µg O_3 is associated with a 49.81% decrease in %CYSS. However, at the 25th quartile of activity level, the decrease weakens to 36.71%, and at the 75th percentile of activity level, a 24.81% decrease from baseline is seen for %CYSS. See Figure 2 for a depiction of this relationship.

Table 5.

Multi-pollutant Models: Associations between Air Pollution, Physical Activity, and Respiratory Response

Outcome	Predictor	Estimated β Coefficient	Standard error	95% CI		Δ for IQR increase in dose	P-value
Log of eNO (n=369*)							
	time	-0.1227	0.1317	-0.3820	0.13660	--	0.352
	$PM_{2.5}$ Dose x time	0.000616	0.001988	-0.0033	0.00453	-9.84%	0.757
	PNT Dose x time	0.00011	0.000047	1.8E-05	0.00020	14.77% ǂ	0.019
	O_3 Dose x time	-0.00008	0.000226	-0.0005	0.00036	-13.96%	0.719
	Activity Level x time	0.001112	0.004786	-0.0083	0.01054	-10.06%	0.817
	PNT Dose x Activity x time	-0.00000282	1.424E-06	-6E-06	-1E-08	--	0.049
Log of %GSSG (n=201*)							
	time	-0.187	0.4881	-1.15410	0.78010	--	0.702
	$PM_{2.5}$ Dose x time	0.009758	0.01126	-0.01255	0.03206	12.21%	0.388
	PNT Dose x time	-0.00016	0.000102	-0.00036	4.1E-05	-43.21%	0.116
	O_3 Dose x time	-0.00074	0.001184	-0.00308	0.00161	-35.77%	0.535
	Activity Level x time	0.019	0.01726	-0.01520	0.05319	10.27%	0.273
Log of %CYSS (n=203*)							
	time	0.2196	0.2348	-0.24550	0.68480	--	0.352
	$PM_{2.5}$ Dose x time	0.00102	0.004131	-0.00716	0.00920	28.56%	0.805
	PNT Dose x time	0.000027	0.000038	-0.00005	0.00010	32.78%	0.480
	O_3 Dose x time	-0.00263	0.000971	-0.00455	-0.0007	-49.81% ǂ	0.008
	Activity Level x time	-0.00682	0.00883	-0.02431	0.01067	12.46%	0.441
	O3 Dose x Activity x time	0.000053	0.000025	3.4E-06	0.00010	--	0.036
Log of %Oxidized (n=203*)							
	time	0.1668	0.2915	-0.41050	0.74410	--	0.568
	$PM_{2.5}$ Dose x time	0.004897	0.006613	-0.00820	0.01800	37.50%	0.461
	PNT Dose x time	-0.00004	0.000062	-0.00016	0.00009	7.47%	0.551
	O_3 Dose x time	-0.00148	0.000717	-0.00290	-6E-05	-29.16%	0.041
	Activity Level x time	0.002249	0.01044	-0.01844	0.02294	22.20%	0.830
FEF_{25-75} (n=317*)							
	time	-0.00228	0.2444	-0.48410	0.47960	--	0.993
	$PM_{2.5}$ Dose x time	-0.00252	0.004778	-0.01194	0.00690	-0.0803	0.598
	PNT Dose x time	-0.00007	0.000036	-0.00014	-1E-06	-0.168	0.047
	O_3 Dose x time	0.00005	0.000575	-0.00108	0.00118	0.015	0.930
	Activity Level x time	0.004851	0.008432	-0.01177	0.02148	0.07042	0.566
FEV_1 (n=310*)							
	time	-0.00204	0.09936	-0.19800	0.19390	--	0.984
	$PM_{2.5}$ Dose x time	0.0013	0.001935	-0.00252	0.00512	0.03823	0.502
	PNT Dose x time	0.000001272	0.000014	-0.00003	0.00003	0.00097	0.930
	O_3 Dose x time	-0.00023	0.000232	-0.00069	0.00023	-0.0815	0.323
	Activity Level x time	-0.00072	0.003417	-0.00746	0.00602	-0.0128	0.833
FVC (n=318*)							
	time	-0.07345	0.09686	-0.26440	0.11750	--	0.449
	$PM_{2.5}$ Dose x time	0.001413	0.001896	-0.00233	0.00515	-0.0297	0.457
	PNT Dose x time	0.000019	0.000014	-0.00001	0.00005	-0.0285	0.193
	O_3 Dose x time	-0.0002	0.000228	-0.00065	0.00025	-0.1426	0.382
	Activity Level x time	0.000437	0.003345	-0.00616	0.00703	-0.0669	0.896

Observations with missing data were excluded from the analysis. For each outcome, the model includes terms for all predictors listed beneath the outcome as well as sex, age and BMI.

*n represents the number of measurements included in the analysis out of 494 total measurements.

ǂ For models that include dose x activity x time interactions, the dose x time interaction can only be interpreted as the effect of dose on change in outcome when activity level is zero.

Abbreviations: %CYSS, percent oxidized cysteine; %GSSG, percent oxidized glutathione; %Oxidized, total percent oxidized of measured antioxidants; BMI, body mass index; CI, confidence interval; CYSS, cystine; eNO, exhaled nitric oxide; FEF_{25-75}, forced expiratory flow; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; GSSG, glutathione disulfide; IQR, interquartile range; O_3, ozone; n, number; $PM_{2.5}$, particulate matter 2.5; PNT, particle number total; SE, standard error;

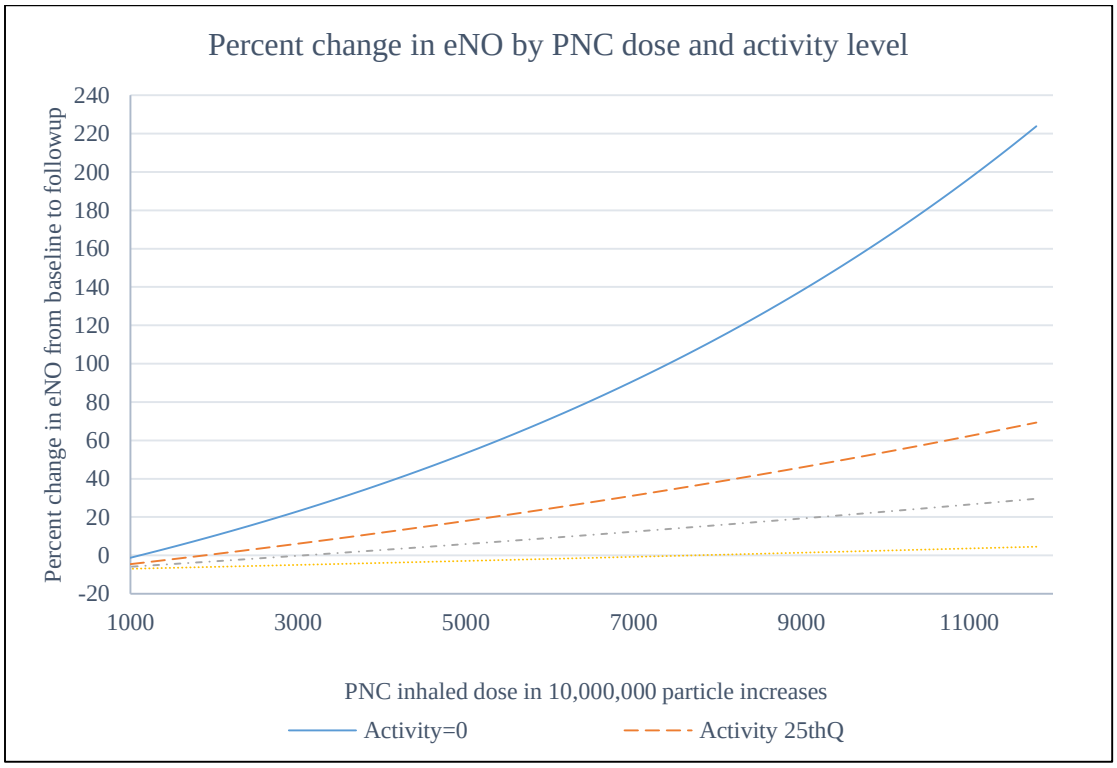

Figure 1. The relationship between PNT and eNO is moderated by activity level

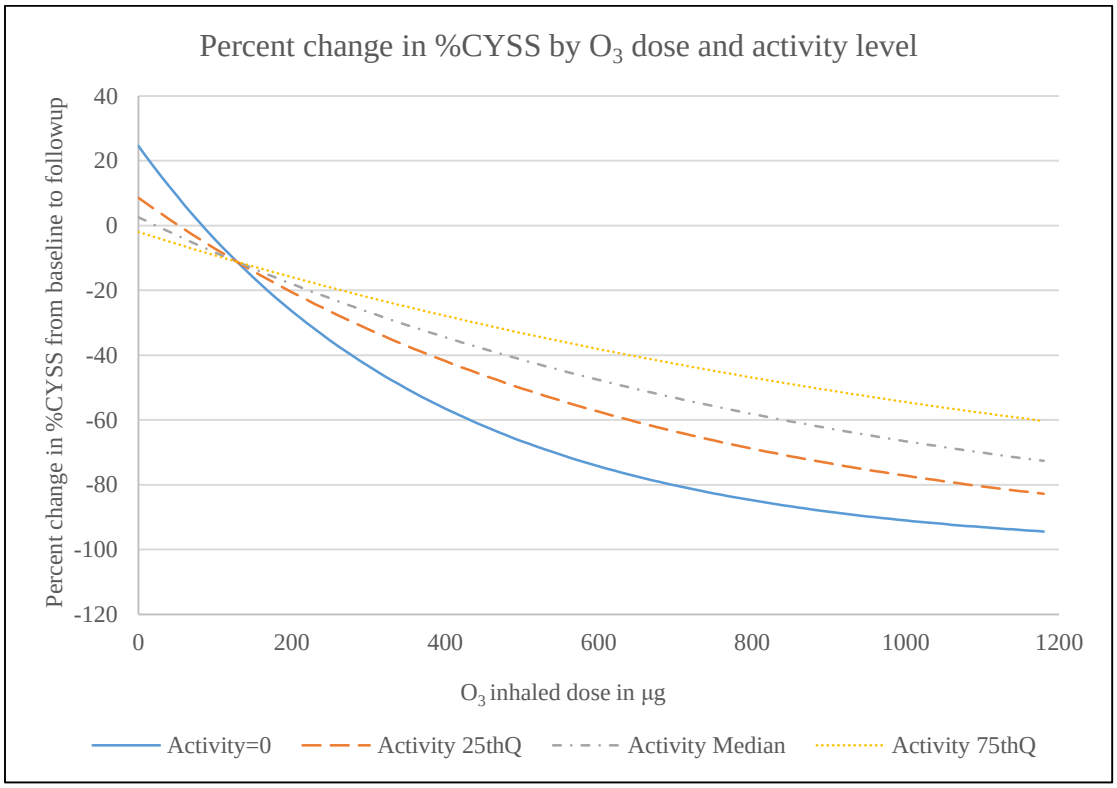

Figure 2. The relationship between 0_3 inhaled dose and %CYSS is moderated by activity level

4.6 Single Pollutant General Linear Mixed Models

The results of all single pollutant models are presented in Table 6. In single pollutant models, significant relationships are observed between different types of air pollution doses and %CYSS, %Oxidized, FEF_{25-75}, and FEV_1. A one IQR increase in $PM_{2.5}$ inhaled dose (i.e. 30.97 μg) is associated with a 6.9% decrease in %CYSS, and a 9.68% increase in %Oxidized, however, at inhaled dose levels of $PM_{2.5}$ higher than 41μg, the relationship between $PM_{2.5}$ and %Oxidized becomes negative. A one IQR increase in PNT (i.e. 23,683,300,000 particle) is associated with a 0.179 L/second decrease in FEF_{25-75}. A one IQR increase in ozone inhaled dose (i.e. 345.64 μg) is associated with a 31.42% decrease in %CYSS, and an 18.16% decrease in %Oxidized. A one IQR increase in black carbon (i.e. 1680 ng) is associated with a 23.35% decrease in %CYSS, a 12.67% decrease in %Oxidized, and a 0.028L decrease in FEV_1.

Table 6.

Single Pollutant Models: Associations between Air Pollution and Respiratory Response

Outcome	Predictor	n*	Estimated β Coefficient	Standard error	95% CI		Δ for IQR increase in dose	P-value
Log of eNO								
	$PM_{2.5}$ Dose x time	418	0.000147	0.00111	-0.0020	0.002324	10.37	0.894
	PNT Dose x time	392	0.000022	1.3E-05	-4.35E-06	0.00005	12.13	0.102
	O_3 Dose x time	395	0.000057	0.00014	-0.00021	0.000326	10.07	0.6751
	BC x time	348	0.000015	1.4E-05	-0.00001	0.000042	8.36	0.2744
Log of %GSSG								
	$PM_{2.5}$ Dose x time	222	-0.00344	0.00672	-0.01673	0.009847	-7.96	0.6093
	PNT Dose x time	218	-0.00018	0.00009	-0.00036	1.29E-06	-49.21	0.0517
	O_3 Dose x time	205	-0.00104	0.00072	-0.00247	0.000392	-17.03	0.1529
	BC x time	183	-0.00019	0.00017	-0.00053	0.000145	-26.52	0.2615
Log of %CYSS								
	$PM_{2.5}$ Dose x time	224	-0.00709	0.00257	-0.01218	-0.002	-6.90	0.0067
	PNT Dose x time	221	-0.00002	3.6E-05	-0.0001	0.000046	-14.73	0.4954
	O_3 Dose x time	206	-0.00087	0.00027	-0.0014	-0.00035	-31.42	0.0014
	BC x time	184	-0.00014	0.00007	-0.00028	-5.98E-07	-23.35	0.049
Log of %Oxidized								
	$PM_{2.5}$ Dose x time	224	-0.00808	0.00372	-0.01544	-0.00072	9.68	0.0316
	PNT Dose x time	221	-0.00009	5.2E-05	-0.0002	0.000011	-12.38	0.0801
	O_3 Dose x time	206	-0.00135	0.00043	-0.00221	-0.0005	-18.16	0.0022
	BC x time	184	-0.00023	9.4E-05	-0.00041	-0.00004	-12.67	0.0167
FEF_{25-75}								
	$PM_{2.5}$ Dose x time	362	-0.00116	0.00262	-0.00633	0.003997	-0.158	0.6573
	PNT Dose x time	339	-0.00006	2.7E-05	-0.00011	-7.86E-06	-0.179	0.0245
	O_3 Dose x time	340	-0.00033	0.00035	-0.00101	0.000353	-0.227	0.3452
	BC x time	311	-0.00005	3.1E-05	-0.00011	0.000011	-0.263	0.1061
FEV_1								
	$PM_{2.5}$ Dose x time	355	-0.00023	0.00105	-0.0023	0.001839	0.019	0.8256
	PNT Dose x time	332	-5.68E-06	1.1E-05	-0.00003	0.000016	-0.004	0.5991
	O_3 Dose x time	333	-0.00005	0.00014	-0.00032	0.000229	0.012	0.7456
	BC x time	304	-0.00002	1.2E-05	-0.00005	-8.63E-07	-0.028	0.0421
FVC								
	$PM_{2.5}$ Dose x time	363	-0.00006	0.00102	-0.00207	0.001945	-0.0026	0.9498
	PNT Dose x time	340	0.00001	1.1E-05	-0.00001	0.000031	-0.0146	0.3392
	O_3 Dose x time	341	0.000028	0.00014	-0.00024	0.000296	-0.0035	0.8361
	BC x time	311	-0.00002	1.2E-05	-0.00004	7.32E-06	-0.0590	0.1805

Observations with missing data were excluded from the analysis. For each outcome, four separate models were run. The models include the single pollutant predictor term listed as well as activity level, sex, age and BMI.

*n represents the number of measurements included in the analysis out of 494 total measurements.

Abbreviations: %CYSS, percent oxidized cysteine; %GSSG, percent oxidized glutathione; %Oxidized, total percent oxidized of measured antioxidants; BC, black carbon; BMI, body mass index; CI, confidence interval; CYSS, cystine; eNO, exhaled nitric oxide; FEF_{25-75}, forced expiratory flow; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; GSSG, glutathione disulfide; IQR, interquartile range; O_3, ozone; n, number; $PM_{2.5}$, particulate matter 2.5; PNT, particle number total; SE, standard error;

4.7 Sensitivity Analysis

Nine observations of eNO were flagged as below the detectable limit during data collection. In multi-pollutant models, significant coefficient estimates for PNT*Time

using imputed values of 2.5 and 5, were not significant for imputed values of 0.00001 and missing. Significant coefficient estimates for PNT*Activity*Time using an imputed value of 2.5 were not significant for imputed values of 0.00001, 5 and missing. Remarkable differences in effect size and standard error were noted for models using an imputed value of .00001. Results of the sensitivity analysis are presented in Table 7.

Table 7.

Sensitivity Analysis for eNO values below the detectable limit

Model term	Imputed value	Estimated β Coefficient	Standard error	95% CI		Δ for IQR increase in dose	P-value
time							
	0.00001	-1.244	0.8058	-2.83100	0.34300	--	0.124
	2.5	-0.122700	0.13	-0.38200	0.13660	--	0.352
	5	-0.050050	0.12	-0.27750	0.17740	--	0.665
	missing	-0.019570	0.12	-0.25200	0.21290	--	0.868
PM2.5 Dose x time							
	0.00001	0.009186	0.01	-0.01544	0.03381	-61.69	0.463
	2.5	0.000616	0.001988	-0.00330	0.00453	-9.84	0.757
	5	0.000141	0.001743	-0.00329	0.00357	-4.47	0.935
	missing	-0.00006	0.001737	-0.00349	0.00336	-2.12	0.971
PNC Dose x time							
	0.00001	0.000352	0.000275	-0.00019	0.00089	-33.67	0.201
	2.5	0.00011	0.000047	0.00002	0.00020	14.77	0.019
	5	0.000091	0.000041	0.00001	0.00017	17.99	0.027
	missing	0.000078	0.000042	-3E-06	0.00016	17.96	0.060
O3 Dose x time							
	0.00001	-0.00035	0.00142	-0.00315	0.00245	-74.46	0.805
	2.5	-0.00008	0.000226	-0.00053	0.00036	-13.96	0.719
	5	-0.00007	0.000198	-0.00046	0.00032	-7.16	0.736
	missing	-0.00005	0.000198	-0.00044	0.00033	-3.62	0.782
Activity Level x time							
	0.00001	0.01297	0.02919	-0.04451	0.07045	-64.99	0.657
	2.5	0.001112	0.004786	-0.00831	0.01054	-10.06	0.817
	5	0.000081	0.004199	-0.00819	0.00835	-4.77	0.985
	missing	-0.00032	0.004236	-0.00867	0.00802	-2.41	0.940
PNC Dose x Activity x time							
	0.00001	-6.7E-06	8.33E-06	-0.00002	0.00001	--	0.420
	2.5	-2.8E-06	1.42E-06	-0.00001	-1E-08	--	0.049
	5	-2.4E-06	1.25E-06	-5E-06	4E-08	--	0.054
	missing	-2.1E-06	1.26E-06	-5E-06	4E-07	--	0.095

Values of eNO that were below the detectable limit were imputed prior to taking a natural log transformation of eNO.
Abbreviations: CI, confidence interval; CYSS, cystine; eNO, exhaled nitric oxide; IQR, interquartile range; O3, ozone; n, number; PM2.5 , particulate matter 2.5; PNT, particle number total; SE, standard error;

Chapter 5. Discussion

We hypothesized that interactions exist between physical activity and air pollution, and that when controlling for physical activity, increased inhaled doses of air pollutants would be associated with a decrease in measures of lung function, an increase in eNO, and an increase in the %GSSG, %CYSS, and %Oxidized as GSH and CYS are oxidized during the course of exposure. In keeping with the hypothesis, we found that in both single and multi-pollutant models, an increase in the particle number total in the inhaled dose (PNT) is associated with a decrease in lung function, FEF_{25-75}, and in multi-pollutant models only, an increase in airway inflammation marked by exhaled nitric oxide. Furthermore, we see that in multi-pollutant models, the relationship between PNT and eNO, as well as the relationship between O_3 and %CYSS are both attenuated by activity level. Contrary to our hypothesis, in multi pollutant models, an increased inhaled dose of O_3 is associated with a decrease in %CYSS and %Oxidized. Likewise, in single pollutant models, increasing inhaled doses of O_3 and BC are associated with a decrease in %CYSS and %Oxidized. An increasing inhaled dose of $PM_{2.5}$, however, is associated with a decrease in %CYSS, but attenuates an increase in %Oxidized, and at doses higher than 41μg is associated with a decrease in %Oxidized. No significant relationships were found in multi-pollutant models between any type of air pollution and %GSSG, FEV_1, or FVC. In single pollutant models, BC was associated with a decrease in FEV_1.

This study has several limitations which warrant consideration and suggest that the results of this study should be interpreted with caution. First, the non-probability sample is not representative of the general population of adolescents in the U.S., thus the results are not generalizable to all healthy adolescents. Second, the data collection process for the Study of Air Pollution and Physical Activity is still ongoing and the study has not yet reached it intended sample size, as such, this analysis may be underpowered. Third, due to the difficulty of measuring multiple outcomes quickly among energetic adolescents in a field setting, as well as repeated air quality monitoring equipment failures, much of the data are missing. While the missingness of the data is unlikely to be correlated with either the predictors or the outcomes, with the exception of observations where eNO is below the detectable limit, there is still a possibility that excluding observations with missing data could have introduced bias. Furthermore, missing data may have been at the source of the estimability problems of the models with random effects for time and time*occurrence. Not including these random effects in the final models may have underestimated the standard error and inflated the possibility of type one error. Fourth, while it is important to acknowledge that a single imputation of the value 2.5 is unlikely to approximate well the actual distribution of values of eNO below the detectable limit, it is clear that leaving these values as missing would ignore important information about the nature of their missingness, and would bias our results towards the null. The value 2.5 represents a best guess, avoiding extremes within the possible range of real values. Given the sensitivity of the eNO model to different imputed values of eNO, the results of this model should be interpreted with caution. In light of these limitations and the present findings, we offer five considerations:

First, that there are no significant relationships observed between air pollution and %GSSG in either multi-pollutant models or single pollutant models is consistent with

similar findings which showed that, in mouse models, combined diesel exhaust particle and house dust mite exposure had significant effects on the CYS redox state but no effect on the GSH redox state, which suggests that the CYS redox state may be a better biomarker for oxidative stress induced by diesel exhaust particles and allergens [39].

Second, the presence of unmeasured factures could have affected the results. the study by Lee et al., suggests that diesel exhaust particles alone do not significantly alter the redox balance among mice, but that in combination with allergens, diesel exhaust can induce oxidative stress and may amplify the cellular inflammatory response [39]. The present study did not measure or control for the presence of allergens and thus the possibility of a synergistic relationship between allergens and pollution exposure could introduce bias. Another unmeasured factor that may lead to variability in redox status after exposure to particulate matter is the oxidative potential of the specific mix of particles inhaled at the time of exposure. Several studies have demonstrated that for a given mass concentration of particulate matter the oxidative potential can vary according to the composition, particularly the presence of redox-active metals, which will be affected by proximity to roadways and other sources of particulate pollution [22], [60], [61].

Third, while the associations between pollutant dose and markers of oxidative stress are the opposite of what was hypothesized, the negative relationship between air pollution and percent of oxidized compounds may signal the predominance of a protective antioxidant response to oxidative stress induced by increasing O_3 dose [22]. These findings are consistent with other research that has shown a nonsignificant increase in CYS, and a corresponding decrease in %CYSS after diesel exhaust exposure in mice when compared to saline exposure [39]. Similarly, Behndig et al. observed an early adaptive increase in the antioxidant GSH in both the bronchial lavage and the alveolar compartment within six hours of diesel exhaust particle exposure. This increase in antioxidants was subsequently overwhelmed and followed by the development of an inflammatory response in the bronchial lavage but not in the alveolar compartment [36]. The authors offer the explanation that within the alveolar compartment, deeper into the airway, the tissue particle doses are lower, and thus the cells' adaptive antioxidant response can cope with the onslaught of oxidants, demonstrating a dose threshold for respiratory response to diesel exhaust [36].

Fourth, with a few exceptions, single pollutant and multi-pollutant models reflected similar significant relationships between air pollutant inhaled doses and outcomes, though varying slightly in effect size. That $PM_{2.5}$ showed significant relationships with markers of oxidative stress in single pollutant models, but not in multi-pollutant models, may reflect that in the single pollutant model, the relationship between $PM_{2.5}$ and oxidative stress is confounded by O_3. The degree of correlation between $PM_{2.5}$ and O_3 is moderate, with Pearson's r=0.67. This suggests that multi-pollutant models may be necessary in order to truly evaluate the separate effects of each air pollutant, holding all other pollutant levels constant. However, this information comes with a cost, namely, the increased number of parameters in multi-pollutant models sacrifice power and increase the chance of a type II error. Thus, it is also possible that both types of air pollution have relationships with oxidative stress but we were not able to measure it. Furthermore, in the multi-pollutant models explored in this analysis, BC was not included because of the high rate of missing data for this pollutant. In addition to black carbon, other types of air

pollutants and interactions between pollutants were not examined in this analysis. Future research that is adequately powered to examine a wider range of pollutants and interactions between pollutants in a single multivariable model would help to tease apart the individual effects of each different pollutant.

Fifth, in the present study an increase in PNT is associated with both an apparent increase in the antioxidant CYS and with airway inflammation marked by an increase in eNO, suggesting that high concentration doses may have overwhelmed the antioxidant response. The present study only considers the total dose over a period of several hours, and as such ignores variability in dose concentration over the exposure period. However, someone who inhales a high cumulative dose despite a low activity level is likely breathing in a higher concentration of air pollution in a shorter period of time than a person who receives the same dose with a high activity level, thus the differences seen according to activity level may actually reflect differences in dose concentration over time. The moderating effects of activity level on eNO and %CYSS suggest that peaks of high concentration inhaled doses of air pollution may overwhelm cells' endogenous redox balance resulting in increased airway inflammation. Further research that examines the relationships between dose peaks at the minute level and oxidative stress and inflammation over time could help to determine whether a high concentration dose over a short period of time has a different effect than a lower concentration dose over a longer period of time.

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