Ambient combustion by-product exposures and exhaled biomarkers of airway inflammation and oxidative stress

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ABSTRACT

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Introduction: Numerous studies have found associations between exposure to airborne particulate matter and respiratory morbidity and mortality. However, the varying composition, dependant on the different sources of particulate matter, and its effect on processes of inflammation and oxidative stress in the airways has not been completely elucidated. The use of airway biomarkers, fractional exhaled nitric oxide (FENO) and exhaled breath condensate (EBC), can provide valuable insight into processes of inflammation and oxidative stress in the airways. In these studies we sought to characterize the associations between airborne pollutant exposures and their sources and airway biomarkers.

Methods: The study population of interest for Chapters III-V is a subset of children currently enrolled in a birth cohort under the Columbia Children's Center for Environmental Health. Chapter III refers to the validation in this population of a method for partitioning FENO contributions from the proximal and distal airways. Chapters IV and V refer to the implementation of this method in the study of ambient metals and residential proximity to relevant sources of particulate matter (PM) and black carbon (BC). Briefly, the children of African-American and Dominican mothers living in low-income NYC neighborhoods had FENO samples collected offline at constant flow rates of 50, 83 and 100 mL/sec at ages 9 and/or 11. Surrogate measures for bronchial flux NO (JNO) and alveolar (Calv) NO concentrations were estimated using a previously validated mathematical model. Wheeze in the last 12 months was assessed by the International Study of Asthma and Allergies in Childhood (ISAAC)

questionnaire. Seroatopy was determined by specific IgE at age 9. For Chapter IV, in order to examine the associations between metal fractions of particulate matter and airway inflammation, ambient measures of Ni, V, Zn and Fe were obtained from a local central monitoring site and averaged over nine days based on three 24 hour measures every third day. Seroatopy was determined by specific IgE at age 7. For Chapter V, residential distance to a major road, truck route and bus stop density, area covered by major roads, stationary point sources (SPS), toxic release inventory sites (TRIS) and commercial buildings, and number of buildings burning residual oil within 250-meters of each child's home were determined. These variables were selected a priori as potential important sources or airborne PM and BC. For Chapter VI, the study population was comprised of seven- and eight-year-old children enrolled in an asthma case-control study in New York City. Seven day averages of domestic levels of particulate matter <2.5 microns ($PM_{2.5}$), BC and environmental tobacco smoke (ETS) were measured. Urea and 8-isoprostane were measured by liquid chromatography tandem mass spectrometry (LC/MS/MS) in EBC collected during home visits. All data were analyzed with SPSS.

Results: In our first study, children with seroatopy had significantly higher median JNO $p<0.001$) when compared to non-seroatopic children; however, median Calv was not significantly different between these two groups ($p=0.644$). Children with wheeze in the past year had significantly higher median Calv ($p<0.001$), but not JNO (295 vs. 165 pL/s, $p=0.241$) when compared with children without wheeze. In our second study, ambient V and Fe concentrations were associated positively with $FENO_{50}$ ($p=0.018$, $p=0.027$). Ambient Fe was associated positively with JNO ($p=0.017$). Ambient Ni and V concentrations were associated positively with Calv ($p=0.004$, $p=0.018$ respectively). A stronger association of Ni concentrations with Calv was observed among the children with seroatopy. In our third study,

there were no significant associations between any of the air pollution indicator variables and FENO parameters in adjusted models.

In our final study, $PM_{2.5}$ and BC, but not ETS, were significantly associated with increases in 8-isoprostane ($p<0.05$ for both) after adjustment for covariates. In a co-pollutant model including $PM_{2.5}$, BC and ETS, only BC remained a statistically significant predictor of 8-isoprostane.

Conclusions: Recent exposure to airborne pollutants was associated with increased levels of biomarkers of airway inflammation and oxidative stress measured in exhaled breath. The metal and BC fractions of PM might be more relevant to the understanding adverse respiratory outcomes related to air pollution exposure.

Table of Contents

List of figures and tables iv
List of abbreviations vi
Acknowledgements viii
Dedication ix
Preface x
Chapter I. Introduction 1
Chapter II. Background 2
Particulate matter and combustion by-products 2
Geographic information systems (GIS) based variables 2
Airway inflammation: Fractional exhaled Nitric Oxide (FENO) 2
Biological role of NO 2
Exhaled NO in epidemiological research 2
Exhaled breath condensate (EBC) 2
8-isoprostane as a marker of oxidative stress 2
References 2
Chapter III. Fractional exhaled nitric oxide exchange parameters among nine year-old inner-city children 3
Abstract 3
Introduction 3
Materials and Methods 3
Study Population 3
Procedures 3
Data Analysis 3
Results 3
Correlations between flow independent and dependent NO parameters 3
Flow dependent and independent NO parameters by demographics, seroatopy and wheeze . 3
Multivariable models 3
Exclusion of non-linear flow independent parameters 3
Discussion 3
References 3

Chapter IV: Association of recent exposure to ambient metals with fractional exhaled nitric oxide in 9-11 year old inner-city children 4
Abstract 4
Introduction 4
Materials and Methods 4
Study population 4
Measures of exposure 4
FENO parameters: $FENO_{50}$, JNO and Calv 4
Statistical analyses 4
Results 4
Characteristics of cohort and pollutant concentrations 4
Associations between Ni, V, Zn and Fe and FENO parameters 4
Stratification by seroatopy 4
Discussion 4
References 4
Chapter V: GIS indicators of traffic and stationary sources of air pollution and parameters of airway inflammation 5
Abstract 5
Introduction 5
Materials and Methods 5
Study population 5
FENO parameters: $FENO_{50}$, JNO and Calv 5
Data analysis 5
Results 5
Discussion 5
References 5
Chapter VI: Domestic airborne black carbon levels positively associated with 8-isoprostane in exhaled breath condensate among children in New York City 6
Abstract 6
Introduction 6
Materials and Methods 6

Study population 6
Exposure Assessment 6
Statistical Analyses 6
Results 6
Discussion 6
References 6
Chapter VI: Conclusions and future directions 7

List of Tables and Figures

Chapter II

Figures

Figure 1. Two-compartment model of nitric oxide exchange parameters 9

Figure 2. Respiratory droplets released and collected in condenser 10

Chapter III

Figures

Figure 1. Correlation between calculated (1a) bronchial fluxes and alveolar concentrations (1b) using the Hogman and Pietropaoli methods.....22

Figure 2. Receiver Operation Characteristics (ROC) curves for (a) seroatopy and (b) current wheeze. 24

Figure 3. Bronchial flux comparison (a) and alveolar concentration comparison (b) by current wheeze and seroatopy status. 30

Tables

Table 1. Study cohort demographic information 21

Table 2. Flow dependent and independent NO parameters medians (25^{th}-75^{th} percentile) by demographic characteristics. 26

Table 3. Flow dependent and independent parameter medians (25^{th} – 75^{th} percentile) by medication use, respiratory symptoms and seroatopy.....27

Table 4. Flow dependent and independent parameter medians (25^{th} – 75^{th} percentile) by medication use, respiratory symptoms and seroatopy including only steroid naïve subjects …28

Table 5. Adjusted logistic regression models for flow dependent and independent parameters ….....29

Table S1. Adjusted logistic regression models for flow dependent and independent parameters.. 39

Table S2. Predictors of current wheeze symptoms 39

Chapter IV

Figures

Figure 1. Association between ambient Ni and FENO parameters modified by seroatopic status.....52

Figure S1. Associations between ambient Ni, V, Zn, and Fe concentrations and $FENO_{50}$ 66

Figure S2. Associations between Ni, V, Zn and Fe concentrations and bronchial flux (JNO)..67

Figure S3. Associations between Ni, V, Zn and Fe concentrations and alveolar NO (Calv) 68

Tables

Table 1. Selected cohort characteristics 49

Table 2. Adjusted linear regression models for associations between ambient pollutants and FENO measures β (95% CI)....51
Table S1. Spearman correlation matrix for pollutants....65
Table S2. Spearman correlation matrix for NO measurements....65

Chapter V

Tables

Table 1. Comparison of demographic characteristics between included and excluded participants....78
Table 2. Comparison of GIS variables during prenatal period between included and excluded participants....79
Table 3. Multivariable models for airborne pollutant source variables and FENO parameters [β (95% CI)]....81
Table 4. Multivariable models for airborne pollutant source variables and FENO parameters stratified by sex [β (95% CI....82
Table 5. Multivariable models for airborne pollutant source variables and FENO parameters stratified by race/ethnicity [β (95% CI)]....83
Table 6. Multivariable models for airborne pollutant source variables and FENO parameters stratified by seroatopy [β (95% CI)]....84
Table 7. Multivariable models for airborne pollutant source variables and FENO parameters stratified by wheeze [β (95% CI)]....85
Table S1. Multivariable GEE models for airborne pollutant source variables and FENO parameters [β (95% CI)]....94
Table S2. Spearman correlation matrix for FENO parameters†....95

Chapter VI

Figures

Figure 1. Scatterplots for all domestic pollutant measures....105
Figure 2. Associations between BC, $PM_{2.5}$ and ETS domestic measures and 8-isoprostane in EBC (log-transformed) in a multivariable model....106
Figure 3. Associations between BC, $PM_{2.5}$ and ETS domestic measures and 8-isoprostane in EBC (log-transformed) in a multivariable co-pollutant model....107

Tables

Table 1. Demographic characteristics....104
Table 2. Generalized estimating equations models for associations between selected GIS variables, neighborhood pollutant averages and 8-isoprostane....108
Table S1. Selected cohort characteristics....116

List of Abbreviations

BC: Black carbon

Calv: Alveolar nitric oxide

CCCEH: Columbia Center for Children's Environmental Health

DEP: Diesel exhaust particles

EBC: exhaled breath condensate

EC: Elemental carbon

EPA: Environmental Protection Agency

ETS: Environmental tobacco smoke

FENO: Fractional exhaled nitric oxide

GEE: Generalized estimating equations

GIS: Geographic information systems

HIP: Health Insurance Plan of New York

ICS: Inhaled corticosteroids

IgE: Immunoglobulin E

ISAAC: International Study of Asthma and Allergies in Childhood

JNO: Bronchial flux

LC/MS/MS: Liquid chromatography tandem mass spectrometry

NO: Nitric oxide

NYCCAS: New York City Community Air Survey

NYC NAAS: New York City Neighborhood Asthma and Allergy Study

PAH: Polycyclic aromatic hydrocarbons

$PM_{2.5}$: Particulate matter <2.5 microns in diameter

ROC: Receiver operation characteristics

SPS: Stationary point sources

TRIS: Toxic release inventory sites

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Dedication

Para mi mamá

Preface

The use of fractional exhaled nitric oxide (FENO) and biomarkers measured in exhaled breath condensate (EBC) allows for the non-invasive sampling of the airways. These biomarkers can be used as proxy measures that can provide valuable insight into processes of inflammation and oxidative stress in the airways which are relevant to the study of environmental exposures. This dissertation centers on the use of FENO and EBC in the study of the effects of ambient environmental exposures in New York City. It is comprised of an introduction that includes hypotheses, specific aims and a general background, four separate studies addressing each of the stated specific aims, a conclusion and a future directions section. The first study (Chapter 3) addresses the feasibility of implementing a collection method of FENO, a biomarker of airway inflammation, to partition NO contributions from the proximal and distal airways in pediatric subjects enrolled in a prospective birth cohort. After assessing feasibility, the second objective of this study is to describe the association between these parameters of airway inflammation and allergic and respiratory outcomes in the cohort.

The second and third studies (Chapters 4 and 5) utilize this method of partitioning NO contributions in the study of different airborne pollutant exposures in this same prospective cohort. In Chapter 4, I analyze the associations between metal fractions of airborne fine particulate matter, measured at a central site, and these parameters. I also examine the potential for effect modification in these associations by seroatopic status. In Chapter 5, I focus on geographic information systems (GIS) variables as surrogate markers of airborne pollutant exposure sources and their association with NO parameters. In Chapter 6, I determine the association between domestic measures of black carbon and a marker of oxidative stress 8-isoprostane, measured in EBC in children enrolled in a case-control study of asthma.

The background chapter of the dissertation provides information on the exposures of interest, biology and relevance of nitric oxide and 8-isoprostane production in humans, and the methods utilized to collect and measure them in epidemiological studies. Each stand-alone manuscript also contains a specific introduction, methods and discussion section. The conclusion section aims to summarize the findings from each study and how they fit into the greater context of environmental health research.

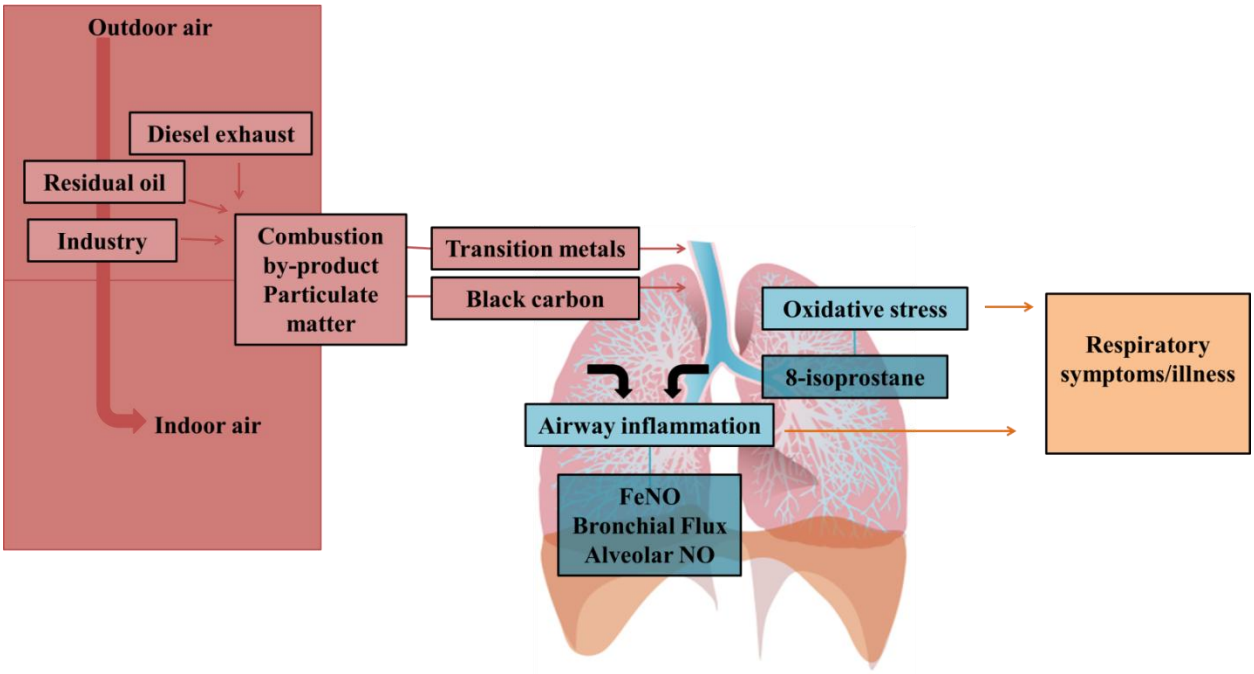

Figure 1. Overall paradigm of dissertation work

Chapter I: Introduction

Statement of hypotheses

This dissertation is an epidemiological investigation of the associations between traffic and other airborne pollutant exposures in New York City and exhaled biomarkers of airway inflammation and oxidative stress in children. Previous epidemiological research has found associations between exposure to airborne particulate matter and respiratory morbidity and mortality. However, the varying composition of particulate matter and its effect on processes of inflammation and oxidative stress in the airways has not been completely elucidated and these studies aim to fill these knowledge gaps. The unifying hypothesis behind this research is: **Current exposure to airborne pollutants will be associated with increased parameters of airway inflammation and oxidative stress measured in exhaled breath.**

Hypothesis 1

We hypothesize that parameters of proximal and distal inflammation will be differentially associated with current respiratory outcomes and seroatopic status.

Specific aim 1a

We will determine the feasibility of implementing a fractional exhaled nitric oxide (FENO) collection method to partition contributions from proximal and distal airways in an inner-city cohort.

Specific aim 1b

We will use logistic regression to determine the association between proximal and distal NO contributions and seroatopic status, by measurement of specific immunoglobulin E (IgE) in sera and questionnaire-based respiratory outcomes.

Hypothesis 2

We hypothesize that recent exposure to transition metals measured in particulate matter <2.5 microns in diameter ($PM_{2.5}$), will be associated with elevated parameters of proximal and distal inflammation. We also predict that the association between ambient metals and inflammation parameters will be modified by seroatopic status.

Specific aim 2

We will build multivariable linear regression models to determine the association between measures of $PM_{2.5}$ fractions of nickel (Ni), vanadium (V), zinc (Zn) and iron (Fe) collected at a central site located in the study area and proximal and distal fractions of FENO. We will also stratify the regression models by seroatopic status in order to determine a potential effect modification on these associations.

Hypothesis 3

We hypothesize that greater concurrent proximity and density of GIS indicator variables of traffic and other airborne pollution sources will be associated with elevated parameters of proximal and distal inflammation. Specifically, we hypothesize that a greater number of buildings burning residual oil will be associated with increased levels of distal inflammation.

Specific aim 3a

We will use generalized estimating equations to determine the association between GIS indicators of traffic-related airborne pollutants: proximity to primary highways and major truck routes, and concurrent truck route density and bus stop counts with parameters of proximal and distal inflammation.

Specific aim 3b

We will build generalized estimating equations models to determine the association between GIS indicators of non-traffic related airborne pollutants: number of buildings burning residual oil, percentage area covered by stationary point sources, toxic release inventory sites and buildings inventoried for commercial use and parameters of proximal and distal inflammation.

Hypothesis 4

We predict that domestic measures of black carbon (BC) will be associated with elevated levels of 8-isoprostane, a marker of oxidative stress, measured in exhaled breath condensate.

Specific aim 4

We will use generalized estimating equations models to determine the association between BC measured in participants' homes and 8-isoprostane levels measured in exhaled breath condensate. We will also examine the association between $PM_{2.5}$ and environmental tobacco smoke (ETS) exposure and 8-isoprostane levels in separate models and in a co-pollutant model with BC.

Chapter II: Background

Numerous studies have found associations between exposure to ambient air pollution and respiratory morbidity and mortality (Jaakkola *et al.* 1991; Kagawa 1994; Morgenstern *et al.* 2008; Samet *et al.* 2000). However, gaps in knowledge remain about the specific components of ambient air pollution that lead to adverse respiratory outcomes. This knowledge is vital in the understanding of biological processes that may consequently lead to respiratory illness. The study of these associations can also provide information required to target interventions aimed at reducing ambient air pollution.

Particulate matter and combustion by-products

Airborne particulate matter (PM) <2.5 microns in diameter ($PM_{2.5}$) is a mixture of liquid droplets and solids that includes nitrates, sulfates, metals and organic compounds and its small size allows it to travel deep into the airways. Variation in the chemical composition of $PM_{2.5}$ has been deemed important in understanding $PM_{2.5}$-associated morbidity and mortality (Bell *et al.* 2007; Zhou *et al.* 2011). The chemical composition of PM is also known to vary spatially, temporally and by emission source (Bell *et al.* 2007; Li *et al.* 2004). Several chemical components of $PM_{2.5}$ have been associated with adverse respiratory outcomes including nickel (Ni), vanadium (V), iron (Fe), zinc (Zn) and black carbon (BC) (Bell *et al.* 2009; Hirshon *et al.* 2008; Ostro *et al.* 2009). These components are of particular interest in the study of adverse health effects in New York City (NYC), given the variety of local sources that emit them.

Residual fuel oils are residual byproducts of refined crude oil, which are burned extensively for heating of commercial and residential buildings throughout NYC. A previous study showed that the major source of Ni in NYC ambient air could be attributed to burning of residual oil

(Peltier *et al.* 2009). Burning of residual oil also contributes to V concentrations in air (Peltier *et al.* 2009). Density of residual oil burners was a significant predictor of wintertime street level BC measured by the NYC Community Air Survey (NYCCAS) and of domestic level BC measures in the NYC Neighborhood Allergy and Asthma Study (NYC NAAS) (Clougherty *et al.* 2013; Cornell *et al.* 2012). Power plants that emit Ni and V during electrical production and upwind emissions from vessel bunkering at Port Elizabeth and Port Newark in nearby New Jersey also contribute to Ni and V air levels in NYC (Peltier *et al.* 2009). Industrial land use was also a significant predictor of wintertime community levels of BC (Clougherty *et al.* 2013). Sources of ambient iron (Fe) in $PM_{2.5}$ include road dust and re-suspended soils (Li *et al.* 2004).

Motor vehicle emissions are also an important source of $PM_{2.5}$. Ambient Zn is produced through motor oil combustion and has been measured in vehicle emissions (Li *et al.* 2004). Total traffic was also significantly associated with wintertime BC as reported by the NYCCAS (Clougherty *et al.* 2013). DEP is mostly comprised of elemental carbon (EC), and BC has been proposed as are more suitable marker of diesel exhaust particles (DEP) than $PM_{2.5}$ given previous research that found associations with truck, but not car traffic and BC levels (Patel *et al.* 2009). In the NYC NAAS, truck route density in a 500-m radius was a significant predictor of domestic airborne BC (Cornell *et al.* 2012).

Geographic information systems (GIS) based variables

Geographic information systems (GIS) based variables, like roadway density and distance to major roadway, can serve as surrogate indicators of traffic and other air pollution exposures. Increased roadway density and increased proximity to roads have been shown to be associated with asthma and allergy (McConnell *et al.* 2006) (Baumann *et al.* 2011). Previous work in our

prospective cohort found significant associations between concurrent density of 4-way intersections and commercial building use and increased odds of wheeze (Patel *et al.* 2011). Concurrent proximity to highway and density of commercial building use also were associated with increased levels of total IgE (Patel *et al.* 2011). Even though GIS variables have been consistently used in the study of traffic related exposures, there are limited data on other potential sources of airborne pollution and their association with adverse respiratory outcomes. Other potentially relevant sources include toxic release inventory sites (TRIS) and stationary point sources (SPS). TRIS are required to provide information to the EPA on the release and disposal of over 650 toxic chemicals. SPS are comprised of facilities that release emissions containing any of the six Criteria Air Pollutants: ozone, particulate matter, carbon monoxide, nitrogen oxides, sulfur dioxide and lead (source EPA).

Airway inflammation: Fractional exhaled Nitric Oxide (FENO)

Biological role of NO

Nitric oxide (NO) is an inorganic free radical gas produced in the body by three different NO synthases from the amino acid L-arginine. Neuronal NO synthase (nNOS) and endothelial NO synthase (eNOS) are the calcium and calmodulin-dependent constitutive forms. nNOS is expressed in brain tissue and involved in nonadrenergic and noncholinergic neural responses while eNOS is expressed in endothelial cells and mediates vasodilation (Knowles and Moncada 1994). The third enzyme is the calcium and calmodulin independent inducible NO synthase (iNOS) which is expressed in a variety of cells in the airways: epithelial cells, alveolar macrophages, fibroblasts, endothelial cells, neutrophils and chondrocytes (Ricciardolo *et al.* 2004). NO production can be induced by the release of pro-inflammatory cytokines like

interferon (IFN)-γ (Barnes and Liew 1995). The combination of IFN-γ and interleukin (IL)-4 has been shown to stimulate the expression of iNOS in primary human epithelial cells (Guo *et al.* 1997). Transcriptional activation of iNOS has also been demonstrated after stimulation with exogenous factors like allergens, viral infections and environmental pollutants (Ricciardolo *et al.* 2004). NO is thought to function as a signaling molecule between macrophages and T cells, to aid in nonspecific host defense against pathogens (Barnes and Liew 1995). Even though the role of endogenous NO in the body is generally beneficial, in the context of asthmatic inflammation and allergic disease, NO production has been hypothesized to amplify and perpetuate the inflammatory response in the airways (Barnes and Liew 1995). There is some evidence that through the production of peroxynitrite, NO in the airways might act as a pro-inflammatory mediator (Dweik *et al.* 2001).

Exhaled NO in epidemiological research

NO was first measured through a chemiluminescence technique in the exhaled breath of rabbits, guinea pigs and humans in the early 1990s (Gustafsson *et al.* 1991). Subsequently, NO in exhaled breath was shown to be significantly higher in allergic, asthmatic subjects than non-allergic healthy controls (Alving *et al.* 1993). NO measured in exhaled breath is predominantly the product of iNOS activity in the airway epithelium (Lane *et al.* 2004). The hypothesis that NO helps amplify and perpetuate Th2 mediated inflammation is supported by previous work showing exhaled NO to be significantly correlated with IgE levels in blood and with eosinophilic inflammation measured in blood, bronchoalveolar lavage and sputum (Ho *et al.* 2000; Jatakanon *et al.* 1998; Silvestri *et al.* 1999; Warke *et al.* 2002).

Collection of FENO has been standardized for both adult and pediatric populations (Dweik *et al.* 2011). FENO can be collected "online", referring to breathing into a device that immediately produces a result or "offline" in which a breath sample is collected in a reservoir (typically a balloon) to be measured at a later time. Levels of NO in exhaled breath are determined by the various cells producing NO in the airways, NO diffusion into capillary circulation and alveolar ventilation and bronchial airflow (Hyde *et al.* 1997). A vast amount of research has utilized FENO in the study of asthma, but it has also been a useful tool in the study of other respiratory diseases like chronic obstructive pulmonary disease (COPD) (Maziak *et al.* 1998). Furthermore, exposure to a variety of indoor and outdoor airborne pollutants, including environmental tobacco smoke (ETS), black carbon (BC) and phthalates, have been associated with significant changes in levels of FENO (Cornell *et al.* 2012; Just *et al.* 2012; Nadif *et al.* 2010). FENO can be a reliable biomarker of eosinophilic inflammation and useful in the study of exposures associated with sub-clinical changes in airway inflammation.

The use of FENO can be further improved by its collection at multiple flow rates. NO concentrations in exhaled breath are highly dependent on exhalation flow rate; the higher the flow rate, the lower the FENO concentration. Taking advantage of this phenomenon, a two-compartment mathematical model of nitric oxide exchange dynamics was developed (Figure 1)(George *et al.* 2004). Exhaled NO is predicted as contributions from the alveolar region (compartment #1) and the airway region (compartment #2). NO from the alveolar region represents the balance between locally produced or inhaled NO and NO destroyed or diffused that reaches a steady state after a prolonged exhalation (>8 s) (George *et al.* 2004). The NO from the airway region represents the NO contribution as this alveolar air is convected through the airways during exhalation (George *et al.* 2004). By plotting the association between exhaled NO

output and exhalation flow rate we can estimate the NO derived from the alveoli (Calv, alveolar NO concentration) and the conducting airways (JNO, bronchial NO flux).

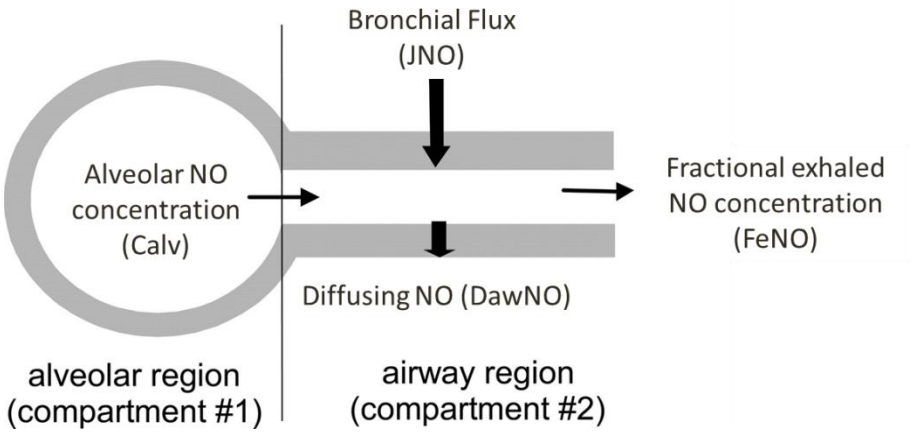

Figure 1. Two-compartment model of nitric oxide exchange parameters (Adapted from George *et al.* 2004)

Exhaled breath condensate (EBC)

Exhaled breath condensate (EBC) allows for the non-invasive collection of biomarkers that reflect the composition of bronchoalveolar extracellular lining fluid (Rosias *et al.* 2004). Respiratory droplets that form on the surface of the airways can be collected through exhalation into a condenser. Bronchoalveolar extracellular lining fluid droplets become aerosolized during exhalation. These droplets can then be captured along with water vapor in the condenser. However, there is extreme and variable dilution of droplets from lung lining fluid (Effros *et al.* 2002). The concentration of these droplets can only be estimated in EBC if an appropriate measurement of dilution by water vapor is made. Urea, which exists under homeostatic regulation in the body, has been used as a marker of dilution in EBC previously (Effros *et al.* 2003).

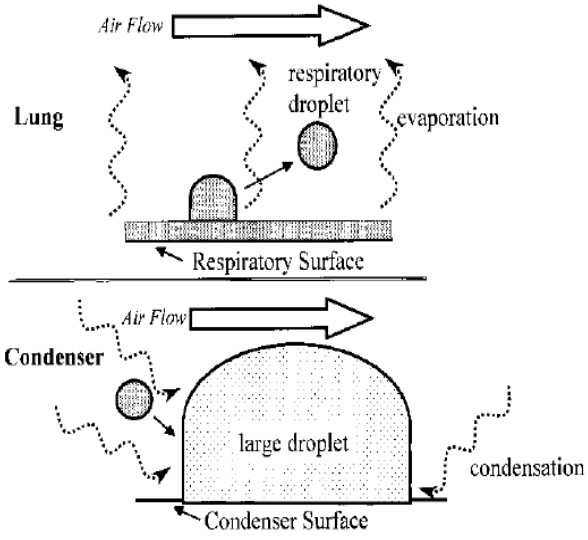

Figure 2. Collection of respiratory droplets through condenser (Effros *et al.* 2002)

8-isoprostane as a marker of oxidative stress

The measurement of a variety of biomarkers in EBC has been reported in the literature. These include biomarkers of oxidative stress, *i.e.* aldehydes, peroxide and isoprostanes and nitrosative stress, *i.e.* nitrite and nitrate. Isoprostanes are molecules that are structurally similar to prostaglandins and both types of molecules are generated from arachidonic acid. The F2 family of isoprostanes, which includes 8-isoprostane, are generated *in vivo* under oxidative stress conditions and have been shown to be relatively stable in EBC (Voynow and Kummarapurugu 2011). Because of the structural similarities between isoprostanes and prostaglandins, isoprostanes can activate prostanoid receptors and induce airway smooth muscle contraction (Shiraki *et al.* 2009). 8-isoprostane levels in EBC have been shown to be elevated in the presence of asthma (Baraldi *et al.* 2003a; Baraldi *et al.* 2003b) and after exposure to ambient air pollution (Patel *et al.* 2012).

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Chapter III: Fractional exhaled nitric oxide exchange parameters among nine year-old inner-city children

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Abstract

Objectives and hypothesis To determine the feasibility of using a multiple flow off-line fractional exhaled nitric oxide (FENO) collection method in an inner-city cohort and determine this population's alveolar and conducting airway contributions of NO. We hypothesized that the flow independent NO parameters would be associated differentially with wheeze and seroatopy.

Methods As part of a birth cohort study, nine-year old children (n=102) of African-American and Dominican mothers living in low-income NYC neighborhoods had FENO samples collected offline at constant flow rates of 50, 83 and 100 mL/sec. Seroatopy was defined as having measurable ($\geq$0.35 IU/ml) specific IgE to any of the five inhalant indoor allergens tested. Current wheeze (last 12 months) was assessed by ISAAC questionnaire. Bronchial NO flux (JNO) and alveolar NO concentration (Calv) were estimated by the Pietropaoli and Hogman methods.

Results Valid exhalation flow rates were achieved in 96% of the children. Children with seroatopy (53%) had significantly higher median JNO (522 vs. 161 pL/s, $p<0.001$) when compared to non-seroatopic children; however, median Calv was not significantly different between these two groups (5.5 vs. 5.8, $p=0.644$). Children with wheeze in the past year (21.6%) had significantly higher median Calv (8.4 vs. 4.9 ppb, $p<0.001$), but not JNO (295 vs. 165 pL/s, $p=0.241$) when compared with children without wheeze. These associations remained stable after adjustment for known confounders/covariates.

Conclusions The multiple flow method was easily implemented in this pediatric inner-city cohort. In this study population, alveolar concentration of NO may be a better indicator of current wheeze than single flow FENO.

Introduction

Fractional exhaled nitric oxide (FENO) is a useful non-invasive marker of airway inflammation in cohort studies assessing respiratory diseases. Currently, the accepted FENO collection method utilizes one constant exhalation flow rate, and both online and offline collection methods have been standardized by the American Thoracic Society (1999). However, this method fails to address relative contributions of NO from the peripheral and distal airways. Source attribution is important because FENO is reduced by inhaled corticosteroids (ICS) use and tobacco smoke exposure and more importantly, is elevated with atopy, reducing the specificity of this measure for respiratory symptoms in population based studies (Jones *et al.* 2002; Yates *et al.* 2001).

NO concentration in exhaled breath is highly dependent on exhalation flow rate; the higher the flow rate, the lower the FENO concentration. Taking advantage of this phenomenon, a mathematical model of nitric oxide exchange dynamics was developed that utilizes collection of breath at multiple exhalation flow rates to estimate the NO derived from the alveoli (Calv, alveolar NO concentration) and the airway (JNO, bronchial NO flux) (Tsoukias and George 1998). This new method has proved useful in identifying NO production sources in the airways of individuals with cystic fibrosis, chronic obstructive pulmonary disease and asthma (Brindicci *et al.* 2007; Brindicci *et al.* 2005; Suri *et al.* 2007). In a study of asthmatic and non-asthmatic children in the UK, when compared to healthy controls, the alveolar concentrations of NO were found to be higher among children with severe or poorly controlled asthma, but not among mild asthmatic or atopic, non-asthmatic children (Paraskakis *et al.* 2006).

The first objective of this study was to demonstrate the feasibility of using a multiple flow off-line collection method in nine-year-old children participating in an inner-city cohort

study. The second objective was to determine alveolar and conducting airway contributions of NO to exhaled breath in this population-based cohort. We hypothesized that the flow independent NO parameters would be associated differentially with wheeze in the past 12 months and seroatopy.

Materials and Methods

Study Population

Participants were enrolled in a prospective birth cohort study conducted by the Columbia Center for Children's Environmental Health (CCCEH). Enrollment has been described previously (Goldstein *et al.* 2005; Miller *et al.* 2001; Perzanowski *et al.* 2006). Briefly, non-smoking, healthy women ages 18-35, who self-identified as being of Dominican ethnicity or African American race and were living in Northern Manhattan and the South Bronx were recruited during pregnancy. Children who had a baseline home visit and a blood sample (cord, maternal or both) were considered fully enrolled (n=725). Among the children fully enrolled in CCCEH who have reached age 9 (n=181), 106 children participated in this study (i.e. 41% lost-to-follow-up for this analysis). All Columbia University Institutional Review Board procedures for consent and assent were followed.

Procedures

Breath samples were collected from children using a modified Sievers bag collection and sampling kit (GE Analytical Instruments, Boulder, CO), which allows for inhalation of air from an NO scrubber and exhalation against a back pressure to prevent contamination from ambient NO and the upper airways, respectively (Perzanowski *et al.* 2008). Samples were collected at three different flow rates, 50, 83 and 100 mL/s, selected because they were considered

achievable by a majority of the nine-year old children in this cohort. The lowest and highest flow rate samples were collected in duplicate and the middle flow rate samples in triplicate. Children were instructed to inhale NO free air through a scrubber. After inhalation, children exhaled for as long possible while maintaining the desired stable pressure. To account for dead space, the first part of the breath (approximately 1-3 seconds) was discarded using the divert valve. The rest of the breath was collected in a 1.5 liter Mylar® balloon. The approximate pressure measured in centimeters of water of each breath sample collection was recorded and converted to a flow rate in mL/s using a conversion equation provided by the device's manufacturer (flow in mL/s=21.127 x pressure in cm $H_2O^{\wedge 0.53306}$) (Sievers Instruments 2001). NO was measured with a chemiluminescent analyzer (NOA 280 i-2; Sievers Instruments; Boulder, CO). All balloons were read within 6 hours of collection time.

Mothers were asked if the child had taken any medication on the day of collection or in the previous 3 months. Questionnaires on detailed asthma and allergy related symptoms in the previous year were administered concurrently. Blood from the child was collected the same day. Specific IgE to cat, mouse urine, dog, *Dermatophagoides farinae* and cockroach were measured as previously described using the Immunocap system (Phadia, Uppsala, Sweden) (Donohue *et al.* 2008). Children were considered seroatopic if they had measureable IgE (>0.35 IU/ml) specific to any of the indoor allergens tested. For the 32 children with missing IgE values at age nine, age seven data were available on 23 children, and were used in the analyses. Data on specific IgE antibodies to mold, grass and ragweed at age seven were also available for 84 of the subjects.

Data Analysis

NO independent parameters were calculated using two previously published methods, the Pietropaoli and Hogman methods (Hogman *et al.* 2000; Pietropaoli *et al.* 1999). Due to a non-normal and non-log-normal distribution of the NO parameters, medians with 25% and 75% are reported and differences between medians were tested by the Mann-Whitney test. Correlations were tested by Spearman Rank test. Parameters also were compared using logistic regression models, adjusting for potential confounders and covariates. For the adjusted models, FENO collected at 50 ml/s ($FENO_{50}$), bronchial flux and alveolar concentrations were dichotomized into highest quartile and the three remaining quartiles. Maternal education, dichotomized on completion of high school at the time of the child's birth, was used as a proxy for socioeconomic status. Children with reported inhaled corticosteroid (ICS) use the day of the test were excluded from these analyses, because these medications are known to decrease both flow dependent and independent exhaled NO concentrations (Kharitonov *et al.* 1996; Paraskakis *et al.* 2006). Analyses of the associations were also conducted by excluding children who had a report of inhaled or oral steroid use in the previous 3 months but not on the day of the test. Data was analyzed using Microsoft Excel (Redmond, WA) and SPSS Version 16 (Chicago, IL).

Results

Study subject demographics are detailed in Table 1. Ninety-six percent of children (104/106) achieved a valid test as determined by inhalation through the collection device and exhalation at the desired flow rates. Two children reported ICS use the day of the test and were excluded from the analyses, resulting in 102 children for analyses. Seven additional subjects

reported inhaled (n=7) or oral (n=4) steroid use the past three months but not on the day of the test were included in the analyses (unless otherwise noted).

Table 1. Study cohort demographic information

Average age in years (min - max)	9.0 (8.9 -9.7)
Male sex, n (%)	45/102 (44)
Ethnicity/Race	
Dominican, n (%)	52/102 (51)
African American, n (%)	50/102 (49)
Mother had not completed high school at enrollment, n (%)	35/102 (35.4)
Maternal asthma, n (%)	22/102 (21.6)
Child current wheeze[a], n (%)	19/101 (18.8)
Child wheezed in the past 2 weeks, n (%)	2/101 (2.0)
Child seroatopic[b], n (%)	49/93 (53)

[a]Current wheeze was defined as reported wheeze in the past 12 months by the ISAAC questionnaire.
[b]Seroatopy was defined as specific IgE $\geq$ 0.35 IU/mL to cockroach, mouse urine, *d. farinae*, cat dander or dog.

Correlations between flow independent and dependent NO parameters

Despite employing different mathematical models, the Hogman and Pietropaoli methods provided estimates of the flow independent NO parameters that correlated well with one another (Figure 1). Therefore, only Hogman method parameters are reported in the subsequent analyses. $FENO_{50}$ correlated with both JNO (r=0.877, p=<0.001) and Calv (r=0.388, p=<0.001); however, there was no correlation between JNO and Calv (r=-0.003, p=0.997).

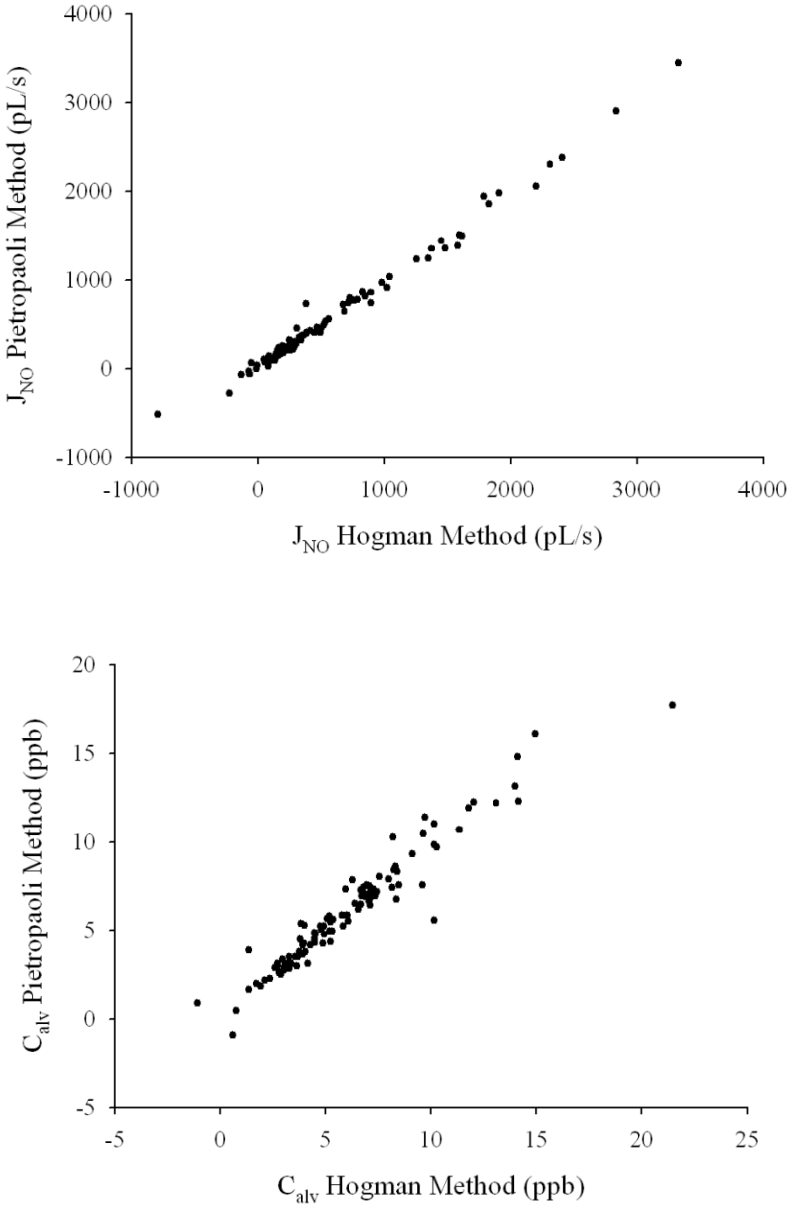

Figure 1. Correlation between calculated (1a) bronchial fluxes and alveolar concentrations (1b.) using the Hogman and Pietropaoli methods. There was a significant correlation for estimations of both bronchial fluxes and ($R=0.989$, $P=0.000$) and alveolar concentrations ($R=0.962$, $P=0.000$).

Flow dependent and independent NO parameters by demographics, seroatopy and wheeze

No significant differences in flow dependent or independent NO parameters were observed by sex or ethnicity/race. Children whose mother reported having asthma had significantly decreased JNO as compared with those children whose mothers did not report having asthma (Table 2).

Children with seroatopy had significantly elevated FENO levels at all three flow rates and elevated JNO when compared to children without seroatopy; however, there was no difference in Calv between these two groups (Table 3). Children with reported current wheeze had significantly elevated FENO at every flow rate when compared to children with no reported wheeze. In contrast to the findings for seroatopy, children with current wheeze had significantly elevated Calv when compared to children with no current wheeze, but there was no difference in JNO. Calv was also significantly elevated in children with reported use of asthma controller medications (e.g. inhaled corticosteroids, leukotriene modifiers) in the past year, and in those who had wheezed with exercise as compared with those who did not. Receiver operation characteristic curves (ROC) further demonstrate the lack of association between atopy and alveolar NO, but show Calv as a better predictor of current wheeze than FENO or bronchial NO flux (Figure 2).

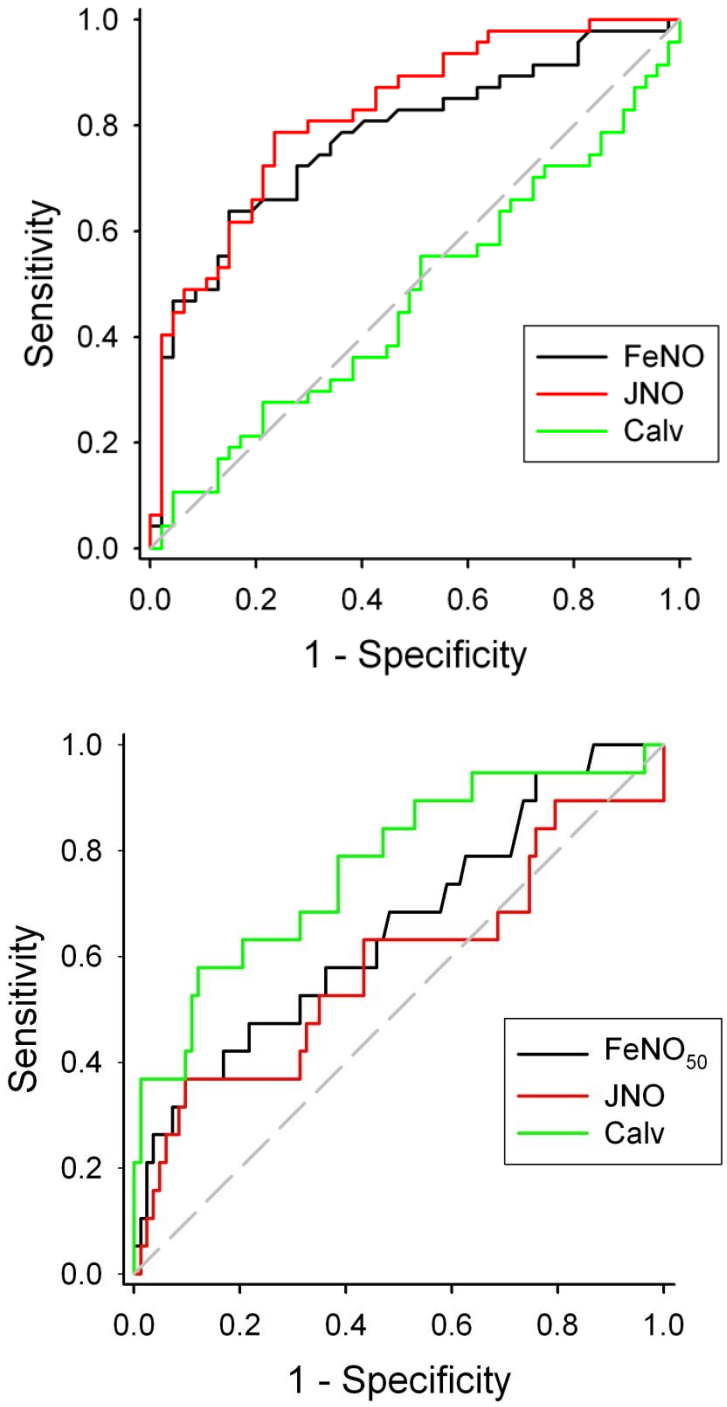

Figure 2. Receiver Operation Characteristics (ROC) curves for (a) seroatopy and (b) current wheeze. For seroatopy, the areas were 0.78 (P<0.001), 0.82 (P<0.001) and 0.47 (P=0.66), for $FENO_{50}$, JNO and Calv, respectively. For wheeze the areas under the curve were 0.66 (P=0.034), 0.58 (P=0.29), and 0.77 (P<0.001) for $FENO_{50}$, Bronchial NO flux (JNO) and alveolar NO (Calv), respectively.

The distributions of the FENO and Calv levels among the 7 children who had a report of steroid use in the past 3 months, but not on the day of the test were similar to the 'wheeze in the past 12 months' population in general with 3/7 and 4/7 having levels above the median for the wheeze group for $FENO_{50}$ and Calv, respectively. Similarly, the JNO among this group was similar to that for the seroatopic children with 3/7 having levels above the median for the seroatopic group. The presence or absence of significant differences in NO parameters by the symptoms in Table 3 for the most part were consistent after exclusion of these children from the analyses and are shown in Table 4, with the notable exception that JNO was statistically significantly higher among children with a report of cough at night when compared to those without (428 vs. 269 pL/s unit, p=0.005). Of the 84 children on whom data on specific IgE to mold, grass or ragweed was available, only three that were not previously classified as allergic were allergic to these allergens. These three children all had $FENO_{50}$ levels below the median for both the non-wheezers and the children without IgE to the indoor inhalant allergens,

JNO was significantly elevated in both wheezing and non-wheezing atopic children when compared to their non-atopic counterparts (Figure 3a). Calv medians were not significantly different between atopic and non-atopic children when stratified by reported wheeze (Figure 3b).

Table 2. Flow dependent and independent NO parameters medians (25^{th}-75^{th} percentile) by demographic characteristics.

			FENO by Flow Rate (ppb)			Flow independent NO[a]	
		n	50 mL/s	83 mL/s	100 mL/s	JNO (pL/s)	Calv (ppb)
Sex	Male	45	10.7 (7.9-22.8)	9.6 (6.5-17.2)	9.5 (6.5-14.1)	326 (137-810)	5.3 (3.7-7.5)
	Female	57	9.9 (7.9-21.5)	8.9 (7.9-21.5)	8.9 (6.0-14.5)	273 (141-695)	5.8 (3.8-7.9)
Ethnicity/Race	Dominican	52	10.4 (8.0-20.1)	8.7 (6.6-12.7)	8.8 (6.5-13.4)	265 (135-635)	6.2 (4.5-8.2)
	African American	50	12.5 (7.5-22.6)	9.9 (6.0-18.4)	9.8 (5.7-14.9)	302 (153-859)	4.7 (3.1-7.5)
Maternal Asthma	No	80	11.8 (8.0-22.1)	9.4 (6.3-16.1)	9.8 (6.3-15.4)	308 (172-782)*	5.2 (3.8-7.4)
	Yes	22	9.2 (6.7-13.9)	8.7(6.5-12.3)	8.8(5.8-11.0)	143 (52-469)	6.5 (4.5-8.3)
Mother completed high school	No	35	9.9 (8.0-16.5)	7.6 (6.0-12.9)	7.6 (6.3-10.7)	265 (158-685)	4.8 (3.3-6.7)
	Yes	64	11.8 (7.9-24.5)	9.6 (6.6-19.3)	9.9 (6.3-16.9)	304 (141-884)	6.0 (3.9-8.3)*

*P value $\leq$ 0.05 †P value $\leq$ 0.01 ‡P value $\leq$ 0.001

[a]Flow independent NO parameters calculated by the Hogman method (Hogman *et al.* 2000).

Table 3. Flow dependent and independent parameter medians (25^{th} – 75^{th} percentile) by medication use, respiratory symptoms[a] and seroatopy.

			Flow Dependent Parameters: FENO (ppb) by Flow Rate			Flow Independent Parameters	
		N	50 mL/s	83 mL/s	100 mL/s	JNO (pL/s)	Calv (ppb)
Current wheeze	No	82	10.2 (7.7-19.6)	8.6 (6.2-13.9)	8.2 (5.9-13.2)	269 (135-675)	4.9 (3.5-7.1)
	Yes	19	16.2 (9.1-41.1)*	13.2(8.6-28.2)†	17.0 (9.5-26.5)‡	389 (140-1595)	8.4 (5.9-13.1)‡
Asthma controller medication[b]	No	90	10.7 (7.9-20.4)	8.8 (6.3-14.6)	8.8 (6.1-13.3)	280 (155-693)	5.2 (3.7-7.2)
	Yes	11	10.6 (7.9-32.8)	12.1 (6.6-25.0)	17.0 (7.9-19.5)*	306 (107-1595)	8.4 (5.2-14.1)*
Woken up at night by cough	No	85	10.4 (7.8-20.7)	8.7 (6.3-14.7)	8.4 (6.0-13.4)	273 (141-679)	5.2 (3.7-7.4)
	Yes	13	16.8 (9.2-40.4)*	13.8 (9.6-28.9)*	16.5 (10.9-23.1)†	522 (137-1681)	6.7 (4.8-10.3)
Wheeze after exercise	No	84	10.6 (7.9-21.5)	9.1 (6.3-15.7)	8.9 (6.1-14.1)	295 (159-727)	5.3 (3.7-7.2)
	Yes	14	10.0 (7.8-34.5)	10.5 (6.7-25.8)	10.9 (6.6-21.2)	165 (103-1488)	6.9 (4.9-10.2)*
Seroatopy[c]	No	44	9.0 (7.3-11.5)	7.4 (6.1-9.7)	7.5 (5.8-10.1)	161 (80-271)	5.5 (3.8-7.8)
	Yes	49	19.5 (10.2-30.4)‡	13.8 (7.3-22.9)‡	12.3 (7.4-19.0)‡	522 (269-1360)‡	5.8 (3.2-8.2)

[a]All symptom Data were assessed through ISAAC questionnaire. Current wheeze was defined as reported wheeze in the past 12 months in ISAAC questionnaire.[b]Asthma controller medications include leukotriene modifiers, inhaled or oral steroids. [c]Seroatopy was defined as specific IgE ≥ 0.35 IU/mL to cockroach, mouse urine, *d. farinae*, cat dander or dog. Among the 92 children with IgE measured at age 7 and age 9 years, total IgE values for the same children correlated well (R=0.829, P<0.001) and 88% of the children considered seroatopic at age nine also were considered seroatopic at age 7. *P value ≤ 0.05, †P value ≤ 0.01 , ‡P value ≤ 0.001

Table 4. Flow dependent and independent parameter medians (25^{th} – 75^{th} percentile) by medication use, respiratory symptoms[a] and seroatopy including only steroid naïve subjects

			Flow Dependent Parameters: FENO (ppb) by Flow Rate			Flow Independent Parameters	
		N	50 mL/s	83 mL/s	100 mL/s	JNO (pL/s)	Calv (ppb)
Current wheeze	No	81	9.9 (7.7-18.1)	8.6(6.2-13.8)	8.2 (5.9-12.8)	265 (134-618)	4.9 (3.5-7.1)
	Yes	13	21.5 (9.4-44.1)*	15.9(8.9-29.0)†	18.1 (8.2.5-27.6)†	467 (244-1620)*	8.3 (5.6-12.6)†
Asthma controller medication[b]	No	90	10.7 (7.9-20.4)	8.8 (6.3-14.6)	8.8 (6.1-13.3)	280 (155-693)	5.2 (3.7-7.2)
	Yes	4	15.6 (7.0-49.5)	14.4 (5.7-35.4)	12.9 (4.8-30.7)	401 (181-1858)	8.4 (2.3-14.2)
Woken up at night by cough	No	82	9.9 (7.7-20.1)	8.5 (6.2-14.1)	8.2 (5.9-13.2)	269 (143-590)	5.2 (3.7-7.4)
	Yes	9	27.7 (14.9-40.4)†	19.6 (11.9-28.9)†	16.5 (11.0-23.1)†	1256 (427-1828)†	5.9 (4.2-7.2)
Wheeze after exercise	No	82	10.5 (7.9-21.5)	8.8 (6.3-15.4)	8.8 (6.1-13.9)	295 (160-721)	5.2 (3.7-7.2)
	Yes	9	10.8 (7.4-28.9)	10.4 (6.6-21.0)	9.6 (6.1-19.2)	183 (125-987)	6.0 (4.8-9.0)
Seroatopy[c]	No	40	8.7 (7.1-12.3)	7.1 (5.9-9.6)	7.4 (5.7-10.0)	180 (85-291)	5.2 (3.8-7.2)
	Yes	46	16.6 (9.9-28.1)‡	13.3 (7.2-21.9)‡	12.0 (6.9-18.2)‡	482 (261-1094)‡	5.8 (3.1-8.2)

[a]All symptom data was assessed through ISAAC questionnaire Current wheeze was defined as reported wheeze in the past 12 months in ISAAC questionnaire. [b]Asthma controller medications include leukotriene modifiers, inhaled or oral steroids. [c]Seroatopy was defined as specific IgE ≥ 0.35 IU/mL to cockroach, mouse urine, *d. farinae*, cat dander or dog. Among the 92 children with IgE measured at age 7 and age 9 years, total IgE values for the same children correlated well (R=0.829, P<0.001) and 88% of the children considered seroatopic at age nine also were considered seroatopic at age 7. *P value ≤ 0.05, †P value ≤ 0.01 , ‡P value ≤ 0.001

Multivariable models

Seroatopy was significantly associated with having an $FENO_{50}$ in the highest quartile, an association which remained after adjustment for wheeze, sex, ethnicity/race, maternal asthma and education, and ambient NO levels (Model 1, Table 5). Seroatopy remained significantly associated with $FENO_{50}$ when the analyses were limited to the steroid naïve children (P=0.02). In a model controlling for the same covariates, including wheeze, seroatopy was significantly associated with elevated JNO (Table 5), an association was also observed among the steroid naïve children (P=0.001). In a third model with Calv in the highest quartile as the dependent variable, only current wheeze was a statistically significant predictor (Table 5), and this association remained when only the steroid naïve children were examined (P=0.019). Calv in the highest quartile was a significant predictor of current wheeze (OR 7.3; 95% CI 2.4-22.7; P<0.001), after adjustment for seroatopy, JNO and other covariates.

Table 5. Adjusted logistic regression models[a] for flow dependent and independent parameters

Covariates	Model 1 $FENO_{50}$ OR (95%CI)	Model 2 JNO OR (95%CI)	Model 3 Calv OR (95%CI)
Current wheeze	3.3 (0.76-14.7)	2.0 (0.50-8.4)	5.8 (1.7-19.6)†
Seroatopy	40.0 (4.7-338.6)†	19.2 (3.9-94.6)‡	1.0 (0.34-2.9)
Sex	1.2 (0.36-4.0)	1.5 (0.49-4.8)	0.7 (0.22-2.0)
Ethnicity/Race	3.3 (0.97-11.4)	3.4 (1.1-10.8)*	1.1 (0.39-3.2)
Maternal Asthma	0.6 (0.11-3.3)	0.57 (0.11-2.8)	1.1 (0.28-4.0)
Maternal education at enrollment	2.1 (0.53-8.4)	1.2 (0.33-4.2)	2.6 (0.72-9.0)
Ambient NO	1.0 (1.0-1.0)	1.0 (1.0-1.0)	1.0 (1.0-1.0)

[a]All models adjusted for all listed covariates.
*P value ≤ 0.05, †P value ≤ 0.01 , ‡P value ≤ 0.001

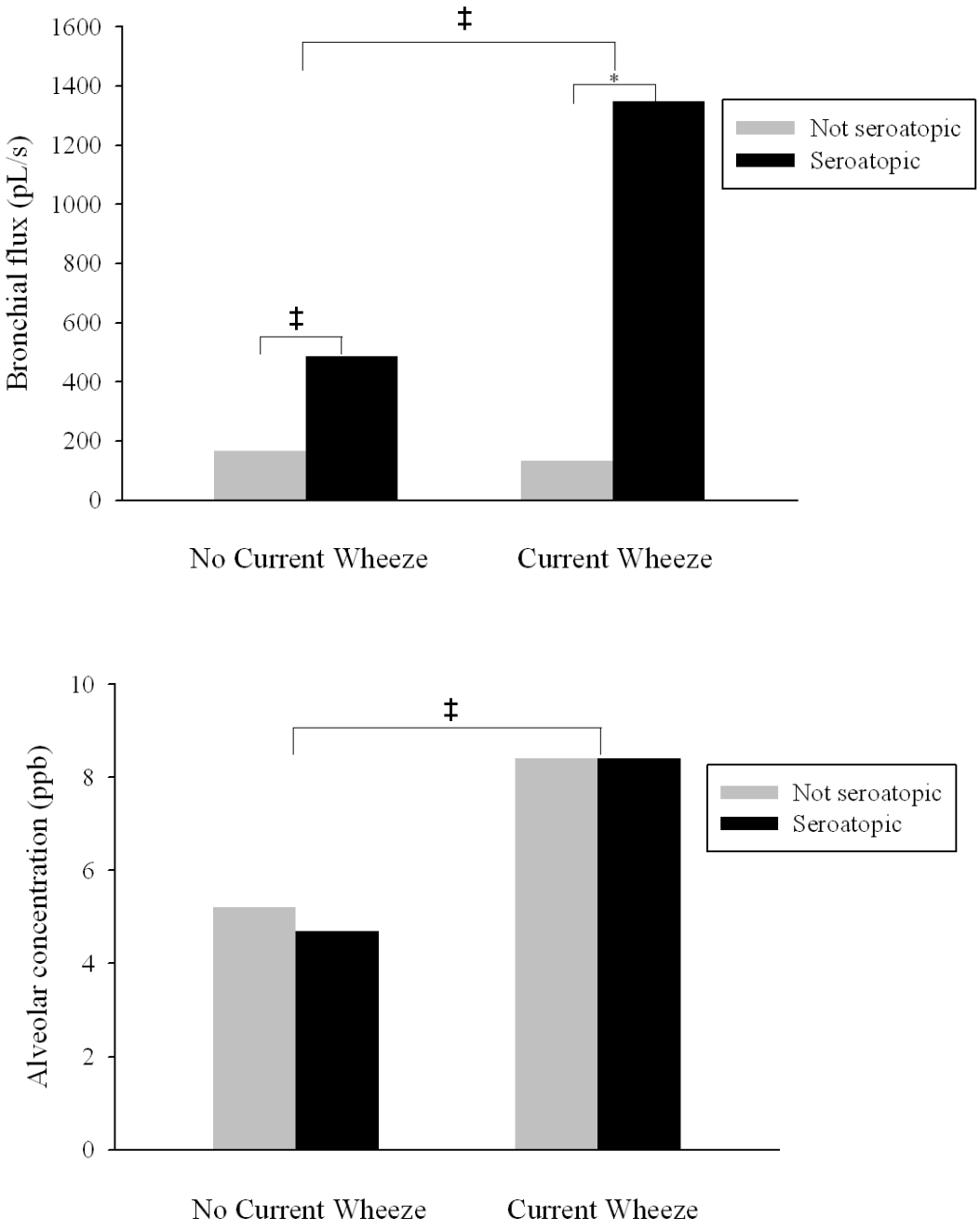

Figure 3. Bronchial flux comparison (a) and alveolar concentration comparison (b) by current wheeze and seroatopy status. *P value ≤ 0.05 †P value ≤ 0.01 ‡P value ≤ 0.001

Exclusion of non-linear flow independent parameters

Even though the majority of the children in our cohort successfully performed the multiple flow maneuvers, some of the measurements did not exhibit the expected positive linear relationship as described by Hogman and Pietropaoli. We chose an r^2 value of greater than 0.8 as a cutoff for our definition of linearity. Twenty-two percent (i.e. 22/102) of the children had Hogman models with an r^2 less than 0.8. Having a linear model with an r^2 <0.8 was significantly more common for children with high (>20ppb) as compared with low (<20 ppb) $FENO_{50}$ (36.7% vs. 15.35%, respectively, p=0.017). To determine whether this nonlinearity affected our findings, the adjusted logistic regression models were performed excluding children with an r^2< 0.8. The magnitudes of the associations were similar and in the same direction (online supplement tables E-1 and E-2).

Discussion

In this inner-city cohort of 9 year-old children, we found that the multiple-flow offline collection method was easily implemented, with a majority of the children (96%) achieving the target flow rates. Even though the Hogman and Pietropaoli methods utilize different calculations to estimate bronchial flux and alveolar concentration, their parameters correlated well. All FENO values were highly correlated with JNO and to a lesser degree with Calv. However, we found that there was no correlation between Calv and JNO values, which has been shown previously in pediatric studies of asthma and cystic fibrosis studies (Paraskakis *et al.* 2006; Suri *et al.* 2007). FENO values at every flow rate were associated with both seroatopy and respiratory symptoms. JNO rates were strongly associated with seroatopy and less so with respiratory symptoms. In contrast, alveolar NO concentrations were only associated with wheeze and not with seroatopy. These associations held after adjusting for sex, ethnicity/race, maternal asthma and education,

seroatopy and ambient NO. Furthermore, Calv was a better predictor of current wheeze than seroatopy or bronchial NO flux in a multivariate model. The lack of correlation between JNO and Calv and the differential associations between these parameters and seroatopy and respiratory symptoms strengthen the proposal that JNO and Calv provide distinct, clinically relevant outcomes for cohort studies.

Our findings of elevated alveolar NO among children with respiratory symptoms but not among children with atopy replicate those from a study of children in the UK (Paraskakis *et al.* 2006). Novelties of our findings come from the population-based study design and their recruitment from high asthma prevalence neighborhoods in New York City. With respect to the former study characteristic, while the prevalence of wheeze in this cohort was relatively high (i.e. 19%), the majority of the asthmatics appeared to be mild asthmatics (e.g. although 90% had a physician diagnosis of asthma, only 2% of the children had wheezed in the past 2 weeks). In the UK study, elevated Calv was observed only among the severe and uncontrolled asthmatics and not the mild asthmatics. Similarly, a clinic based study of adult asthmatics in the UK observed elevated Calv among refractory asthmatics and not among mild asthmatics when compared with controls (Berry *et al.* 2005). So our findings from this inner-city community are in contrast to UK findings since we observed significantly higher alveolar NO among relatively mild asthmatics. While future studies are needed to confirm these differences, they suggest that the level of distal airway inflammation may be higher in the inner-city U.S. mild asthmatic population than in other urban mild asthmatic populations where the prevalence of asthma is also high.

Many studies have demonstrated the importance of distal airway inflammation to asthma morbidity, including its association with airway hyper-responsiveness, exacerbation of

symptoms and tissue remodeling (Kraft *et al.* 1996; Kraft *et al.* 2001; Martin 2002). Support for alveolar NO as a marker of distal airway inflammation comes from studies showing an association between the number of eosinophils in bronchial alveolar lavage (BAL) and alveolar NO in contrast to the number of eosinophils in sputum and bronchial fluid that have been more closely correlated with conducting airway NO (Berry *et al.* 2005). Further evidence of the association between alveolar airway inflammation and distal airway pathology comes from a study of children with refractory asthma, that found a correlation between alveolar NO and lung function expiratory mid-flows (MEF25-75) (Mahut *et al.* 2004). This same study found differential associations between the flow independent parameter of NO and markers of airway remodeling. Alveolar NO correlated with TGF-β in BAL fluid, while airway NO correlated with basement membrane thickness (Mahut *et al.* 2004). With all of these studies, the cross-sectional design and study populations consisting of only relatively severe asthmatics limit the interpretation of the role of NO in airway inflammation and remodeling, but do suggest a relevance of anatomical location of the source of NO (i.e. distal vs. proximal airways).

The partitioning of the NO into proximal and distal airway derived NO can provide a measure of inflammation more closely associated with atopy (JNO) and respiratory symptoms (Calv). Because these measures are modeled and not directly measured, we are not certain whether the alveolar NO has solely alveolar sources or also includes NO from the transition zone airways. Given the importance of this latter part of the airway to asthma and the association in our study and others between Calv and respiratory symptoms, it is compelling that the measure of Calv may represent a combination of the alveolar and transition zone airways.

In our study population, none of the FENO values differed by gender, ethnicity/race, maternal asthma or maternal educational level. While bronchial flux levels were lower among

children whose mother had reported asthma and alveolar NO concentrations were higher among children whose mother did not have a high school education, these associations appeared to be driven by associations between maternal asthma and atopy and maternal education and children with wheeze, respectively. The relationships were no longer statistically significant when adjusted for other covariates in a multivariable model.

In our study we collected steroid (inhaled and oral) medication use for asthma for the day of the test and the previous 3 months. Given the findings that steroid medication in the previous week can affect exhaled nitric oxide levels, it is a limitation that we did not ascertain steroid use specifically for that time period (Kharitonov *et al.* 2002). For the current analyses we *a priori* excluded the children who had taken mediation on the day of the test, but not those that only reported steroid use in the previous 3 months. Based on reports of non-compliance to controller medication use in similar inner-city populations, we believed that it was more likely that children who had not taken steroid medication on the day of the test would also not have taken it in the previous week (Rohan *et al.* 2009). The children with a report of steroid use in the past 3 months, but not on the day of the test had similar FENO and Calv measures to the children with a report of wheeze and JNO levels to the seroatopic children who did not have a report of steroid medication use in the past 3 months. That we did not have a sufficient sample size to test the steroid naïve and steroid exposed groups separately in the current analyses is also a limitation of the current study. We did test differences in the associations with the NO parameters by symptoms after excluding the 7 children (Table 4), and for the most part the associations were similar to those with the 7 children. The two notable exceptions were an emergence of a significant difference in JNO between the children with and without nighttime cough and wheeze. Given the small number of children with nighttime cough or wheeze and no steroid

medication use, we are reluctant to draw substantive conclusions from this finding. However this difference in children with and without a history of steroid use could be related to steroid medication use reducing JNO among children with symptoms. Clearly, it will be important in the future to examine the response of these NO parameters to steroid medication treatment in this population.

There are several other limitations to our study. The use of the offline method does not currently allow for an automated recording of flow rate over the breath collection. We estimated flow rates by recording the approximate average pressure during collection. In general, these nine-year old children were able to maintain a constant pressure at all of the selected flow rates. The offline method has been shown to be subject to contamination by ambient NO. Nevertheless, adjusting for ambient NO in multivariate models did not alter the associations. Furthermore, to ensure exclusion of ambient NO contamination we had modified the device as to ensure confirmation that the children actually inhaled through the filter before collecting their breath (Perzanowski *et al.* 2008). The online methodology for FENO collection has been recommended over the offline in clinic settings; therefore, our use of the offline methods may be seen as a limitation. However, we believe that a strength of our study was demonstrating the feasibility of offline multiple flow collection, because unlike currently available online equipment, offline methods can be used to collect samples a field setting. Our method could be best described as a modified Hogman method (Hogman *et al.* 2000). Nevertheless, we did not utilize the same flow rates stated by this study, but selected flow rates that were easily achievable by the children in our cohort. The Hogman method assumes a linear association between NO output and exhalation flow rate, so the replication of the exact flow rates published in other studies should be less important as long as they are made in the range expected to be linear. Another limitation

was that current wheeze was defined broadly as any wheeze within the past year, which would have resulted in children with only one virally associated wheeze episode categorized the same as children with moderate or severe asthma. A more detailed history and the inclusion of a more severe asthmatic population could have allowed us to better understand the association between alveolar NO and asthma severity and control in this inner-city population. Even though we did not correct for multiple comparisons, *a priori* we only chose two independent health predictors, wheeze and seroatopy, and three FENO parameters, $FENO_{50}$, Calv and JNO. Our main health outcome findings of significant differences in JNO by seroatopy and Calv by wheeze were both highly significant ($P<0.001$), limiting the likelihood that they were due to chance.

In summary, the multiple flow collection of FENO proved to be an easily implemented and reproducible method for a study of nine-year old children living in inner-city communities. The bronchial flux parameter appeared to be conditioned by seroatopy, while alveolar concentrations were only elevated in the presence of respiratory symptoms in the past year. In contrast to previous studies that only found elevated alveolar NO among severe and uncontrolled asthmatics, children with relatively mild asthma symptom frequencies in this inner-city community had higher alveolar NO than those without symptoms. The specificity of alveolar NO for respiratory symptoms and not atopy potentially make it a more suitable outcome than single flow FENO in cohort studies of pediatric asthma.

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Appendix 1: Supplementary tables

Table E-1. Adjusted logistic regression models[a] for flow dependent and independent parameters

Covariates	Model 1 $FENO_{50}$ OR (95%CI)	Model 2 JNO OR (95%CI)	Model 3 Calv OR (95%CI)
Current wheeze	9.0 (0.93-87.0)	5.0 (0.70-36.1)	9.3 (2.1-41.4)†
Seroatopy	33.0 (2.3-481.1)†	9.2 (1.5-58.1)*	1.4 (0.39-5.1)
Gender	3.0 (0.56-16.5)	2.9 (0.62-13.3)	1.1 (0.30-4.0)
Ethnicity/Race	5.7 (1.0-31.7)*	4.2 (0.94-18.8)	1.9 (0.52-6.8)
Maternal Asthma	0.74 (0.10-5.5)	0.77 (0.13-4.6)	1.0 (0.24-4.5)
Maternal education at enrollment	2.7(0.37-19.4)	0.73 (0.14-3.8)	1.8 (0.40-8.3)
Ambient NO	0.98 (0.96-1.0)	0.98 (0.94-1.0)	1.0 (1.0-1.0)

*P value ≤ 0.05, †P value ≤ 0.01, ‡P value ≤ 0.001

[a]All models adjusted for all listed covariates.

Table E2. Predictors of current wheeze symptoms

Predictor	OR (95% CI)
Seroatopy	0.85 (0.19-3.7)
JNO	1.9 (0.36-10.6)
Calv	11.2 (2.8-45.0)†

*P value ≤ 0.05, †P value ≤ 0.01, ‡P value ≤ 0.001

Chapter IV: Association of recent exposure to ambient metals with fractional exhaled nitric oxide in 9-11 year old inner-city children

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Abstract

Background: Exposure to ambient metals in urban environments has been associated with wheeze, and emergency room visits and hospitalizations due to respiratory illness. However, the effect of ambient metals exposure on airway inflammation has not been determined. We sought to characterize the associations between ambient concentrations of Ni, V, Zn and Fe averaged over 9 days and measures of sub-acute airway inflammation, as determined by exhaled NO. We also hypothesized that these associations would be modified by seroatopy.

Methods: As part of the Columbia Center for Children's Environmental Health (CCCEH) birth cohort study, 9-11 year old children (n=192) were evaluated. Ambient measures of Ni, V, Zn and Fe were obtained from a local central monitoring site and averaged over nine days based on three 24 hour measures every third day. Fractional exhaled nitric oxide (FENO) samples were obtained at constant flows of 50 ($FENO_{50}$), 83 and 100 mL/sec, and used to determine surrogate measures for proximal (JNO) and alveolar (Calv) inflammation. Seroatopy was determined by specific IgE at age 7. Data were analyzed using multivariable linear regression.

Results: Ambient V and Fe concentrations were associated positively with $FENO_{50}$ (p=0.018, p=0.027). Ambient Fe was associated positively with JNO (p=0.017). Ambient Ni and V concentrations were associated positively with Calv (p=0.004, p=0.018 respectively). A stronger association of Ni concentrations with Calv was observed among the children with seroatopy.

Conclusions: These results suggest that ambient metals are associated differentially with different fractions of FENO production, and this relationship may be modified by seroatopy.

Introduction

Exposure to particulate matter <2.5 microns in diameter ($PM_{2.5}$) has been associated with asthma development and acute exacerbations of symptoms (McConnell *et al.* 2006; Morgenstern *et al.* 2008). The U.S. Environmental Protection Agency (EPA) currently utilizes particle diameter to establish mass-based standards for air quality regulation of fine inhalable particles that comprise $PM_{2.5}$. However, $PM_{2.5}$ consists of complex mixture that includes metals, organic compounds, sulfates and nitrates among other compounds and this composition may vary temporally, spatially and by source (Bell *et al.* 2007). Mass-based standards fail to account for varying compositions and their differential effects on respiratory health. Therefore, it is important to understand which particular compounds may be associated with increased toxicity.

Despite growing research on some of the components, including polycyclic aromatic hydrocarbons (PAH) and elemental carbon (EC) (Jung *et al.* 2012; Miller *et al.* 2004), few studies have focused on the contribution of ambient transition metals. In New York City (NYC), a large number of apartment buildings burn residual oil for heating, and the burning is the major source of ambient nickel (Ni) and vanadium (V) in $PM_{2.5}$ (Peltier *et al.* 2009). Ni also is emitted during smelting processes, alloy production and other industrial activities ((IPCS) 1991). Motor vehicle emissions are drivers of ambient zinc (Zn) and to a lesser extent Ni and V ambient concentrations (Li *et al.* 2004). Additionally, burning of other fossil fuels such as coal and petroleum coke, used in electrical power generation, releases soot and fly ash containing V (Costigan *et al.* 2001). Sources of ambient iron (Fe) in $PM_{2.5}$ include road dust, oil combustion, and re-suspended soils (Chillrud *et al.* 2004; Li *et al.* 2004). Children are not only exposed to these pollutants outdoors, as these outdoor sources have been shown to penetrate indoors and contribute to indoor ambient levels (Kinney *et al.* 2002). Given the variety in sources of

exposure, it is important to understand how the individual airborne metals impact respiratory health.

Emerging evidence implicates several individual airborne metals and adverse respiratory effects. For example, central site levels of ambient Zn were associated with increases in emergency department (ED) visits and hospitalizations for asthma in a pediatric population in Baltimore (Hirshon *et al.* 2008). Risk of cardiovascular and respiratory hospitalizations following short-term exposure to $PM_{2.5}$, was higher in US counties with higher measured levels of Ni and V (Bell *et al.* 2009). In California, increased ambient levels of Fe and Zn were associated with respiratory hospital admissions among children (Ostro *et al.* 2009). In NYC, ambient measures of Ni and V were associated with increased probability of wheeze, and higher levels of Fe were associated with cough at age 24 months in our Columbia Center for Children's Environmental Health (CCCEH) birth cohort (Patel *et al.* 2009).

Measurement of fractional exhaled nitric oxide (FENO), typically collected at 50 ml/s as recommend by the American Thoracic Society (ATS)(Dweik *et al.* 2011), provides an indication of airway inflammation that has been associated with asthma development (Caudri *et al.* 2010) and exacerbations (van der Valk *et al.* 2012). Higher levels have been detected following recent environmental exposures (Just *et al.* 2012). Personal and central site exposure to both EC and NO_2 was associated with increments in FENO in California school children (Delfino *et al.* 2006). In a study of French children, levels of $PM_{2.5}$, formaldehyde and acetaldehyde measured in schools were associated positively with FENO (Flamant-Hulin *et al.* 2009). Another study in California school children found significant associations between ambient cumulative lagged averages over 1-8 days of $PM_{2.5}$ and elevated levels of FENO (Berhane *et al.* 2011). In NYC, domestic levels of black carbon (BC)/soot, a tracer of combustion emissions of residual fuel oil

and traffic, were associated with higher FENO levels among children without seroatopy (Cornell *et al.* 2012).

Furthermore, the collection of FENO at multiple flow rates allows the apportionment of NO derived from the alveolar/peripheral and bronchial/central compartments through the use of a mathematical model (George 2003). This method has proved useful in identifying sources of NO production in subjects with different asthma severity (Brindicci *et al.* 2007). In our cohort we found that bronchial NO (JNO) was associated with seroatopy while alveolar NO (Calv) was associated only with report of current wheeze (Rosa *et al.* 2011). Furthermore, this method has been used to the determine the association between ambient exposures such as environmental tobacco smoke (ETS) and airway inflammation (Malinovschi *et al.* 2006). For example, non-asthmatic silica-exposed workers in Finland had significantly higher alveolar NO (Calv), but not bronchial NO (JNO), compared to healthy volunteers after adjustment for age, body mass index (BMI) and smoking history (Sauni *et al.* 2012).

Previously, it has been shown that the association between PM_{10}, $PM_{2.5}$, O_3 and NOx and FENO may possess a time lag of several days (Berhane *et al.* 2011; Delfino *et al.* 2006; Obeng 2010). Berhane *et al* reported significant associations between cumulative lagged averages over 1-8 days between $PM_{2.5}$ and FENO. In order to account for weekly pollutant and weather patterns (Cerveny *et al.* 1998; Clevelan *et al.* 1974) and due to results from preliminary analyses, we sought to characterize the association between 9 day averages of airborne ambient metal concentrations (based on three 24 hour measurements each 3rd day for Ni, V, Zn and Fe) on NO derived from alveolar and bronchial compartments measured from children aged 9-11 years enrolled in the CCCEH birth cohort in NYC. Given previously observed differential associations between proximal and distal fractions of NO in our cohort, we hypothesized that airborne

concentrations of Ni, V, Zn and Fe would be associated differentially with proximal and distal fractions of NO. We also hypothesized that the association between ambient metal exposure and FENO parameters would be modified by seroatopy.

Material and Methods

Study population

Participants were enrolled in the prospective birth cohort study (n=727) conducted by the CCCEH and enrollment has been described previously (Miller *et al.* 2004). Non-smoking, healthy pregnant women aged 18-35, who self-identified as African-American or Dominican and were living in Northern Manhattan and the South Bronx were enrolled. Of the 727 fully enrolled participants, 225 (30.9%) had at least one valid set of multiple flow measures from which distal and proximal fractions could be calculated, at ages 9 or 11. Of those 225 children, 192 (85.3%) had corresponding ambient metal concentrations collected within nine days of the FENO collection (Table 1). Only children that resided within the study catchment area at the time of collection were included in the analyses. Children were assigned their mother's reported race/ethnicity. Informed consent was obtained in accordance with the Institutional Review Board at Columbia University. Socio-demographic information was obtained from baseline (prenatal) questionnaires. Specific IgE against cockroach, *D. farinae*, mouse, dog, cat, mold, tree mix, grass and ragweed were measured by Immunocap (Phadia, Uppsala, Sweden) in sera collected at age 7 (n=171) as previously described (Donohue *et al.* 2008).

Measures of exposure

Twenty-four hour average $PM_{2.5}$ concentrations were collected daily using a filter based method (Rupprecht & Patashnick TEOM 1400ab and 2025 Partisol) by the New York State Department of Environmental Conservation (DEC) in accordance to EPA guidelines. Average 24

hour ambient concentrations of $PM_{2.5}$ fractions of Ni, V, Zn and Fe were measured every third day using a Rupprecht & Patshnick 2300 Partisol and the most complete, validated dataset from the Intermediate School 52 (IS52) in Bronx county, was requested. This site was picked due to the availability of metals data and its location within the study catchment area of Northern Manhattan and the South Bronx which has been previously described (Horton *et al.* 2011; Jung *et al.* 2010). Given the limited number of EC measurements available, EC was excluded as a potential predictor. In these analyses the available data spanned from January 2008 to June 18, 2010 until the monitoring site was shut down

FENO parameters: $FENO_{50}$, JNO and Calv

FENO was collected at ages 9 or 11 years utilizing a previously modified offline NO collection method (Perzanowski *et al.* 2008). Children were instructed to inhale through an NO scrubber. FENO samples were obtained in duplicate at 50 and 100 mL/sec and in triplicate at 83 mL/s. $FENO_{50}$ was defined as FENO collected at 50 ml/sec. Bronchial flux (JNO) and alveolar NO (Calv) were calculated using a previously described modified Hogman method that utilized FENO collected at these 3 different flow rates (Hogman *et al.* 2002; Rosa *et al.* 2011). Even though the Hogman method utilizes higher flow rates, previously we described good correlations for both JNO and Calv using values calculated with the Hogman and the Pietropaoli methods (Rosa *et al.* 2011). JNO and Calv were defined as surrogate measures of NO emanating from 2 compartments described by the Hogman mathematical model, the proximal airway region and the alveolar region respectively.

Statistical analyses

Data were analyzed using linear regression. In order to examine the potential temporal variation in these associations, three different metal exposure assignments were modeled.

Because metals data was only collected every third day, the first model assigned exposure based on the closest available measurement, either same day as FENO collection, one day or 2 days prior. The second exposure assignment utilized the average of two measurements within 6 days of the FENO collection and the third exposure assignment used the average of three measurements within 9 days of the FENO collection. In these models, the metal measurements averaged had to be collected the day of or prior to the day of FENO collection. The final model utilized the natural log of the average of three measurements three days apart because these models were able to capture 7-day weather trends. Metal concentrations were log transformed due to a large amount of relative variation and to improve fit (Gelman and Hill 2007). Exploratory analyses also were conducted for daily $PM_{2.5}$ concentrations and 1-9 day lag day values. The outcomes of interest were $FENO_{50}$, JNO and Calv. NO outcomes were found to be log normally distributed; therefore they were log transformed. Log transformations of outcome and predictor variables require β coefficients be interpreted as percent increases.

During FENO collections, parents also were asked about whether the child had a cold or respiratory infection that day and about use of asthma medication, including current inhaled corticosteroid (ICS) use. Parents were also asked if the children had eaten anything in the 2 hours prior to the FENO collection. Multivariable models were adjusted for race/ethnicity, sex, cold/influenza season, report of current respiratory infection or cold, report of smoker in home, report of the child eating within 2 hours of the FENO collection, and ambient NO levels. Report of any recent ICS use was uncommon among the participants (<10%) and only 5 children had report of ICS use the day of the FENO collection. Effect estimates did not vary significantly after exclusion of the children with report of ICS use; consequently these children were included in final analyses. NOx species (4-day lag) was included as a co-pollutant in all models due a

previously seen association with FENO (Obeng 2010). Due to a high degree of correlation, Ni, V, and Zn were all analyzed in separate models. Fe was highly correlated with V and Zn but not with Ni, so Ni and Fe were also examined in a co-pollutant model. Stratified analyses were carried out to determine if seroatopy, defined as any specific IgE ≥0.35 IU/mL to cockroach, mouse, *Dermatophagoides farinae*, dog, cat, mold, tree, grass or ragweed at age 7 modified the association between ambient metals exposure and FENO parameters.

Results

Characteristics of cohort and pollutant concentrations

Demographic characteristics comparing CCCEH participants included in analyses (n=192) with those excluded (n=539) are shown (Table 1). There were no significant differences in maternal education and maternal asthma. As expected, the median age of the excluded children was significantly lower since the majority had not reached age 9. The percentage of males in the included cohort was slightly lower than those in the excluded cohort (41% vs. 51%). There was a significant difference in prenatal ETS exposure, with 42% in the included cohort and 32% in excluded cohort. Medians and 25^{th} and 75^{th} percentiles for averaged metal concentrations were as follows: Ni 5.25 ng/m^3 [3.30, 9.96], V 2.33 ng/m^3 [1.35, 3.43] Zn 27.8 ng/m^3 [22.1, 41.7] and Fe 93.8 ng/m^3 [74.6, 116]. Correlation coefficients among V, Zn and Fe measures were all significant ($p<0.01$, Table S1, online supplement). Ni measures correlated significantly with V and Zn but not with Fe. The median distance to the IS52 site was 3.93 km (25^{th}-75^{th} percentile 3.15-4.53 km). NO measurements all correlated with one another ($p<0.01$, Table S2, online supplement). The proportion of the selected children that reported wheeze in the past 12 months in response to the ISAAC questionnaire was 45/192 (23.4%). The proportion of children that had seroatopy was 78/171 (45.6%).

Table 1. Selected cohort characteristics

Characteristic	Included n=192	Excluded n=539	p-value
Mother's age[a] (IQR)	25 (21-29)	25 (21-29)	0.395
Child's age[a]	9.0 (9.0-9.2)	8.1 (5.8,10)	<0.001
Mother's race/ethnicity			<0.001
African American (%)	48	30	
Dominican (%)	52	70	
Maternal education[b] (%)	64	64	0.903
Maternal asthma[c] (%)	28	24	0.295
Child's sex (% male)	41	51	0.021
Prenatal ETS[d] exposure (%)	42	32	0.013

[a] Age at delivery, geometric mean, Mann Whitney U test
[b]Mother completed high school, General Educational Development (GED) test or greater
[c]Mother reported asthma in prenatal or 3 month questionnaire.
[d]Prenatal ETS exposure defined as maternal or cord blood cotinine level ≥ 15 ng/ml or report of smoker in the home from prenatal questionnaire.
#Differences in categorical variables tested using Pearson Chi-Square
333/727 (45.8%) children excluded because they had not yet reached at least age 9 by June 24th, 2010, the last day of available central site monitoring data.
169/394 (42.9 %) children were excluded because they did not have available FENO data.
3/225 (1.3%) were excluded due to ambient NO levels greater than 100 ppb and the potential for contamination from ambient NO.
30/222 (13.5%) were excluded because they resided outside of study catchment area.

Associations between Ni, V, Zn and Fe and FENO parameters

In univariate models (Figure S1), V and Fe, but not Ni or Zn, averaged over 9 days were significant predictors of $FENO_{50}$ (β=0.157, 95% CI [0.007, 0.306] for V and β=0.375, 95% CI [0.053, 0.696] for Fe). Of all 4 metals, only Fe was a significant predictor of JNO levels

(β=0.607, 95% CI [0.074, 1.139]). Ni (β=0.185, 95% CI [0.035, 0.335]) and V (β=0.179, 95% CI [0.044, 0.313]), and not Fe, were significant predictors of Calv.

In multivariable models, after adjusting for covariates, V and Fe remained significant positive predictors of $FENO_{50}$ (β=0.188, 95% CI [0.033, 0.343]; β=0.396, 95% CI [0.045, 0.748] for V and Fe respectively) (Table 2). Fe also remained a significant predictor of JNO (β=0.673, 95% CI [0.122, 1.225]). Ni and V remained significant predictors of Calv (β=0.252, 95% CI [0.081, 0.424] for Ni and (β=0.172, 95% CI [0.030, 0.315] for V). $PM_{2.5}$ measures were examined as same day measurements and as 1-9 day lags. None of the $PM_{2.5}$ measures were associated significantly with any FENO parameter nor changed any parameter estimates for the metals when included as a covariate in the multivariable model. Additionally, heating season was examined as a potential covariate to be included in the multivariable model instead of cold and flu season (which substantially overlaps with heating season), but inclusion of cold and flu season provided a better model fit (results not shown).

Stratification by seroatopy

Previous studies have shown a strong association between allergic sensitization and FENO levels (Ludviksdottir *et al.* 1999; Nordvall *et al.* 2005; Patelis *et al.* 2012; Rosa *et al.* 2011). Therefore, we proceeded to analyze a potential effect modification by seroatopy. Interaction terms for each metal and seroatopy were tested in the final multivariable model and a significant interaction was found between Ni and seroatopy. Ni appeared to have a stronger positive association on Calv among those children classified as seroatopic (β=0.435, 95% CI [0.119, 0.751]) than those who were not seroatopic (β=0.057, 95% CI [-0.177, 0.290]). Figure 1 shows a regression plot for the univariate Ni model stratified by seroatopic status. The

association between V, Zn, Fe and FENO parameters did not appear to vary across strata of seroatopy (data now shown).

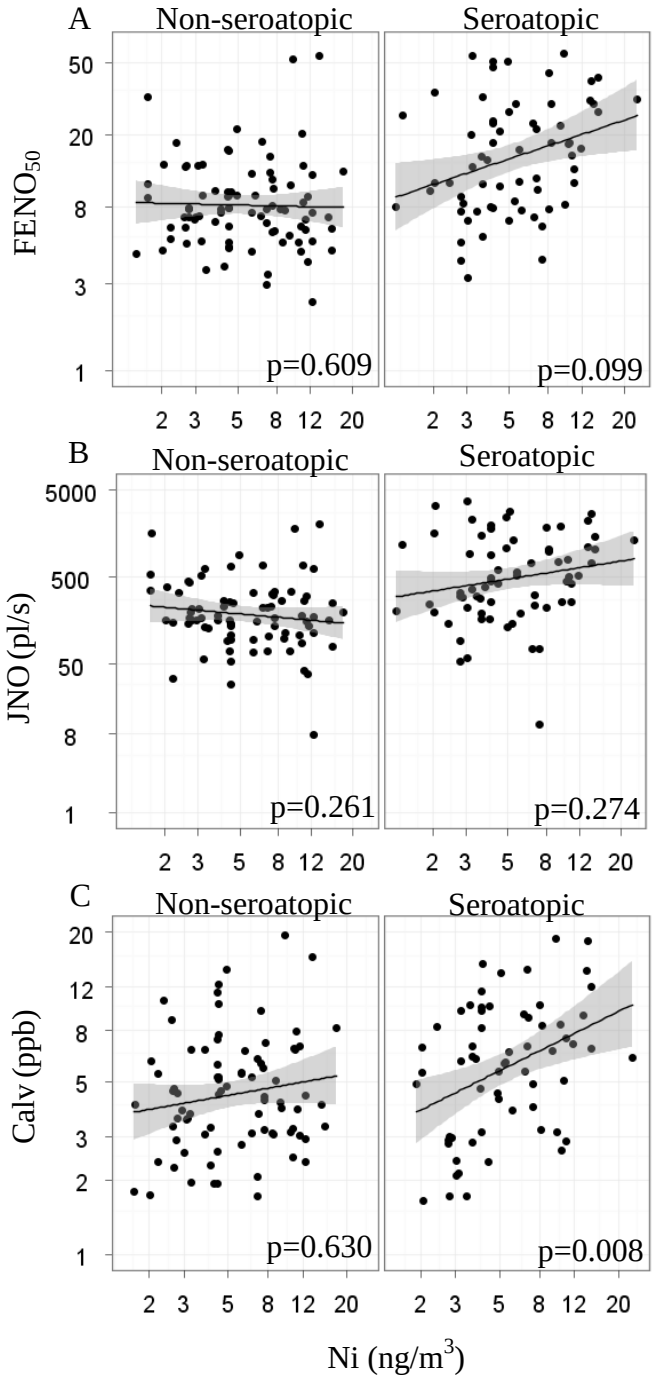
Table 2. Adjusted[a] linear regression models for associations between ambient pollutants and FENO measures β (95% CI)[b]

Pollutant	$FENO_{50}$	JNO	Calv
Ni	0.105 (-0.080,0.290)	0.038 (-0.260,0.336)	0.248† (0.079,0.417)
V	0.198* (0.046,0.349)	0.226 (-0.014, 0.465)	0.171* (0.030,0.312)
Zn	-0.003 (-0.236,0.230)	-0.036 (-0.406,0.334)	-0.058 (-0.277,0.162)
Fe	0.406* (0.061,0.750)	0.679* (0.134,1.223)	0.025 (-0.301,0.352)

[a]Models adjusted for race/ethnicity, sex, cold/flu season, report of cold or respiratory infection the day of the FENO collection, current ETS (report of a smoker in home), and mean ambient NO levels, report of the child eating within 2 hours of the FENO collection and NOx 4-day lag
[b]Log transformations of outcome and predictor variables require β coefficients be interpreted as percent increases, *i.e.* a 1% increase in Ni is associated with a 25.2% increase in Calv
*$p<0.05$, †$p<0.01$

In order to examine if Ni and Fe were independent predictors of FENO parameters, a multivariable model that included both as predictors was tested. Results show that Ni and Fe appeared to act independently from one another. Fe remained a significant predictor of both $FENO_{50}$ (β=0.373, 95% CI [0.009, 0.737], p=0.045) and JNO (β=0.700, 95% CI [0.129, 1.271], p=0.017) after inclusion of Ni in the model, but the result from the regression model ANOVA table was not significant (p=0.063). Ni also remained a predictor of Calv (β=0.263, 95% CI [0.085, 0.440], p=0.004) after Fe was included in the multivariable model.

Figure 1. Association between ambient Ni and FENO parameters modified by seroatopic[a] status.



[a]Seroatopy defined as any specific IgE ≥0.35 IU/mL to cockroach, mouse, *D. farinae*, dog, cat, mold, tree, grass or ragweed at age 7. (A) univariate linear regression model for Ni predicting $FENO_{50}$ by seroatopic status. (B) univariate linear regression model for Ni predicting JNO by seroatopic status . (C) univariate linear regression model for Ni predicting Calv by seroatopic status. All x and y axes plotted on the natural log scale. P-values shown for multivariable models.

Discussion

The objective of this study was to characterize the association between measures of ambient Ni, V, Zn and Fe averaged over nine days and FENO parameters differentially representative of NO sources from proximal and distal airways in a cohort of children living in NYC. To our knowledge, this is the first paper to utilize the multiple flow method to determine such associations. We found that Ni and V concentrations were associated with increased levels of Calv, an indicator of distal airway inflammation, and additionally V concentrations were associated with increased levels of $FENO_{50}$, presumed to indicate inflammation emanating from all compartments of the airways. Fe concentrations were associated with increased levels of $FENO_{50}$ and JNO, and not Calv, indicating more proximal sources of inflammation. Zn concentrations were not associated with FENO. This study provides evidence that relatively short-term exposure to ambient transition metal fractions of $PM_{2.5}$ may lead to an increase in airway inflammation, and that these metals may exert differential effects on the localized production of NO in the airways.

Associations between metal concentrations and FENO parameters were found when metal measures taken 3 days apart were averaged over longer time periods, which is consistent with previously published data on PM and EC (Berhane *et al.* 2011; Delfino *et al.* 2006). A study of French schoolchildren found a significant association between 5-day average concentrations of $PM_{2.5}$ measured in classrooms and increased NO in children with and without asthma (Flamant-Hulin *et al.* 2009). In another study of California school children, the strongest associations were seen between cumulative lagged models of $PM_{2.5}$ over 1-8 days and FENO, rather than with models utilizing only the previous 24 hour average of $PM_{2.5}$ (Berhane *et al.* 2011).

These results showed that Ni, V and Fe exerted differential effects on fractions of exhaled NO, despite the significant correlations between all outcome measures. Only a few studies have used the multiple flow method to partition NO contributions with mixed results. In a study of wood smoke exposure, healthy adult subjects who were exposed experimentally in a chamber to wood smoke for 4-hours, had significantly elevated alveolar NO (Calv), but neither $FENO_{50}$ nor bronchial NO (JNO) were increased (Barregard *et al.* 2008). Another study of healthy adult subjects showed that after 30 minutes of exercise in an area with high ambient UPM concentrations, alveolar NO decreased without change in bronchial NO (Rundell *et al.* 2008). Finally, in a study of adult asthmatic and healthy subjects, experimental exposure to nebulized UPM through a mouthpiece was not associated with any FENO parameter (Pietropaoli *et al.* 2004). A possible explanation for the differential effects of Ni, V and Fe on fractions of FENO is that each may affect the production of NO in the airways through a different pathway. There is some evidence that both iNOS and nNOS contribute to alveolar NO (Brindicci *et al.* 2009). Another explanation might be the relative distribution of metals on particles of different sizes. In another study, Ni was found in fine, intermediate and coarse airborne particles while Fe was mainly found in particles with a diameter >2.7μm.(Samara *et al.* 2005) These findings suggest that Fe might not travel as small enough particles to reach the alveolar region and exert an effect on Calv levels.

We observed no association between Zn concentrations and any of the 3 FENO parameters. This finding was not entirely unexpected because there is evidence from rodents that intratracheal instillations of zinc sulfate induced pulmonary neutrophilic inflammation (Kodavanti *et al.* 2002). Previous studies have shown that $FENO_{50}$ is elevated in the setting of eosinophilic, not neutrophilic, inflammation and correlated with sputum eosinophil counts

(Schleich *et al.* 2010). There is also evidence that the addition of Zn to murine macrophages stimulated with interferon (IFN)-γ and tumor necrosis factor (TNF)-α had no demonstrable effect on NO production (Tian *et al.* 1996).

There are only a few studies that help elucidate the mechanism through which ambient metal exposure leads to airway inflammation, and presumably subsequent airway disease and disease exacerbation. *In vivo* instillation of ambient particles rich in Ni, Zn, Cd and Cu increased secretion of pro-inflammatory cytokines interleukin (IL)-6 and TNF-α and generated elevated levels of oxidant radicals in bronchoalveolar lavage fluid (BAL) (Schaumann 2004). TNF-α, in conjunction IFN-γ, may activate nuclear factor-kappa B (NF-κB) in tumorigenic mouse lung epithelial cells, leading to downstream activation of STAT1 and STAT3, ultimately responsible for iNOS expression (Tyagi *et al.* 2012). Another potential mechanism through which these metals may lead to elevated NO levels involves the dysregulation of Fe homeostasis in the lungs. Inhaled Ni, V and Fe may compete with the uptake of endogenous Fe, leading to elevated levels of available Fe and release of reactive oxygen species (ROS) (Ghio *et al.* 2005; Prophete *et al.* 2006). NF-κB can be activated by oxidative stressors (Rahman *et al.* 1998) and its activation has been shown to induce iNOS gene expression in mouse macrophages treated with lipolysaccharide (LPS) (Xie *et al.* 1994).

The association between ambient measures of Ni and elevated Calv levels was strongest among seroatopic children. These results agree with previous research that found stronger associations between classroom levels of $PM_{2.5}$, acetaldehyde and formaldehyde and elevated $FENO_{50}$ levels among atopic children, suggesting atopic children may be more susceptible to the airway effects of these ambient pollutants (Flamant-Hulin *et al.* 2009). These results also are consistent with a murine model of allergic asthma, where intratracheally administered UPM with

high fractions of transition metals and metalloids, enhanced ovalbumin (OVA)-induced eosinophil recruitment in the alveoli and increased levels of eosinophil-relevant cytokines and chemokines, IL-5 and monocyte chemotactic protein-3 (MCP-3) in BAL (He *et al.* 2010). However, the association for Ni and FENO is not consistent with Cornell et al (2012) that found that nonseroatopic children had a relationship between BC and $FENO_{83}$ (FENO collected at 83 ml/s). This is also conflicting because one of the major sources of BC is residual fuel oil that is also a major source of Ni in NYC.

There are some limitations to this study. First, the use of central site measurements to assign exposure may not adequately reflect the spatial heterogeneity of individual exposure. However, a previous study found that 1 week average concentrations of Ni, V and Zn measured at IS-52 site were highly correlated with the same metals measured at the New York Botanical Gardens site in Bronx county, also located within our study catchment area, approximately 3 miles away from the IS-52 site (Patel *et al.* 2009). It is also possible that the ambient metals may be surrogates for other component of $PM_{2.5}$. As seen in table S1, the metals were highly correlated and we were unable to examine Ni and V in a co-pollutant model because of multi-collinearity, thus making it difficult to disentangle the individual effects of these metals on airway inflammation. Nevertheless, Ni and Fe were not strongly associated and we observed differential associations with FENO parameters when both metals were tested in the same model providing evidence of an independent effect. Another potential limitation is the fact that we did not control for cumulative exposure. FENO measures fluctuate daily within subjects, making it a more suitable biomarker for the study of responses to recent exposures. However, one-time measurements have been associated with environmental exposures and clinical outcomes. $PM_{2.5}$ averaged over 5 days that was measured in school-yards and classrooms was associated with

elevated levels of FENO in children living in France (Flamant-Hulin *et al.* 2010). Domestic black measures averaged over 7 days were associated with increased levels of FENO in children without seroatopy living in NYC (Cornell *et al.* 2012). Elevated FENO levels measured in pre-school aged children were associated with increased risk of respiratory tract illness at one-year follow-up (Beigelman *et al.* 2009). We also averaged metal concentrations over 9 days to assess lag effects after sub-acute exposure instead of fully evaluating lag effects of various durations to pinpoint the relevant time period, and instead of measuring cumulative exposures. Sample size was also a limitation that did not allow us to analyze other potential effect modifiers such as current wheeze and asthma diagnosis, nor to include other covariates such as heating season; however, all models were adjusted for cold and flu season that substantially overlaps with heating season. Another limitation was the cross-sectional nature of the analyses, given the daily fluctuation of FENO measures.

Even though each lung compartment was not sampled separately in this study, the robustness of the mathematical model has been tested previously and the multiple flow method has been shown to reflect NO arising from different compartments (Verbanck *et al.* 2009). In a previous study in the CCCEH cohort, we showed that seroatopy was closely related to JNO while report of wheeze in the past 12 months was only closely related to Calv (Rosa *et al.* 2011). A previous study also found elevated Calv levels among severe and uncontrolled asthmatic children when compared with corresponding healthy controls (Paraskakis 2006). Distal lung inflammation also has been associated airway hyper-responsiveness, symptom exacerbation and tissue remodeling (Kraft *et al.* 2001; Martin 2002). These studies suggest that the anatomical location of the NO source (*i.e.* distal vs. proximal airways) is relevant and consequently Calv may serve as a better predictor in the study of air pollution-induced exacerbations.

The associations between recent concentrations of airborne Ni, V and Fe and increased levels of NO suggest that these metal fractions of $PM_{2.5}$ lead to airway inflammation in the proximal and distal regions of the lungs. Our findings provide a better understanding of how different inhaled metals contribute to airway inflammation that may consequently lead to asthma exacerbation. The use of mass-based standards of fine particulate matter in air quality regulation by the EPA may not be sufficient to protect children from the immunotoxic effects of transition metals found in PM. Knowledge of the differential effects of different pollutants may lead to more targeted interventions directed at their sources *i.e.* switching to cleaner oils or natural gas for heating and setting better traffic emission standards.

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Appendix 1: Supplemental tables and figures

Table S1. Spearman correlation matrix for pollutants

	Ni	V	Zn	Fe	NOx
Ni	1.000	0.493**	0.625**	0.090	-0.085
V		1.000	0.423**	0.556**	-0.004
Zn			1.000	0.239**	-0.010
Fe				1.000	0.115
NOx					1.000

**p<0.01

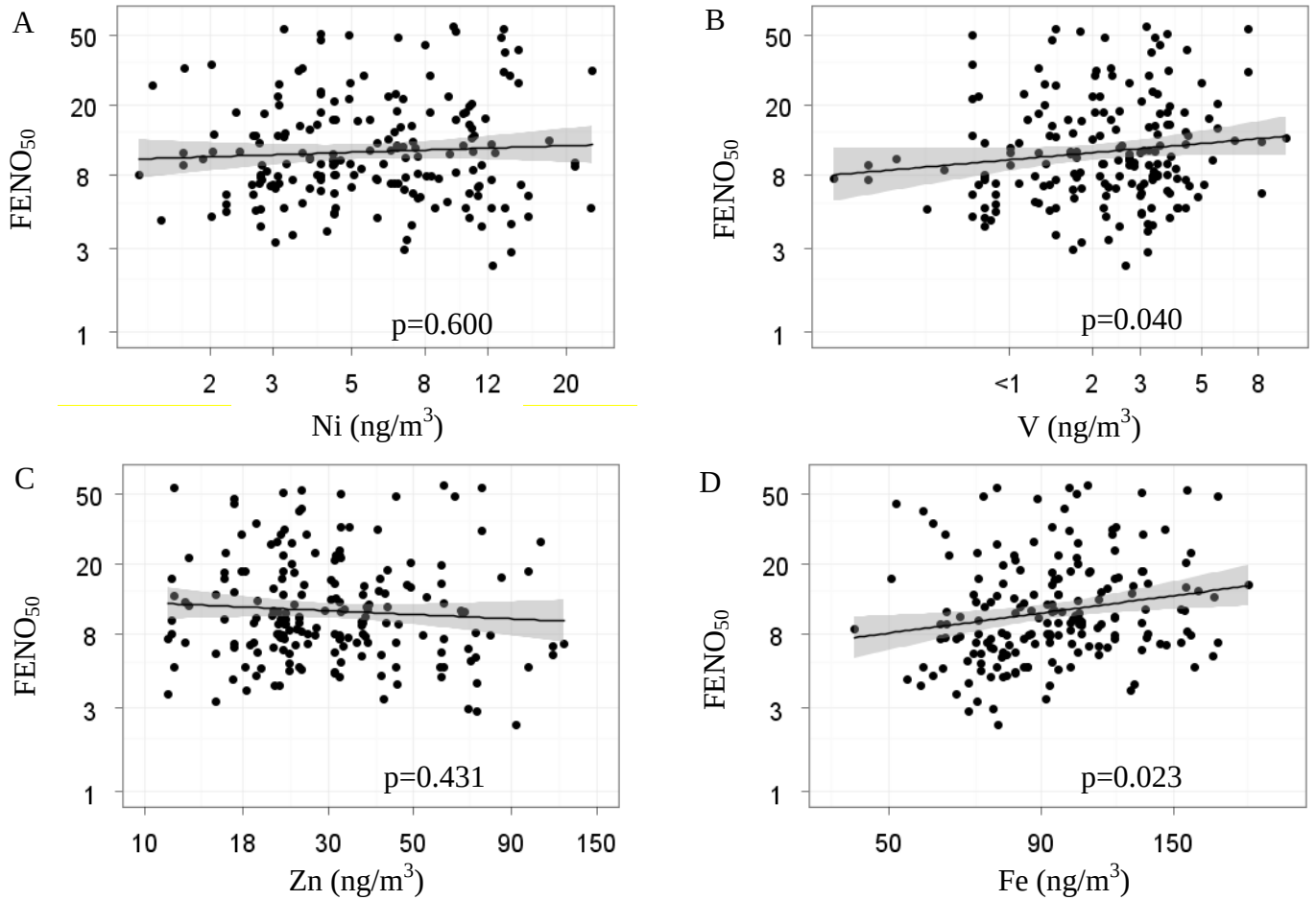
†All coefficients, except for NOx, refer to correlations for average of 3 measures. NOx represents NOx species with a 4-day from FENO collection.

Table S2. Spearman correlation matrix for NO measurements

	$FENO_{50}$	JNO	Calv
$FENO_{50}$	1.000	0.816**	0.577**
JNO		1.000	0.120
Calv			1.000

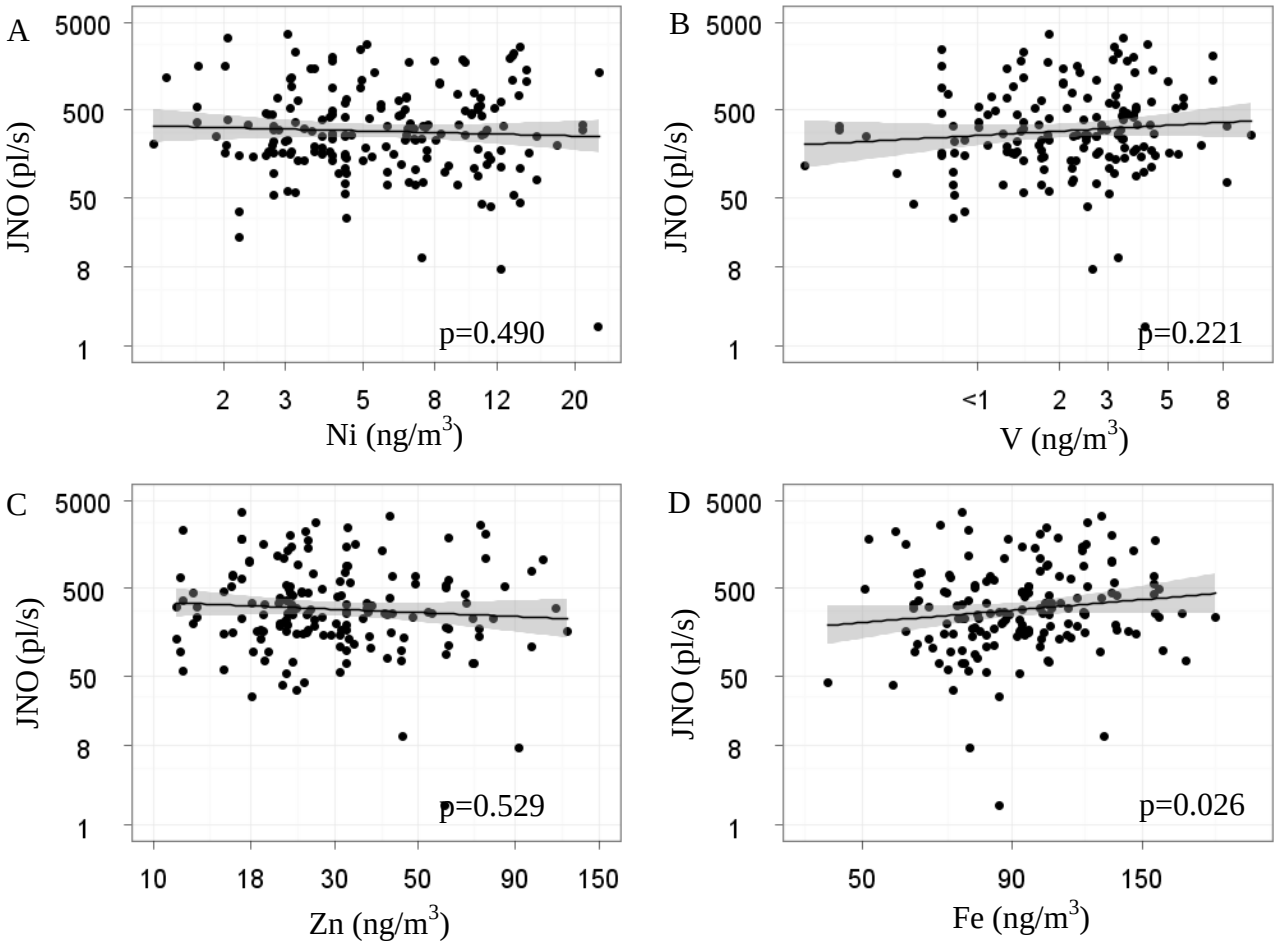
**p<0.01

Figure S1. Associations between ambient Ni, V, Zn, and Fe concentrations and $FENO_{50}$



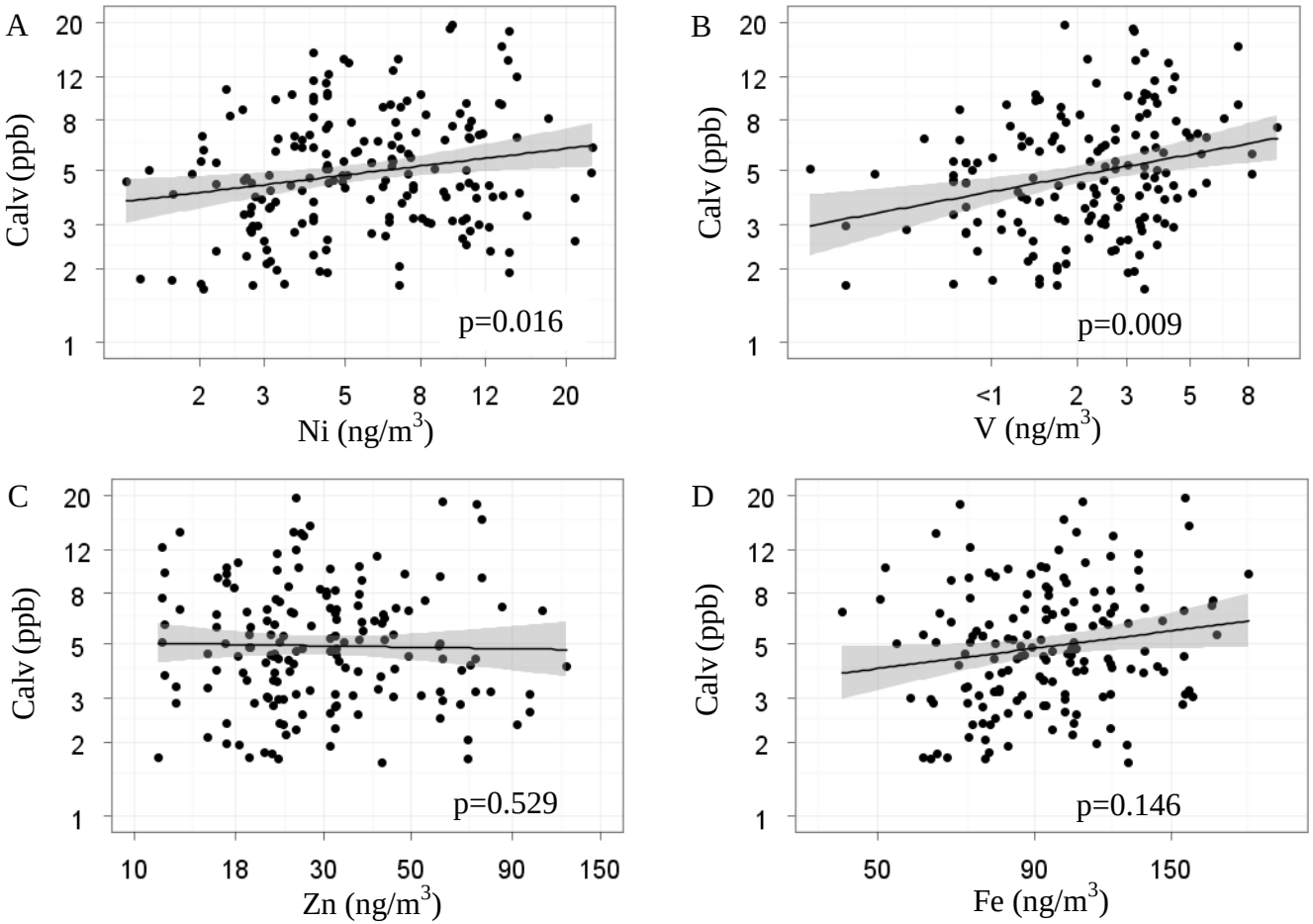
Panel A, Univariate linear regression model for Ni predicting $FENO_{50}$. Panel B, Univariate linear regression model for V predicting $FENO_{50}$. Panel C, Univariate linear regression model for Zn predicting $FENO_{50}$. Panel D, Univariate linear regression model for Fe predicting $FENO_{50}$. For all panels, both x and y axis plotted on the natural log scale. Values for each metal represent the average value of 3 ambient measurements taken closest in time to day of FENO collection. $FENO_{50}$ values represent the average of all acceptable FENO collections at 50 ml/s (at least 1, up to 3).

Figure S2. Associations between Ni, V, Zn and Fe concentrations and bronchial flux (JNO)



Panel A, Univariate linear regression model for Ni predicting JNO. Panel B, Univariate linear regression model for V predicting JNO. Panel C, Univariate linear regression model for Zn predicting JNO. Panel D, Univariate linear regression model for Fe predicting JNO. For all panels, both x and y axis plotted on the natural log scale. Values for each metal represent the average value of 3 measurements taken closest in time to day of FENO collection.

Figure S3. Associations between Ni, V, Zn and Fe concentrations and alveolar NO (Calv)



Panel A, Univariate linear regression model for Ni predicting Calv. Panel B, Univariate linear regression model for V predicting Calv. Panel C, Univariate linear regression model for Zn predicting Calv. Panel D, Univariate linear regression model for Fe predicting Calv. For all panels, both x and y axis plotted on the natural log scale. Values for each metal represent the average value of 3 measurements taken closest in time to day of FENO collection.

Chapter V: GIS indicators of traffic and stationary sources of air pollution and parameters of airway inflammation

Abstract

Background: Living in close proximity to major roadways has been associated with increased risk of asthma, allergy and respiratory symptoms. However, there are limited data on the effect of other measures of traffic and non-traffic sources of airborne pollution on airway inflammation, a hallmark of asthma. In this study we sought to characterize the associations between sources of traffic and non-traffic related airborne pollutants and fractional exhaled nitric oxide (FENO) parameters, as surrogate markers of airway inflammation, in children living in New York City.

Methods: As part of the Columbia Center for Children's Environmental Health (CCCEH) birth cohort study, children were evaluated at ages 9 and/or 11 years old (n=423). We determined residential distance to a major road, truck route and bus stop density, area covered by major roads, stationary point sources (SPS), toxic release inventory sites (TRIS) and commercial buildings, and number of buildings burning residual oil within 250-meters of each child's home using geographic information systems (GIS). FENO samples were obtained at constant flows of 50 ($FENO_{50}$), 83 and 100 mL/sec, and used to determine surrogate measures for proximal (JNO) and alveolar (Calv) inflammation. Seroatopy was determined by specific IgE measured at age 9. Current wheeze was reported through questionnaire. Data were analyzed using generalized estimating equations.

Results: In univariate analyses, there were significant associations between percentage area covered by major trucking routes and Calv (β=-0.168, 95% CI [-0.333, -0.003] and also between area covered by TRIS and JNO levels (β=0.139, 95% CI [0.001, 0.276]) as determined by GIS variables. However these associations were diminished and no longer significant after adjustment

for covariates. Overall, there were no significant associations between any of the air pollution indicator variables and FENO parameters in adjusted models. In secondary analyses for effect modification, we found few significant interactions between the hypothesized effect modifiers, sex, seroatopy and report wheeze, and pollution indicator variables.

Conclusions: Geographic indicators of airborne pollutants were not associated with FENO parameters. FENO parameters may be better indicators of short-term airborne pollutant exposure.

Introduction

Exposure to traffic-related air pollution has been associated with increased respiratory morbidity (Kim *et al.* 2004; Penard-Morand *et al.* 2010; Venn *et al.* 2001). Traffic emissions are significant contributors to airborne concentrations of fine particulate matter ($PM_{2.5}$), carbon monoxide, nitrogen oxides and other pollutants deemed relevant to the study of respiratory health (Ross *et al.* 2011; Sahsuvaroglu *et al.* 2006). Residential proximity to a major roadway and traffic density has been used as surrogate indicators of traffic sources of airborne pollutant in the study of respiratory illness. For example, living within 75 meters of a major road was associated with higher odds of prevalent asthma and wheeze among schoolchildren in Southern California (McConnell *et al.* 2006). In a study in northern California, proximity to roadway was also associated with a significant decrease in lung function in adults with asthma (Balmes *et al.* 2009). In a study of 10 European cities, 14% of all childhood asthma cases were attributable to closer proximity to roads with high vehicle traffic (Perez *et al.* 2013). In addition to distance to a major road, the traffic type has also been deemed important in the study of health effects related to traffic emissions. Exposure to emissions from diesel vehicles, *i.e.* trucks and buses, may be a more important risk factor in the development and exacerbation of respiratory disease than exposure to emissions from gasoline vehicles (Ryan *et al.* 2005).

The use of geographic information systems (GIS) allows the determination of not only variables related to traffic-related pollution, but other sources that might be important contributors to airborne pollution. In New York City (NYC), burning of residual oil for heating purposes is the main source of airborne nickel (Peltier *et al.* 2009). The number of buildings burning residual oil was also a significant predictor of domestic levels of black carbon (BC) (Cornell *et al.* 2012) and a significant contributor to the spatial variation of $PM_{2.5}$, elemental

carbon (EC) and SO_2 within NYC (Clougherty *et al.* 2013). Previous studies have also shown that truck traffic density and proximity to roadway are strong predictors of $PM_{2.5}$ and BC concentrations in NYC (Clougherty *et al.* 2013; Spira-Cohen *et al.* 2011). Because of the associations between these GIS variables and airborne pollutants, GIS variables may serve as suitable surrogates of chronic exposure to these pollutants.

Measurement of fractional exhaled nitric oxide (FENO) at a single flow has been used as an indicator of airway inflammation that has been associated with asthma development and exacerbations (Caudri *et al.* 2010; van der Valk *et al.* 2012). FENO has also been found to be elevated following recent exposures to airborne pollutants like black carbon (BC) and fine particulate matter ($PM_{2.5}$) (Barraza-Villarreal *et al.* 2008; Cornell *et al.* 2012). A few studies have examined the association between GIS variables and FENO measured at a single flow with mixed results. Residential proximity to roadway and road density, but not school levels of pollutants, was associated with elevated FENO levels in asthmatic children living in Ciudad Juarez, Mexico (Holguin *et al.* 2007). Among elementary-school age children in Ontario, FENO was positively and significantly related to increased roadway density, while FENO concentrations were not significantly associated with land-use regression (LUR)-estimated NO_2, SO_2, black smoke, coarse PM, or $PM_{2.5}$ (Dales *et al.* 2008). However, in a follow-up study of these children, traffic exposure was redefined as the sum of the annual volume of vehicles on all roadways and no association with FENO levels was found.(Cakmak *et al.* 2012) In a study of adolescents in a periurban shantytown in Lima, Peru, distance to roadway was associated with increased odds of atopy and asthma symptoms but was not significantly associated with FENO (Baumann *et al.* 2011). In another study of asthmatic children in the Atlanta area, distance

between residence and major roadway was associated with increased frequency of wheezing, medication use and hospitalizations, but not FENO (Brown *et al.* 2012).

All these studies relied on FENO collected at a single flow rate, which is a combination of NO produced from both the proximal and the distal airways. The measurement of FENO at multiple flow rates, allows the apportionment of NO contributions coming from these two compartments. This method can provide a better picture of underlying airway inflammation and has been used in the study of several respiratory illnesses. Therefore, we sought to characterize the association between surrogate variables for traffic-related exposures and proximal and distal inflammation measures in a cohort of New York City children. Given the limited data on other important non-traffic sources of airborne pollutants, we also sought to examine the association between stationary sources and these measures of inflammation. We also examined whether the associations between these exposure variables and FENO parameters were modified by sex, race/ethnicity, heating season, seroatopy and report of recent wheeze. We hypothesized that GIS indicators of airborne pollutant sources would be associated with elevated parameters of airway inflammation.

Methods

Study Population

Participants (n=727) were enrolled in a prospective birth cohort study conducted by the Columbia Center for Children's Environmental Health (CCCEH) and enrollment has been described previously (Goldstein *et al.* 2005; Miller *et al.* 2001; Perzanowski *et al.* 2006). Among the children fully enrolled in CCCEH, 562 (77.3%) had reached at least age 9 by the end of the FENO collection period (February, 23rd, 2013) and were eligible for FENO collection at multiple

flows. Of 562 children who were eligible, 359 had at least one set of measures from which distal and proximal fractions could be calculated (i.e. 36% lost-to-follow-up for this analysis). Of those children, 261 (73%) had ambient NO levels <100 ppb, all corresponding GIS variables and necessary covariates for multivariable analyses. 159 children had 2 sets of valid FENO measures and 1 child had 3 sets of valid FENO measures, with a total of 423 cases available for analyses. Informed consent was obtained in accordance with the Institutional Review Board at Columbia University. Socio-demographic information was obtained from baseline (prenatal) questionnaires.

GIS variables

Home addresses for the children were geocoded using Geosupport and LION geocoding services produced by the New York City Department of City Planning. Proximity to roadway and roadway density variables were calculated using the street centerline GIS layer from the New York State Office of CyberSecurity. Distance to primary highway (A1, mostly interstates) with limited access roads, was examined as a surrogate measure of traffic-related airborne pollution, including diesel emissions. Truck route density, density of unique bus stops and percentage area covered by major trucking routes also were used as surrogate measures for diesel exposure. This last variable was based on the percentage of measurement geography (*i.e.* 250-m) covered by a 300-meter buffer of major trucking routes following the methodology of a previously published study (Maantay 2007). This methodology was also used to determine the percentage area covered by 0.25-mile buffers of stationary point sources (SPS) and 0.50-mile buffers toxic release inventory sites (TRIS). SPS were identified by the National Emissions Inventory and consist of facilities that discharge non-fugitive emissions and have annual

emission data for ozone, particulate matter, carbon monoxide, nitrogen oxides, sulfur dioxide or lead. The number of building burning residual oil was also determined. Data were obtained from the NYC Department of Environmental Protection database that includes buildings with permits to use boilers that are renewed annually. The 250-meter buffer was selected a priori as a relevant distance of density of pollutants. A 500-m buffer was also tested in secondary analyses.

FENO parameters: $FENO_{50}$, JNO and Calv

FENO was collected at ages 9 and/or 11 years utilizing a previously modified offline NO collection method (Perzanowski *et al.* 2008). Children were instructed to inhale through an NO scrubber. FENO samples were obtained in in triplicate at 83 mL/s and in duplicate at 50 and 100 mL/sec and. $FENO_{50}$ was defined as FENO collected at 50 ml/sec. Bronchial flux (JNO) and alveolar NO (Calv) were calculated using a previously described modified Hogman method (Hogman *et al.* 2002; Rosa *et al.* 2011). Negative Calv levels were registered for 6 tests (1.4%) and JNO levels were negative in 58 tests (13.7%). In order to make use of these data, the distributions for these two variables were shifted by adding the minimum value for each variable plus 1 and were natural log transformed.

Data Analysis

Data were analyzed using generalized estimating equations (GEE) models with an autoregressive (AR1) working correlation matrix. $FENO_{50}$, JNO and Calv were each examined as separate outcomes for each of the GIS predictor variables. Models were adjusted for sex, race/ethnicity, report of cold or respiratory infection on the day of FENO collection, heating season (defined as October 1^{st}-April 30^{th}) and natural log of mean ambient NO on the day of the FENO collection. Effect modification was examined in stratified models. Models were stratified by sex, heating season, race/ethnicity, seroatopy and current wheeze. Models with interaction

terms were ran if the p-value within any stratum was less than 0.1 and the effect sizes appeared to differ by stratum. Specific IgE against cockroach, *D. farinae* and mouse were measured by Immunocap (Phadia, Uppsala, Sweden) in sera collected at age 9 (n=303) as previously described. (Donohue *et al.* 2008) Seroatopy was defined as a specific IgE ≥0.35 IU/ml to any of these three allergens. Current wheeze was defined as report of wheeze in the past 12 months on the International Study of Asthma and Allergies in Childhood asthma module (ISAAC). Data was analyzed using SPSS Version 18 (Chicago, IL).

Results

Demographic characteristics comparing CCCEH participants included in analyses (n=261) with those excluded (n=466) are shown (Table 1). There were no significant differences in maternal age, education or asthma. There were also no significant differences in the child's sex and in prenatal ETS exposure. The percentage of African Americans in the included cohort (44%) was significantly higher than those in the excluded cohort (30%). Values for the GIS variables of interest during the prenatal period were also compared (Table 2). There were no significant differences between the included and excluded participants, except in the number of buildings burning residual oil which was lower for the included participants (16 vs. 21). The proportion of cases with seroatopy was 112/303 (37%) and proportion with current wheeze was 93/418 (22.2%) in the included cohort. For those included in the analyses, the mean (±SD) distance from a child's home to a major road was 552 ± 442 m (median: 420-m). The mean (±SD) truck route density was 2.63±1.98 km/km^2 and the mean (±SD) area covered my major truck routes was 0.597±0.285%. The mean (±SD) density of unique bus stops per km^2 was 29.3±17.6 and the mean number of buildings burning residual oil and 14.8±14.8 respectively.

Mean (±SD) area covered by TRI sites, SPS and commercial buildings was 0.198±0.335, 0.187±0.2945 and 7.77±9.01 respectively.

In univariate analyses, there were no significant associations between truck route density, distance to primary highway and density of unique bus stops and any of the FENO parameters. There were also no significant associations between number of buildings burning residual oil, percentage area covered by SPS and percentage area used for commercial purposes and $FENO_{50}$, JNO or Calv. However, there was a significant inverse association between percentage area covered by major trucking routes and Calv (β=-0.168, 95% CI [-0.333, -0.003]. There was also a significant association between area covered by TRI sites and JNO levels (β=0.139, [0.001, 0.276]).

Table 1. Comparison of demographic characteristics between included and excluded participants

Characteristic	Included n=261	Excluded n=466	p-value
Mother's age[a] (SD)	25 (5.1)	25 (4.8)	0.597
Child's race/ethnicity			<0.001
African American (%)	44	30	
Dominican (%)	56	70	
Maternal education[b] (%)	64	64	0.895
Maternal asthma[c] (%)	24	26	0.645
Child's sex (% male)	44	51	0.088
Prenatal ETS[d] exposure (%)	37	33	0.300

[a]Mother's age at time of child's birth, Mann Whitney U Test
[b]Mother completed high school, General Educational Development (GED) test or greater
[c]Mother reported asthma in prenatal or 3 month questionnaire.
[d]Prenatal ETS exposure defined as maternal or cord blood cotinine level ≥ 15 ng/ml or report of smoker in the home from prenatal questionnaire. #Differences in categorical variables tested using Pearson Chi-Square

Table 2. Comparison of GIS variables during prenatal period between included and excluded participants

Characteristic	Included n=261	Excluded n=466	p-value
Truck route density (km/km^2)	2.51	2.52	0.591
Distance to primary highway (km)	0.428	0.443	0.868
Major truck routes (% area)	0.699	0.705	0.450
Density of unique bus stops	30.6	30.6	0.396
Buildings burning residual oil (#)	16	21	0.010
SPS (% area)	0.025	0.022	0.915
TRI sites (% area)	0.000	0.000	0.669
Commercial buildings (% area)	6.22	6.34	0.961

250-meter buffers were drawn around each subject's home to calculate all GIS variables
#Medians shown. Differences were tested using Mann-Whitney U Test.

In multivariable models, after adjustment for sex, race/ethnicity, report of cold or respiratory infection on the day of FENO collection, heating season and natural log of mean ambient NO there were no significant associations between any of the GIS variables and FENO parameters (Table 3). The effect sizes for the significant associations seen in univariate analyses were smaller and no longer significant after adjustment for covariates.

For the majority of analyses for effect modification, there were no significant differences across strata (Tables 4-7). There was a significant interaction between sex and percent area covered by major truck routes in the model predicting $FENO_{50}$ (interaction p-value 0.038). We also found significant interactions between sex and area covered by SPS for models predicting $FENO_{50}$ and Calv levels (interaction p-values 0.038 and 0.031 respectively). In analyses for effect modification by heating season, there was only a significant interaction between percent area covered by TRIS and heating season in the $FENO_{50}$ model (interaction p-value=0.012). Greater percentage area covered by TRIS was associated with higher $FENO_{50}$ only during the non-heating season (β=0.394, 95% CI [0.088, 0.701], p=0.012). Race/ethnicity and seroatopy and were also examined as potential effect modifiers but interaction models did not yield any significant results (Tables 5 and 6). There was a significant interaction between distance to a major highway and report of current wheeze in models predicting $FENO_{50}$ and JNO (interaction p-value=0.001 for both). Models stratified by report of current wheeze are shown in Table 7. Greater distance from major roadway was associated with lower levels of $FENO_{50}$ and JNO only in children with no report of current wheeze. Results for all models using a buffer of 500-m to determine exposure variables are presented in the appendix.

Table 3. Multivariable models for airborne pollutant source variables and FENO parameters [β (95% CI)]

Variable	$FENO_{50}$	JNO	Calv
Truck route density (km/km^2)	-0.009 (-0.039, 0.021) p=0.546	0.007 (-0.018,0.033) p=0.583	-0.009 (-0.028, 0.010) p=0.347
Distance to primary highway (km)	0.002 (-0.196, 0.199) p=0.987	-0.183 (-0.535, 0.169) p=0.308	0.043 (-0.054, 0.141) p=0.382
Major truck routes (% area)	-0.067 (-0.306, 0.172) p=0.581	0.150 (-0.113, 0.413) p=0.263	-0.139 (-0.299, 0.021) p=0.089
Density of unique bus stops	0.000 (-0.004, 0.003) p=0.881	0.000 (-0.002,0.002) p=0.790	0.000 (-0.002, 0.003) p=0.732
Buildings burning residual oil (#)	0.002 (-0.003, 0.006) p=0.471	0.003 (0.000, 0.005) p=0.070	0.000 (-0.003, 0.003) p=0.988
SPS (% area)	-0.087 (-0.296, 0.122) p=0.416	0.062 (-0.074,0.197) p=0.371	-0.091 (-0.214, 0.032) p=0.146
TRI sites (% area)	0.130 (-0.071, 0.330) p=0.204	0.119 (-0.030, 0.268) p=0.116	-0.071 (-0.208, 0.066) p=0.309
Commercial buildings (% area)	-0.004 (-0.011, 0.003) p=0.267	0.000 (-0.004, 0.004) p=0.967	-0.004 (-0.010, 0.001) p=0.128

250-meter buffers were drawn around each subject's home to calculate all GIS variables
Adjusted for sex, race/ethnicity, report of cold or respiratory infection on the day of FENO collection, heating season and natural log of mean ambient NO.

Table 4. Multivariable models for airborne pollutant source variables and FENO parameters stratified by sex [β (95% CI)]

Variable	$FENO_{50}$		JNO		Calv	
	Male	Female	Male	Female	Male	Female
Truck route density (km/km^2)	-0.039 (-0.095, 0.017)	0.009 (-0.026,0.045)	-0.006 (-0.037, 0.024)	0.022 (-0.021, 0.065)	-0.024 (-0.054, 0.005)	0.001 (-0.025, 0.027)
Distance to primary highway (km)	-0.089 (-0.360, 0.182)	0.045 (-0.204, 0.294)	-0.219 (-0.739, 0.302)	-0.107 (-0.224, 0.009)	0.046 (-0.140, 0.232)	0.040 (-0.072, 0,151)
Major truck routes (% area)	-0.469* (-0.929, -0.009)	0.159 (-0.090, 0.407)	-0.066 (-0.304, 0.171)	0.321 (-0.110, 0.752)	-0.302* (-0.583,-0.021)	-0.044 (-0.228, 0.140)
Density of unique bus stops	-0.001 (-0.008, 0.005)	0.000 (-0.005, 0.004)	0.000 (-0.003, 0.003)	0.000 (-0.002, 0.002)	0.000 (-0.004, 0.005)	0.001 (-0.002, 0.003)
Buildings burning residual oil (#)	0.005 (-0.004, 0.013)	-0.001 (-0.005, 0.004)	0.004* (0.000,0.008)	0.002 (-0.002, 0.005)	0.000 (-0.006, 0.005)	0.000 (-0.003, 0.003)
SPS (% area)	-0.397* (-0.736, -0.058)	0.106 (-0.158, 0.370)	-0.042 (-0.201, 0.116)	0.176 (-0.048, 0.399)	-0.259† (-0.433, -0.085)	0.037 (-0.118, 0.193)
TRI sites (% area)	0.178 (-0.176, 0.533)	0.075 (-0.157, 0.307)	0.177 (-0.031, 0.385)	0.088 (-0.154, 0.330)	-0.159 (-0.380, 0.061)	-0.025 (-0.191. 0.142)
Commercial buildings (% area)	-0.008 (-0.020, 0.004)	0.003 (-0.007, 0.014)	-0.003* (-0.006, 0.000)	0.009 (-0.004, 0.021)	-0.004 (-0.011, 0.003)	-0.006 (-0.013, 0.001)

Adjusted for sex, race/ethnicity, report of cold or respiratory infection on the day of FENO collection, heating season and natural log of mean ambient NO. *****$p<0.05$, †$p<0.01$. Models in gray had significant interaction p-values

Table 5. Multivariable models for airborne pollutant source variables and FENO parameters stratified by race/ethnicity [β (95% CI)]

Variable	$FENO_{50}$		JNO		Calv	
	African American	Dominican	African American	Dominican	African American	Dominican
Truck route density (km/km^2)	-0.011 (-0.083, 0.062)	-0.007 (-0.039, 0.024)	0.046 (-0.037, 0.130)	-0.003 (-0.091, 0.088)	-0.018 (-0.060, 0.023)	-0.006 (-0.027, 0.015)
Distance to primary highway (km)	-0.017 (-0.319, 0.285)	0.058 (-0.148, 0.264)	-0.287 (-0.807, 0.233)	0.044 (-0.068, 0.157)	0.074 (-0.050, 0.198)	-0.025 (-0.167, 0.117)
Major truck routes (% area)	-0.115 (-0.555, 0.325)	0.11 (-0.265, 0.287)	0.409 (-0.245, 1.063)	0.049 (-0.105, 0.202)	-0.179 (-0.469, 0.112)	-0.107 (-0.291, 0.078)
Density of unique bus stops	0.003 (-0.004, 0.011)	-0.002 (-0.006, 0.002)	0.001 (-0.003, 0.005)	-0.001 (-0.002, 0.001)	0.001 (-0.003, 0.006)	0.000 (-0.003, 0.002)
Buildings burning residual oil (#)	0.001 (-0.01, 0.013)	0.001 (-0.003, 0.006)	0.006 (-0.005, 0.017)	0.002 (-0.001, 0.004)	0.003 (-0.004, 0.010)	-0.001 (-0.004, 0.002)
SPS (% area)	-0.163 (-0.529, 0.203)	-0.076 (-0.301, 0.149)	0.095 (-0.135, 0.325)	0.002 (-0.136, 0.141)	-0.173 (-0.374, 0.027)	-0.040 (-0.184, 0.103)
TRI sites (% area)	0.113 (-0.172, 0.398)	0.256 (-0.010, 0.522)	0.217 (-0.020, 0.454)	0.055 (-0.145, 0.256)	-0.148 (-0.324, 0.027)	0.048 (-0.162, 0.258)
Commercial buildings (% area)	-0.010 (-0.022, 0.0010	0.001 (-0.007, 0.010)	0.002 (-0.008, 0.011)	0.000 (-0.003, 0.003)	-0.008 (-0.015, -0.002)	-0.002 (-0.008, 0.005)

Adjusted for sex, race/ethnicity, report of cold or respiratory infection on the day of FENO collection, heating season and natural log of mean ambient NO. *****$p<0.05$, †$p<0.01$.

Table 6. Multivariable models for airborne pollutant source variables and FENO parameters stratified by seroatopy [β (95% CI)]

Variable	$FENO_{50}$		JNO		Calv	
	Non-seroatopic	Seroatopic	Non-seroatopic	Seroatopic	Non-seroatopic	Seroatopic
Truck route density (km/km^2)	-0.003 (-0.035, 0.029)	-0.020 (-0.070, 0.030)	-0.006 (-0.020. 0.009)	-0.008 (-0.049, 0.033)	0.002 (-0.026, 0.029)	-0.026 (-0.073, 0.021)
Distance to primary highway (km)	0.115 (-0.132, 0.361)	0.022 (-0.361, 0.405)	0.055 (-0.071, 0.181)	-0.064 (-0.341, 0.213)	0.053 (-0.053, 0.159)	0.061 (-0.127, 0.248)
Major truck routes (% area)	-0.039 (-0.309, 0.230)	-0.050 (-0.472, 0.371)	0.005 (-0.089, 0.099)	0.016 (-0.305, 0.337)	-0.069 (-0.279, 0.141)	-0.177 (-0.475, 0.121)
Density of unique bus stops	0.000 (-0.005, 0.005)	-0.006 (-0.012, 0.000)	0.000 (-0.001, 0.002)	-0.004 (-0.008, 0.001)	-0.001 (-0.004, 0.002)	0.001 (-0.003, 0.005)
Buildings burning residual oil (#)	0.000 (-0.007, 0.007)	-0.005 (-0.012, 0.003)	0.001 (-0.001, 0.003)	0.000 (-0.006, 0.005)	-0.002 (-0.006, 0.002)	-0.002 (-0.008, 0.004)
SPS (% area)	-0.055 (-0.310, 0.200)	-0.140 (-0.545, 0.265)	-0.001 (-0.114, 0.111)	0.025 (-0.259, 0.309)	-0.020 (-0.196, 0.155)	-0.148 (-0.383, 0.088)
TRI sites (% area)	0.008 (-0.223, 0.240)	0.323 (-0.073, 0.718)	-0.013 (-0.111, 0.086)	0.313* (0.037, 0.589)	-0.007 (-0.207, 0.194)	-0.299* (-0.584, -0.013)
Commercial buildings (% area)	-0.006* (-0.012, -0.001)	-0.002 (-0.027, 0.023)	0.000 (-0.003, 0.002)	0.003 (-0.016, 0.022)	-0.002 (-0.007, 0.002)	-0.012 (-0.028, 0.005)

Adjusted for sex, race/ethnicity, report of cold or respiratory infection on the day of FENO collection, heating season and natural log of mean ambient NO. Seroatopy was defined as a specific IgE ≥0.35 IU/ml to cockroach, mouse or *D. farinae*. *****p<0.05, †p<0.01.

Table 7. Multivariable models for airborne pollutant source variables and FENO parameters stratified by wheeze [β (95% CI)]

Variable	$FENO_{50}$		JNO		Calv	
	No wheeze	Wheeze	No wheeze	Wheeze	No wheeze	Wheeze
Truck route density (km/km^2)	-0.002 (-0.038, 0.033)	0.006 (-0.018, 0.030)	-0.001 (-0.020, 0.018)	0.003 (-0.034, 0.039)	-0.007 (-0.028, 0.014)	-0.020 (-0.074, 0.034)
Distance to primary highway (km)	-0.205† (-0.356, -0.054)	-0.184 (-0.400, 0.031)	-0.109† (-0.190, -0.029)	0.157 (-0.025, 0.340)	-0.014 (-0.105, 0.077)	0.079 (-0.095, 0.252)
Major truck routes (% area)	-0.013 (-0.288, 0.262)	0.125 (-0.099, 0.349)	0.040 (-0.104, 0.184)	0.081 (-0.153, 0.315)	-0.131 (-0.301, 0.038)	-0.135 (-0.475, 0.205)
Density of unique bus stops	0.000 (-0.004, 0.004)	0.003 (-0.004, 0.011)	0.000 (-0.002, 0.002)	-0.002 (-0.006, 0.003)	0.000 (-0.002, 0.002)	0.002 (-0.004, 0.007)
Buildings burning residual oil (#)	0.001 (-0.004, 0.006)	0.005 (-0.006, 0.017)	0.002 (-0.001, 0.004)	0.002 (-0.002, 0.007)	-0.001 (-0.004, 0.002)	0.003 (-0.002, 0.009)
SPS (% area)	-0.053 (-0.270, 0.164)	0.072 (-0.300, 0.444)	0.022 (-0.101, 0.146)	0.025 (-0.233, 0.283)	-0.025 (-0.155, 0.104)	-0.293 (-0.616, 0.031)
TRI sites (% area)	0.150 (-0.039, 0.340)	0.566 (-5.53, 6.66)	0.039 (-0.090, 0.169)	0.274 (-0.067, 0.616)	-0.022 (-0.161, 0.117)	-0.181 (-0.513, 0.152)
Commercial buildings (% area)	-0.002 (-0.009, 0.006)	0.020 (-0.017, 0.056)	-0.001 (-0.003, 0.002)	-0.001 (-0.008, 0.006)	-0.002 (-0.008, 0.003)	-0.008* (-0.016, 0.000)

Adjusted for sex, race/ethnicity, report of cold or respiratory infection on the day of FENO collection, heating season and natural log of mean ambient NO. Wheeze classification based on ISAAC question "Has your child had wheezing or whistling in the chest in the past 12 months?" *****$p<0.05$, †$p<0.01$. Models in gray had significant interaction p-values

Discussion

The objective of this study was to characterize the associations between GIS variables, as surrogate measures of chronic exposure to traffic-related and stationary source airborne pollutants and biomarkers of proximal and distal airway inflammation in a cohort of children living in NYC. To our knowledge, this is the first paper to use these proximal and distal markers of inflammation to determine these associations and to include other non-traffic related sources. In our study, we did not find any significant associations between traffic-related or stationary source GIS variables and any of the FENO parameters in multivariable models. In analyses for effect modification, we found that the majority of associations did not vary across strata.

There is substantial evidence linking distance to a major roadway to risk of asthma or respiratory symptoms in children (Baumann *et al.* 2011; Brown *et al.* 2012; Ryan *et al.* 2005; Venn *et al.* 2001). However, some of these studies have not found significant associations between distance to major roadway and elevated FENO parameters. Distance to a heavily transited avenue in Peru was associated with higher odds of asthma and atopy, but was not associated with FENO levels (Baumann *et al.* 2011). However, in this study, indoor and outdoor $PM_{2.5}$ measures were not associated with distance to roadway.

In these analyses, we attempted to study other exposure variables related to diesel traffic since there is epidemiological evidence that exposure to this type of emission is more relevant to the study of respiratory morbidity (Janssen *et al.* 2003; Janssen *et al.* 2001; Ryan *et al.* 2005). Trucks and buses are mostly fueled by diesel, and have been shown to be associated with higher levels of ambient black carbon (BC) in NYC (Lena *et al.* 2002; Richmond-Bryant *et al.* 2009). However, we did not see any significant associations with truck route density, percent area covered by major truck routes or density of unique bus stops.

One potential explanation for the lack of associations might be the buffer used to determine these GIS variables. In the study in Ciudad Juarez, the association between road density and FENO levels was stronger when smaller buffers were used and was only significant with buffers smaller than 150-m (Holguin *et al.* 2007). Other studies have also relied on variables calculated with buffers smaller than 250-m (Cakmak *et al.* 2012; Dales *et al.* 2008). Another potential explanation for the lack of association may be the geographic distribution of our subjects. Studies have found that the associations between distance to roadway and respiratory symptoms are mostly limited to major roadways located within 100-150-m of children's homes (McConnell *et al.* 2006; Ryan *et al.* 2005; Venn *et al.* 2001). This is because pollutants from vehicle exhaust decrease markedly at distances greater than 150-m from the road, diminishing their potential effects (Gilbert *et al.* 2005; Sahsuvaroglu *et al.* 2006). In our cohort, we found that only 50 subjects lived within 150-m of a major road and only half of these subjects lived within 100-m of a major road.

Another possible explanation for the lack of association is that these GIS variables might act as better surrogates for chronic exposure. For example, in the study by McConnell *et al*, the association between distance to major road and prevalent was asthma was strongest among children who were long-term residents when compared to short-term residents (McConnell *et al.* 2006). Because FENO levels have been shown to be elevated after short-term exposure to $PM_{2.5}$ and EC they might not be adequate markers of the potential effects of longer-term exposures (Delfino *et al.* 2006; Sarnat *et al.* 2012). GIS variables were also only determined for the children's home addresses, which might neglect other important exposure locations like schools. Air pollutant measures from school monitors were better predictors of FENO levels in a panel of

children living in the US-Mexico border, than measurements from central site monitors (Sarnat *et al.* 2012).

We attempted to examine the potential increased susceptibility of asthmatics to air pollution exposure by stratifying by current wheeze as a surrogate for asthma diagnosis. We reported significant decreases in $FENO_{50}$ and JNO with increasing distance to primary highway only among children who had *no report* of current wheeze. Previous studies of exposure to traffic related air pollution have also reported sex differences (McConnell *et al.* 2006; Pershagen *et al.* 1995; Venn *et al.* 2001). In our study, we found significant negative associations in boys and positive but non-significant associations in girls in models for associations between SPS and $FENO_{50}$ and Calv. These significant results must be interpreted with caution. Due to the number of comparisons in our analyses, these results might be the product of chance and may not reflect a real association between distance to primary highway and wheeze in the reported stratum.

There are some limitations to this study. Airborne pollutants are known to be affected by weather patterns, and we did not adjust for variables like wind speed, direction and rainfall. Temporality was adjusted through a dichotomous heating season variable. Our cohort was also composed of Dominican and African American children, limiting its generalizability to the rest of the population. However, there are also strengths to this study. We had a large enough sample size to examine several associations. We did not limit our analyses to variables related to traffic-related air pollutants, but we also examined the potential for other stationary sources that might be important contributors to our cohort's exposure. We were also able to examine interactions between several susceptibility factors and these exposure variables.

In conclusion, we saw no associations between several surrogate variables for traffic and other sources of airborne pollutants and FENO parameters. The associations we saw in stratified

analyses should be interpreted with caution. These surrogate variables might be better used markers of chronic exposure in this cohort and FENO parameters are better suited to the study of short-term exposures.

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Supplemental tables

Table S1. Multivariable GEE models for airborne pollutant source variables and FENO parameters [β (95% CI)]

Variable	$FENO_{50}$	JNO	Calv
Truck route density (km/km^2)	-0.002 (-0.048, 0.044) p=0.935	0.020 (-0.028,0.068) p=0.415	0.007 (-0.026, 0.041) p=0.664
Major truck routes (% area)	0.063 (-0.325, 0.451) p=0.752	0.360 (-0.192, 0.911) p=0.201	-0.061 (-0.325, 0.202) p=0.648
Density of unique bus stops	0.001 (-0.004, 0.006) p=0.703	0.001 (-0.002,0.004) p=0.423	0.001 (-0.003, 0.004) p=0.658
Buildings burning residual oil (#)	0.001 (-0.001, 0.002) p=0.459	0.001 (0.000, 0.002) p=0.060	0.000 (-0.001, 0.001) p=0.748
SPS (% area)	-0.230 (-0.503, 0.043) p=0.099	0.058 (-0.132,0.247) p=0.551	-0.169 (-0.345, 0.006) p=0.059
TRI sites (% area)	0.055 (-0.173, 0.283) p=0.635	0.100 (-0.072, 0.271) p=0.254	-0.091 (-0.237, 0.056) p=0.227
Commercial buildings (% area)	-0.001 (-0.008, 0.006) p=0.763	0.001 (-0.004, 0.007) p=0.631	-0.002 (-0.007, 0.003) p=0.447

500-meter buffers were drawn around each subject's home to calculate all GIS variables
Adjusted for sex, race/ethnicity, report of cold or respiratory infection on the day of FENO collection, heating season and natural log of mean ambient NO

Table S2. Spearman correlation matrix for FENO parameters†

	$FENO_{50}$	JNO	Calv
$FENO_{50}$	1.000	0.683**	0.580**
JNO		1.000	-0.066
Calv			1.000

†Natural log transformed values
**p<0.001

Chapter VI: Domestic Airborne Black Carbon Levels Positively Associated With 8-isoprostane In Exhaled Breath Condensate Among Children In New York City

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Abstract

Background: Exposure to airborne black carbon (BC) has been associated with asthma development, respiratory symptoms and decrements in lung function. However, the mechanism through which BC may lead to respiratory symptoms has not been completely elucidated. Oxidative stress has been suggested as a potential mechanism through which BC might lead to adverse health outcomes. Exhaled breath condensate (EBC) allows for the non-invasive collection of airway lining fluid containing biomarkers of oxidative stress like 8-isoprostane, a stable by-product of lipid peroxidation. Therefore, we sought to characterize the association between domestic airborne BC exposure and 8-isoprostane in EBC.

Methods: Seven- and eight-year-old children participated in an asthma case-control study in New York City. During home visits, air samples and EBC were collected. Seven day averages of domestic levels of particulate matter <2.5 microns ($PM_{2.5}$), BC and environmental tobacco smoke (ETS) were measured. Urea and 8-isoprostane were measured by liquid chromatography tandem mass spectrometry (LC/MS/MS) in EBC.

Results: In univariate models, $PM_{2.5}$ and BC, but not ETS, were significantly associated with increases in 8-isoprostane in the EBC (β=0.006 and β=0.106 respectively, $p<0.05$ for both)). These associations remained statistically significant for both $PM_{2.5}$ and BC after adjustment for covariates. In a co-pollutant model including $PM_{2.5}$, BC and ETS, only BC remained a statistically significant predictor of 8-isoprostane.

Conclusions: Our findings suggest the BC fraction of PM might have the largest effect on a biomarker of oxidative stress among airborne combustion by-products.

Introduction

Exposure to airborne particulate matter (PM), diesel exhaust particles (DEP) and combustion by-products has been implicated in asthma development and morbidity (Clark *et al.* 2010; Jung *et al.* 2012). Early life exposure to traffic-related air pollutants and living in proximity to point sources that contribute to airborne PM have been associated with elevated risk of asthma in young children (Clark *et al.* 2010). Proximity to roadway has been associated with increased asthma prevalence and report of wheeze in children living in southern California (McConnell *et al.* 2006). Indoor levels of PM were found to be associated with development of wheeze at ages 5-7 years in an inner-city cohort in New York City (NYC) (Jung *et al.* 2012).

Recently, black carbon (BC) has been proposed as a more suitable surrogate for DEP exposure than PM, given its association with the volume of diesel traffic and not car traffic (Cornell *et al.* 2012; Lena *et al.* 2002). DEP are thought to be responsible for a large portion of the detrimental effects of traffic-associated PM (Sydbom *et al.* 2001). Truck route density was also a strong predictor of wintertime BC and domestic level BC in two different studies (Clougherty *et al.* 2013; Cornell *et al.* 2012). In addition, burning of residual fuel oil, used extensively in apartment and commercial heating in NYC, is a significant source of airborne BC (Clougherty *et al.* 2013; Cornell *et al.* 2012). Previous epidemiological studies have found associations between BC exposure and adverse respiratory outcomes. In a birth cohort in British Columbia, central site exposure to BC, but not $PM_{2.5}$, early in life was associated with increased risk of childhood asthma diagnosis (Clark *et al.* 2010). Local BC levels estimated through a spatiotemporal land-use regression model were associated with decrements in lung function measures in women living in East Boston (Suglia *et al.* 2008). In NYC adolescents, increases in

school levels of BC were associated with acute respiratory symptoms, including increased wheeze, chest tightness and shortness of breath (Patel *et al.* 2010).

Despite growing evidence linking BC exposure and respiratory illness, the mechanism through which BC may lead to these adverse respiratory effects has not been completely elucidated. One of the potential mechanisms is oxidative stress. 8-isoprostane, which belongs to the family of F2-isoprostanes, is a by-product of the free radical-catalyzed peroxidation of arachidonic acid can be measured in exhaled breath condensate (EBC). It has been used as a surrogate marker of oxidative stress in multiple studies, and found to be elevated in the presence of asthma, cystic fibrosis and chronic obstructive pulmonary disease (Baraldi *et al.* 2003; Montuschi *et al.* 2000a; Montuschi *et al.* 1999; Montuschi *et al.* 2000b). Exposure to combustion fly ash particles, as a model for particulate matter exposure, has been shown to induce generation of reactive oxygen species (ROS) in lung murine and primary human macrophages (Fritsch-Decker *et al.* 2011). In this particular study, murine macrophages that were incubated with a low dose of fly ash particles, had a time dependent increase in 8-isoprostane concentrations (Fritsch-Decker *et al.* 2011).

The measurement of 8-isoprostane in the airways also can serve in the study of airborne exposures. Recently, researchers in China found positive associations between central site levels of $PM_{2.5}$ and 8-isoprostane, measured in EBC collected from healthy young adults (Huang *et al.* 2012). In another recent study, 1-5 day averages of BC measured at NYC high schools were associated with increased levels of 8-isoprostane measured in EBC in adolescents enrolled at the schools (Patel *et al.* 2012). Spatial variability and temporality have been shown to be significant predictors of BC concentrations in NYC, stressing the importance of understanding local exposure patterns (Clougherty *et al.* 2013). Therefore, we sought to characterize the association

between short-term domestic measures of BC, and additionally $PM_{2.5}$ and environmental tobacco smoke (ETS), another known pollutant associated with oxidative stress (Kostikas *et al.* 2013; Noakes *et al.* 2007), and 8-isoprostane measured in EBC as a surrogate marker of oxidative stress in a cohort of children living in NYC. We hypothesized that 7-day averages of residential measures of all three of these pollutants will be associated with increased levels of 8-isoprostane in EBC.

Methods

Study cohort

Participants (N=350) were enrolled in the New York City Neighborhood Asthma and Allergy Study (NYC NAAS) and enrollment has been previously described (Olmedo *et al.* 2011). In brief, the NYC NAAS is a case-control asthma study of 7-8 year old children living in NYC whose parents were recruited through the Health Insurance Plan of New York (HIP), a middle-income insurance provider. Neighborhoods were selected based on zip code level asthma prevalence among 5-year-old children as reported by the NYC Department of Health and Mental Hygiene (2003). Neighborhoods in the Bronx, Brooklyn, Queens and Manhattan with asthma prevalence of 3-9% were defined as lower asthma prevalence neighborhoods (LAPN) and those with asthma prevalence of 11-18% as higher asthma prevalence neighborhoods (HAPN). Cases were defined based on parental report of symptoms, including responses to the International Study of Asthma and Allergy in Childhood (ISAAC) wheeze module. Children were classified based on whether the parent reported at least one of the following for the child in the 12 months prior to administration of the questionnaire: 1) wheeze, 2) being woken at night by cough without having a cold, 3) wheeze with exercise or 4) report of asthma medication use. Children

who did not meet any of these criteria were classified as controls. Demographic characteristics were obtained through questionnaires administered during home visits.

Exposure assessment

Average $PM_{2.5}$ was collected by sampling air in the child's home at 1.5 L/min for 7 days. BC and ETS were quantified on the filter using a recently validated multi-wavelength optical absorption technique developed for Teflon filters utilizing a modified Lawless method (Yan *et al.* 2011). The optical device used consisted of a balanced deuterium tungsten halogen light source (DH-2000-BAL), an integrating sphere (ISP-50-8-R), a lab-made filter holder, and an Ocean Optics USB4000-VIS-NIR miniature fiber-optic spectrometer. Children's home addresses were geocoded and linked to a previously described GIS database (Lovasi *et al.* 2009). The density of buildings burning residual oil and the truck route density in a 500-m Euclidean radius were examined as potential predictors due to a previously seen association between these two variables and domestic BC levels (Cornell *et al.* 2012). This buffer geography had been chosen a priori but sensitivity analyses with a 250-m buffer yielded similar results (Cornell *et al.* 2012). In order to examine neighborhood level exposures, annual averages of $PM_{2.5}$ and elemental carbon (EC), which is representative of the same carbonaceous fraction as BC and are considered comparable, were obtained from the NYC Community Air Survey for 2009 and assigned to each participant based on their United Hospital Fund (UHF) ID.

Exhaled breath condensate collection and analysis

EBC was collected using the R-tube system (Respiratory Research Inc., Charlottesville, VA) during the home visit *before* the start of the 7 day air monitoring session due to logistical

issues. Exhaled breath is condensed as it passes through a collection chamber within a cold (-20°C) aluminum sleeve. Children were seated and instructed to form a complete seal around the mouthpiece and breathe at a normal rate for 10 minutes. The breath condensate was aliquoted and stored at -80 degrees until analyses. The measurement of biomarkers in EBC stems from the idea that lung lining fluid droplets become aerosolized during exhalation (Kharitonov and Barnes 2002). However, there is extreme and variable dilution of droplets from lung lining fluid (Effros *et al.* 2003). Urea, which exists under homeostatic regulation in the body, has been previously used as a marker of dilution in EBC (Effros *et al.* 2003). Urea and 8-isoprostane were measured in 150 randomly chosen EBC samples by liquid chromatography tandem mass spectrometry (LC/MS/MS) using a Thermo Finnigan TSQ Quantum system (Thermo Fisher Scientific, Waltham, MA) at the Lamont-Doherty Earth Institute. Due to a transient problem with the assay, 8-isoprostane levels were not detectable in one batch of 14 samples. Because there was not enough EBC volume in storage, these samples could not be assayed again and were excluded from analyses. Of these 14 samples, 6 (42.9%) were from participants classified as controls while the remaining 8 (57.1%) were from participants classified as cases.

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idea that bronchoalveolar extracellular lining fluid droplets become aerosolized during exhalation (Kharitonov and Barnes 2002). However, there is extreme and variable dilution of droplets from lung lining fluid (Effros *et al.* 2003). Urea, which exists under homeostatic regulation in the body, has been previously used as a marker of dilution in EBC (Effros *et al.* 2003). Urea and 8-isoprostane were measured in 150 randomly chosen EBC samples by liquid chromatography tandem mass spectrometry (LC/MS/MS) using a Thermo Finnigan TSQ Quantum system (Thermo Fisher Scientific, Waltham, MA) at the Lamont-Doherty Earth Institute. Due to a transient problem with the assay, 8-isoprostane levels were not detectable in one batch of 14 samples. Because there was not enough EBC volume in storage, these samples could not be assayed again and were excluded from analyses. Of these 14 samples, 6 (42.9%) were from participants classified as controls while the remaining 8 (57.1%) were from participants classified as cases.

Statistical Analyses

Complete data was available for 130 participants. Data were analyzed using generalized estimating equations models with an exchangeable correlation matrix and a robust estimator covariance structure (SPSS 18, Chicago, IL). Children were matched by UHF ID to account for spatial correlation within neighborhoods. All three pollutants were analyzed as continuous predictors. 8-isoprotane and urea concentrations were natural log transformed. Potential covariates were included in the model if their presence produced a greater than 10% change in beta. Final models were adjusted for case control status, sex, African American race, heating season (defined as October 1st-April 30th) and urea concentrations. BC, $PM_{2.5}$ and ETS were analyzed both as separate predictors of 8-isoprostane and in a co-pollutant model.

Results

Selected participants did not differ significantly from participants excluded from these reported analyses (supplemental Table S1), except that the included cohort had a significantly greater proportion of cases to controls (p=0.007). Demographic characteristics for the participants and descriptive measures for each pollutant are shown in Table 1. Almost half (46.9%) of the participants were African American and 36.2% were classified as of Hispanic ethnicity. As shown in Figure 1, domestic measures of BC and $PM_{2.5}$ correlated moderately. BC also significantly correlated with neighborhood annual measures of EC and $PM_{2.5}$. In this subset of participants, we also found that both truck route density (β=0.217, p= 0.015) and number of buildings burning residual oil (β=0.016, p= 0.001) were significant predictors of domestic BC as previously reported in the larger cohort (Cornell *et al.* 2012). Domestic levels of $PM_{2.5}$ were not significantly associated with any neighborhood pollutant measures, however there was a modest association between domestic and neighborhood $PM_{2.5}$ during the heating season (results not shown). BC and ETS were inversely correlated, but upon further exploration, these results appeared to be driven by the samples with non-detectable levels of ETS (57/130, 44%).

$PM_{2.5}$ (β=0.006, 95% CI [0.000, 0.012]) and BC (β=0.106, 95%CI [0.044, 0.167]) but not ETS exposure, were associated with increased 8-isoprostane in the EBC. These associations remained significant after adjustment for case control status, sex, African American race, heating season and urea concentrations for both $PM_{2.5}$ (β=0.008, 95% CI [0.001,0.014]) and BC (β=0.094, 95% CI [0.021, 0.166]). Figure 2 shows adjusted results expressed in terms of percent increase in 8-isoprostane per interquartile (IQR) range increase of each pollutant. An IQR increase in BC was associated with an 11% mean increase in 8-isoprostane and an IQR increase in $PM_{2.5}$ was associated with a mean 6% increase in 8-isoprostane. As shown in Figure 3, in a co-

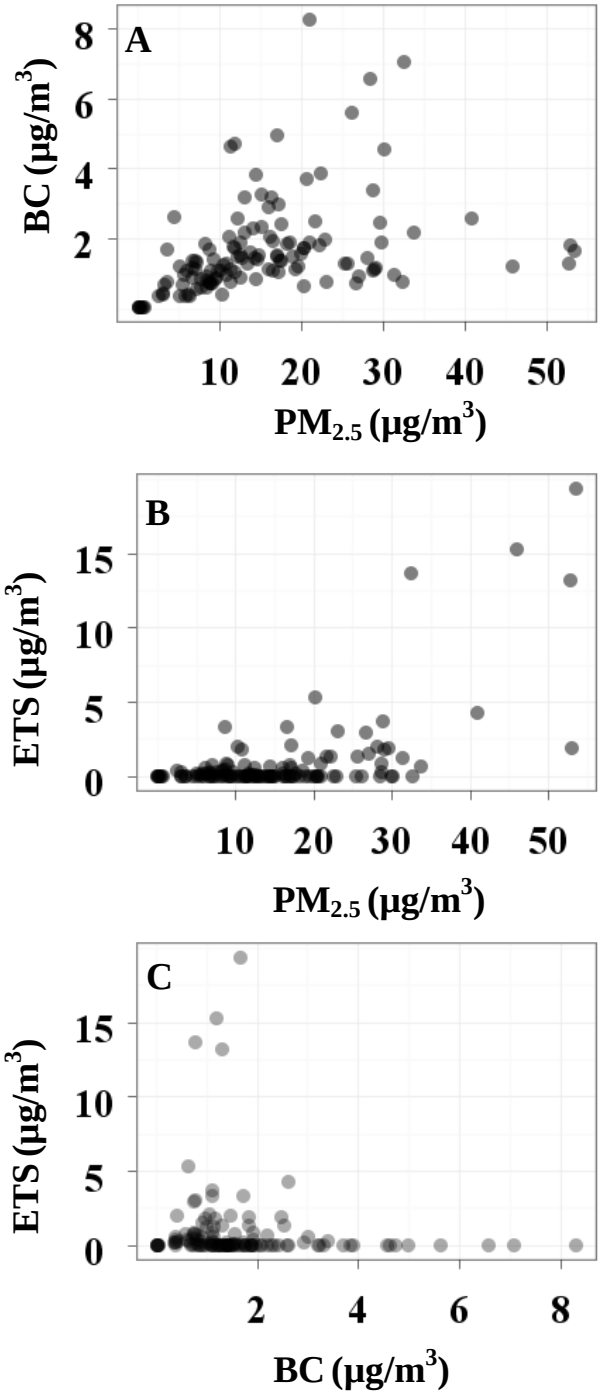
pollutant model including $PM_{2.5}$, BC and ETS, only BC (β=0.099, 95% CI [0.012, 0.187]) remained a statistically significant predictor of 8-isoprostane.

Table 1. Demographic characteristics

Case: Control (n)	88:42
Male sex, n (%)	78/130 (60)
Ethnicity/Race n (%)	
White	22/130 (16.9)
African American	61/130 (46.9)
Asian	15/130 (11.5)
Other/Mixed	29/130 (22.3)
Hispanic ethnicity	47/130 (36.2)
Mother completed high school, n (%)	119/128 (93)
Father completed high school, n (%)	111/121 (91.7)
Household income <25K, n (%)	9/130 (6.9)
Mother has asthma, n (%)	28/128 (21.9)
Father has asthma, n (%)	17/128 (13.3)
Seroatopic	65/125 (52)
$PM_{2.5}$ median (25^{th}-75^{th})	12.97(8.21-20.22)
BC median (25^{th}-75^{th})	1.30 (0.84-1.92)
ETS median (25^{th}-75^{th})	0.07 (0.00-0.70)

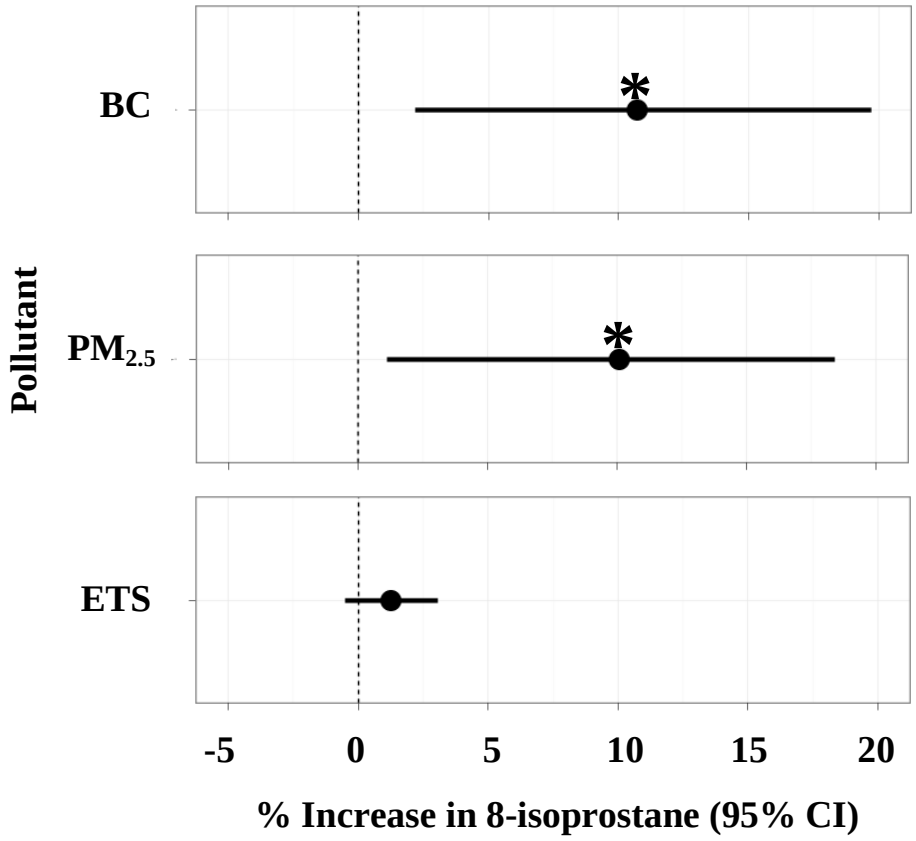
Seroatopy defined as any specific IgE ≥0.35 IU/ml to ragweed, *d farinae*, cockroach, mouse urinary protein, cat, dog, tree mix or grass mix.

Figure 1. Scatterplots for all domestic pollutant measures



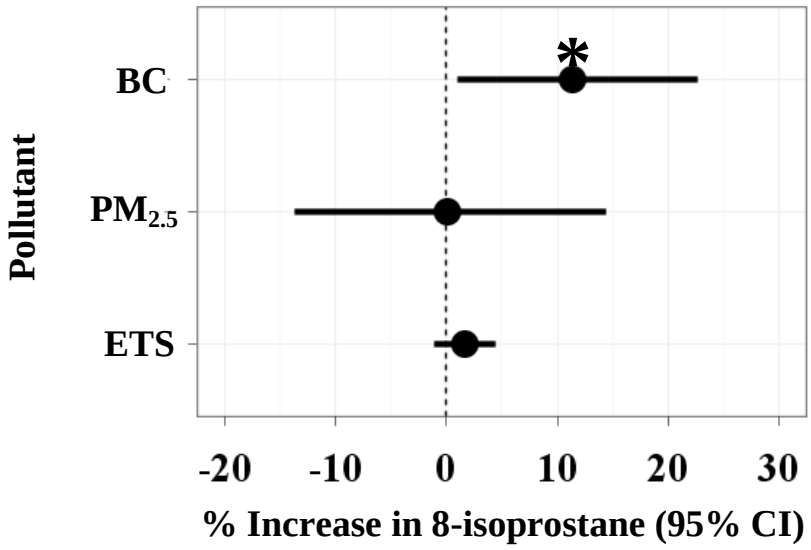
Panel A, scatterplot for domestic measures of $PM_{2.5}$ and BC. Spearman ρ=0.568. Panel B, scatter plot for domestic measures of $PM_{2.5}$ and ETS. Spearman ρ=0.342. Panel C, scatter plot for domestic measures of BC and ETS. Spearman ρ=-0.432. All p-values <0.001.

Figure 2. Associations between BC, $PM_{2.5}$ and ETS domestic measures and 8-isoprostane in EBC (log-transformed) in a multivariable model



Data points and error bars describe the percent change in 8-isoprostane concentrations and 95% CI per an interquartile range increase in the concentrations of each pollutant, adjusted for sex, case-control status, African American race, heating season and urea concentration in EBC (log transformed). *p<0.05

Figure 3. Associations between BC, $PM_{2.5}$ and ETS domestic measures and 8-isoprostane in EBC (log-transformed) in a multivariable co-pollutant model



Data points and error bars describe the percent change in 8-isoprostane concentrations and 95% CI per an interquartile range increase in the concentrations of each pollutant, adjusted for sex, case-control status, African American race, heating season, urea concentration in EBC (log transformed) and with the inclusion of the three pollutants in the model. *$p<0.05$

GIS variables and central site measures also were examined as potential predictors of 8-isoprostane. Truck route density and number of buildings burning residual oil were previously reported as significant predictors of domestic BC measures (Cornell *et al.* 2012), however, neither variable was significantly associated with 8-isoprostane (Table 2). Even though annual averages of $PM_{2.5}$ and EC were significantly correlated with domestic BC measures (Spearman correlations 0.336 and 0.346 respectively, $p<0.001$), these averages were not found to be predictors of 8-isoprostane (Table 2). Furthermore, none of these variables significantly affected the associations between domestic pollutant levels and 8-isoprostane when included in multivariable models.

Table 2. Generalized estimating equations models for associations between selected GIS variables, neighborhood pollutant averages and 8-isoprostane

Variable	Univariate β (95% CI)	Multivariable[#] β (95% CI)
Buildings burning residual oil (#)	0.001 (-0.003, 0.004)	0.000 (-0.004, 0.003)
Truck route density (km/km^2)	-0.015 (-0.110, 0.081)	-0.031 (-0.128,0.066)
Annual $PM_{2.5}$ average (μg/m^3)	0.030 (-0.091, 0.151)	0.011 (-0.111, 0.132)
Annual EC average (μg/m^3)	-0.177 (-0.891, 0.536)	-0.277 (-1.007, 0.453)

#Adjusted for sex, African American race, case-control status, heating season and urea concentration (natural log).

Discussion

The objective of this study was to determine the association between short-term domestic levels of airborne pollutants and 8-isoprostane, a biomarker of oxidative stress, measured in EBC. While we found that in single pollutant models, domestic levels of $PM_{2.5}$ and BC, but not ETS, were associated with increased levels of 8-isoprostane, in a co-pollutant model including all three, only BC remained a significant predictor of increased 8-isoprostane levels. To our knowledge this is the first paper to look at these associations between 8-isoprostane and domestic levels of these airborne pollutants after adjusting for dilution by water vapor. This study provides evidence that the BC fraction of $PM_{2.5}$ may be more important in the study of short-term oxidative stress response.

Few studies have looked at associations between airborne pollutant exposure and 8-isoprostane levels in EBC. In a study of asthmatic children in Canada, levels of $PM_{2.5}$ obtained from central site monitoring were associated with decreased measures of pulmonary function but were not significantly associated with 8-isoprostane levels (Liu *et al.* 2009). Patients with stable coronary disease and healthy volunteers who underwent chamber exposure to concentrated fine and ultrafine particulate matter had significantly higher levels of 8-isoprostane after exposure (Mills *et al.* 2008). Higher BC school levels measured at NYC high schools were associated with increased 8-isoprostane, in adolescents(Patel *et al.* 2012).

There is some experimental evidence that particulate matter exposure can lead to oxidative stress. Diesel exhaust particle exposure has been shown to induce production of reactive oxygen species (ROS) in human airway epithelial cells (Marano *et al.* 2002). In *vitro* DEP exposure of was shown to significantly induce oxidative stress as measured by glutathione (GSH) synthesis genes and on total GSH in endothelial cells and in a co-culture model of mouse macrophages and endothelial cells (Weldy *et al.* 2011). The increased production of 8-isoprostane, *i.e.* increased oxidative stress, may lead to lung injury and downstream inflammation (Voynow and Kummarapurugu 2011).

Even though tobacco smoke is a known oxidant (Howard *et al.* 1998; Kosecik *et al.* 2005), and ETS was detectable in 56% of the filters collected at the participants home, we found no significant association between domestic ETS measures and 8-isoprostane. Previous studies have not reported consistent associations between ETS exposure and oxidative stress biomarkers measured in EBC. After a one hour experimental exposure to ETS, healthy young adults had significantly higher levels of H_2O_2 in EBC (Kostikas *et al.* 2013). In another study, healthy children who had one or two parents who were smokers, did not have significantly higher levels

of H_2O_2 in EBC when compared to children who were not exposed to ETS (Doniec *et al.* 2005). BC exposure might also be the more important contributor to oxidative stress, explaining the lack of any statically significant association. This hypothesis is supported by our results, in which ETS was not a significant independent predictor of 8-isoprostane either in the single or co-pollutant models, while the BC association remained consistent across analyses.

Because burning of residual oil for heating and truck traffic are associated with indoor BC levels in this cohort, we also sought to determine if these variables were associated with 8-isoprostane levels. Heating season, during which burning of residual oil happens more frequently, was a significant predictor of 8-isoprostane in all models. However, the number of buildings burning residual oil and truck route density in a 500-m Euclidean radius were not associated nor did they change the association between domestic BC and 8-isoprostane. A potential explanation for this lack of association is that these variables might reflect long-term exposure while domestic measures reflect recent exposure. Even though these local sources are somewhat predictive of home levels, weather and non-local sources may also be significant contributors to the variability in domestic measures. However, because these are weekly averaged measures, future studies could aim to compare day to day variability in exposure.

Our study had several strengths. 8-isoprostane was measured using a highly sensitive LC/MS/MS method, and was detectable in the majority of our samples. We were also able to adjust for the variable dilution of droplets from bronchoalveolar extracellular fluid, which has posed a problem in the accurate measurement of biomarkers in EBC (Effros 2010). Urea, which exists under homeostatic regulation in the body, has been used as a marker of dilution in EBC previously and shown to correlate well with other dilution markers (Effros *et al.* 2003). However, there are also some limitations to our study. Domestic exposure was assessed 7 days

after collection of EBC, however we previously showed a good correlation between outdoor central site BC levels averaged over 7 days measured the week prior and the week following the home visit (Cornell *et al.* 2012). The cohort consisted of children belonging to middle-income families, limiting the generalizability to children of other socioeconomic strata. We also had a limited sample size that did not allow the analysis for potential effect modification and we did not adjust for potential indoor sources of airborne pollutants like incense burning. Finally, we cannot completely rule out BC acting as a surrogate for an unmeasured pollutant such as nickel, which is also released in significant quantities from the burning of residual fuel oil (Peltier *et al.* 2009).

The associations between domestic levels of BC and 8-isoprostane, suggest that BC may lead to increased oxidative stress in the airways. Our findings provide a better understanding of whether short-term exposure to different airborne pollutants contributes to airway oxidative stress which might contribute to downstream inflammation and respiratory symptoms. Measurement of BC exposure might be more relevant than $PM_{2.5}$ in the study of the sub-clinical effects of airborne pollutants on the airways.

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Appendix: Supplemental Tables

Table S1. Selected cohort characteristics

Characteristic	Included n=130	Excluded n=220
Case:Control*	88:42	118:102
Sex (% male)	60.0	52.7
Race (%)		
White	16.9	13.2
African American	46.9	47.3
Asian	11.5	10.9
Other/mixed	21.7	24
Hispanic ethnicity	36.2	33.2
Seroatopy	52	50.2
Mother completed high school (%)	93	91.8
Father completed high school (%)	91.7	92.8
Household income <25K (%)	7	11
Mother has asthma (%)	21.9	19.1
Father has asthma (%)	13.3	14.9

*Ratio of case:control significantly different between included and excluded cohorts (p=0.007)
Seroatopy defined as any specific IgE ≥0.35 IU/ml to ragweed, *d farinae*, cockroach, mouse urinary protein, cat, dog, tree mix or grass mix, available for n=125 and n=207 respectively.
Maternal education available for n=128 and n=218 respectively
Paternal education available for n=121 and n=207 respectively
Household income available for n=129 and n=154 respectively.
Maternal and paternal asthma available for n=128 and n=215 respectively.

Chapter VII. Conclusions and Future Directions

The studies that comprise this dissertation attempted to address the following unifying hypothesis: **Current exposure to airborne pollutants will be associated with increased parameters of airway inflammation and oxidative stress measured in exhaled breath.** The main conclusions from each specific aim are as follows:

Hypothesis 1

We hypothesize that parameters of proximal and distal airway inflammation will be differentially associated with current respiratory outcomes and seroatopic status.

Main findings for aim 1

We determined that it was feasible to implement in an inner-city cohort, the multiple flow collection method of FENO that allowed for the partitioning of NO contributions from the proximal and distal airways. In accordance with our main hypothesis, we found that these parameters of proximal and distal inflammation were differentially associated with current respiratory symptoms and seroatopy. Proximal inflammation was most strongly associated with seroatopy while distal inflammation appeared to be driven by recent respiratory symptoms. We concluded that the parameter of distal inflammation might be more appropriate in the study of respiratory symptoms given these associations. This method also was developed further in the subsequent chapters and implemented in the study of various airborne pollutant exposures.

Hypothesis 2

We hypothesize that recent exposure to transition metals measured in particulate matter <2.5 microns in diameter ($PM_{2.5}$), will be associated with elevated parameters of proximal and distal airway inflammation. We also predict that the association between ambient metals and inflammation parameters will be modified by seroatopic status.

Main findings for aim 2

We found that 9-day averages of transition metal fractions of $PM_{2.5}$ collected at a central site within our study catchment were associated with increases in FENO parameters. Both Ni and V were associated with elevated distal airway inflammation. V also was associated with elevated $FENO_{50}$ levels. Fe was associated with elevated $FENO_{50}$ and proximal inflammation levels. Zn was not associated with any parameters of airway inflammation. Furthermore we found that the association between Ni and distal inflammation was stronger for the children who were seroatopic. Based on these results, we concluded that it is important to study the composition of inhalable particulate matter in order to understand what specific components are responsible for the detrimental health effects associated with this exposure. Transition metals in PM may lead to increases in airway inflammation; furthermore, children with seroatopy might be more susceptible to the detrimental effects of these exposures.

Hypothesis 3

We hypothesize that greater concurrent proximity and density of GIS indicator variables of traffic and other airborne pollution sources will be associated with elevated parameters of

proximal and distal airway inflammation. Specifically, we hypothesize that greater number of buildings burning residual oil will be associated with increased levels of distal inflammation.

Main findings for aim 3

Contrary to our main hypothesis, we did not find any associations between any of the GIS indicator variables for airborne pollution sources and any airway inflammation parameter. In secondary analyses for effect modification most variables did not vary across strata. However, previous studies that have utilized FENO parameters as outcomes in the study of the effect of proximity to roadway and traffic density have yielded mixed results. We believe this lack of association in our analyses might be due to the fact that these indicators may better serve as markers of chronic exposure. FENO parameters may be better suited for the study of acute response given its variability. FENO may be downregulated by exposure to certain environmental contaminants, like chronic smoking, however, it is unlikely that chronic exposure to traffic related air pollution may lead to downregulation of FENO. A potential explanation for the lack of associations may be that the size of the buffers used was too large and most of the children in the study did not live within what is thought to be a relevant distance from major highways.

Hypothesis 4

We predict that domestic measures of black carbon (BC) will be associated with elevated levels of 8-isoprostane, as a marker of oxidative stress, measured in exhaled breath condensate.

Main findings for aim 4

We found that domestic levels of BC and $PM_{2.5}$ were associated with elevated levels of 8-isoprostane. More importantly, once these associations were examined with both pollutants in the same model, only the association between BC and 8-isoprostane remained significant. These results lead us to conclude that the BC fraction of PM might be more important in the study of short-term effects of airborne pollution. We also found that even though number of buildings burning residual oil and truck route density were significant predictors of domestic BC measures, they were not independently associated with 8-isoprostane, nor did they change the association when included as a covariate.

Overall conclusions

We can conclude that the partitioning of FENO contributions from distal and proximal airways does provide more specific information about underlying airway inflammation. The use of non-invasive biomarkers can provide important information about inflammatory and oxidative stress processes in the airways. From our results in Chapters IV and VI we can conclude that the use of mass-based standards, like measurement of PM_{10} and $PM_{2.5}$ to monitor particulate matter might be insufficient to protect the population against the detrimental effects of air pollution. The results from Chapters IV, V and VI can also lead us to conclude that these biomarkers better serve the study of the sub-acute effects of recent (7-9 days) exposure as determined by residential or central site measures. Finally, our results in conjunction with previous findings demonstrate the importance of determining the associations between different components of inhalable PM and airway inflammation and oxidative stress.

Future directions

Composition of particulate matter

Previous studies and the findings in this dissertation show that the use mass-based exposure assessment might lead to the underestimation of the effects of airborne pollution on respiratory health. Most studies that utilize personal monitoring have been limited to speciation of the BC or EC fraction of PM that is collected. For example, personal measures of EC, rather than $PM_{2.5}$, had the strongest associations with respiratory symptoms in children in the Bronx (Spira-Cohen *et al.* 2011). Future studies might also aim to determine the Ni, V and Fe fractions of PM collected with personal monitors. The study of Ni might be especially important since Ni, like EC/BC is released by the burning of residual oil. With a larger sample size it might also be interesting to determine if there are any synergistic effects between PM components or if there is a specific component profile that can lead to greater detrimental health effects.

The study of the composition of particulate matter is also important from a public health policy perspective. Current mass-based standards set by the EPA do not account for the composition of particulate matter. Even if PM levels are below the levels required by the EPA, depending on its composition, it might still lead to sub-acute effects in the airways that might lead to downstream respiratory illness. It is also important to address other important sources of airborne pollutants besides traffic. Promotion of clean fuel use might lead to a reduction in the number of buildings that burn residual oil and it might be useful to monitor changes in airway biomarkers to later determine if these reductions lead to decreases in asthma or respiratory symptoms.

Use of airway biomarkers

The ease of implementation and the wealth of data on FENO have made it an important biomarker in the study of respiratory disease. With regards to studies on airborne pollutant exposure, it is a most useful marker of short-term inflammatory response. The two-compartment model allowed further allocation of the sources of inflammation in the airways. Recent findings have reported an association between lack of asthma control and alveolar NO, but no association with bronchial NO, in a cohort of mild asthmatics (Scichilone *et al.* 2013). The measurement of alveolar NO could be used to monitor asthmatics that are exposed to airborne pollutants and might be used to predict future risk of exacerbations.

The use of EBC is still a fertile ground for the discovery of new exhaled biomarkers relevant to the study of both airborne exposures and airway disease. In our study, we focused on only one biomarker, 8-isoprostane. Future studies might use EBC to study several biomarkers in conjunction. An interesting approach would be to look at specific exhaled biomarker profiles and see how these relate the exposure or the disease of interest. The studies might also choose to examine the interaction between biomarkers involved in different processes in the airways *i.e.* nitrosative stress vs. oxidative stress.

Future studies would also benefit from the study of genetic factors that might modify these associations. In our studies we focused on comparing the effects of airborne pollutants on seroatopic vs. non-seroatopic children and between children who wheezed and those who did not. Further examining these subgroups by genetic susceptibility, *i.e.* deficiencies in detoxification enzymes, might provide even more insight. Regarding the use of FENO parameters, examining iNOS expression and transcription levels after recent airborne pollutant

exposures would provide stronger evidence that these exposures are responsible for increases in NO production in the airways. This research would also provide more information about the poorly understood relevance of NO production in the airways.