Exhaled Nitric Oxide: A Useful Biomarker of Acute Respiratory Health Effects Resulting from Exposure to Ambient Air Pollution?
A Pilot Study in Conyers, Georgia

by

Lisa Richards

A thesis submitted to the Faculty of Graduate Studies of the
University of Manitoba
in partial fulfilment of the requirements of the degree of
MASTER OF SCIENCE

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FACULTY OF GRADUATE STUDIES

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ABSTRACT

Ambient ozone and particulate matter (PM) are atmospheric pollutants that comprise 'smog', and can cause a variety of respiratory and non-respiratory health effects. Ozone concentrations are particularly high in metropolitan Atlanta, and exceed health-based standards on more than a third of summer days. Adolescent student athletes often practice during late summer afternoons, when air pollutant levels are highest, making them particularly susceptible to the health effects of these air pollutants. Biological markers of air pollution exposure from exhaled breath, including indicators of neutrophilic inflammation such as exhaled nitric oxide (FeNO), hold promise as non-invasive indicators of respiratory health effects.

A pilot study was undertaken by the Centers for Disease Control and Prevention (CDC) in Conyers, Georgia, downwind of metropolitan Atlanta, from August 16 to 30, 2004, to examine a group of 16 practicing high school athletes. This study involved examining the exhaled nitric oxide (FeNO) data as a subset of the data collected in the CDC pilot study. The purpose of this research was to determine if FeNO is a useful biomarker of the acute respiratory health effects of exposure to air pollution, especially ozone and $PM_{2.5}$, in field epidemiology studies. Exhaled nitric oxide was evaluated for its (1) reliability; (2) validity; and (3) responsiveness to ambient air pollution.

The reliability of the FeNO measurements was evaluated through examining the variability in the FeNO measurements, as well as their reproducibility. There was substantial variability in the FeNO data, with most of this variation (88%) explained by

between-subject variation. Although the intraclass correlation coefficient (ICC) (0.87) suggested that FeNO meets the criterion of acceptable reproducibility (ICC$\geq$ 0.6), the average within-subject coefficient of variation (CV) (25%) and the coefficient of reproducibility (expressed as the mean pooled SD) (7.3) suggested that the reproducibility of FeNO was not optimum.

An assessment of construct validity revealed mixed results. The mean pre-practice FeNO in this sample is somewhat lower that what is reported in the literature (8.3 ppb vs. 13.7 ppb in one study examining the same age group). A variety of factors are known to cause discrepancies in the results between studies, including the use of different analyzers in the collection of FeNO. A comparison of group differences in the baseline FeNO study data to those described in the literature, on the other hand, revealed that the group differences examined (age, race, exposure to home ETS, BMI, asthma/allergy/hayfever status, respiratory symptoms) were largely consistent with the literature. Only a few characteristics, including gender, height and those experiencing symptoms in the past 24 hours, had a difference in mean pre-practice FeNO values that were in a direction contrary to what would be expected.

The responsiveness of FeNO was evaluated by examining the association of FeNO with ambient ozone and $PM_{2.5}$ concentrations among the 16 participants exposed to these air pollutants during vigourous outdoor exercise. Statistically significant associations were observed between post-practice FeNO (natural log-transformed) and: (1) 1-day lagged maximum ozone (1-hr avg.) concentration (natural log-transformed); (2) 1-day lagged $PM_{2.5}$ at 5pm (natural log-transformed) ($p<0.01$), controlling for race and pre-practice

FeNO. No other significant associations were observed with same-day maximum ozone (1-hr avg.) or same-day $PM_{2.5}$ at 5 pm controlling for race and pre-practice FeNO, or when ozone and $PM_{2.5}$ concentrations were lagged by 2 days. Although the estimates of effect were small (<2.8 ppb FeNO per 10 unit increase of pollutant), they were similar to that found in the literature and would suggest that air pollution increases inflammation. Caution is needed in attempting to generalize these results, though, as this was a small convenience sample of healthy student athletes with low power.

It was concluded that, although there are some limitations to using FeNO as a biomarker of effect, this study found evidence to suggest that FeNO has potential as a reasonably reliable, valid and responsive measure that can detect pulmonary inflammation as a result of exposure to ambient air pollution. A sensitive biomarker of effect, such as FeNO, may prove to be a useful tool to identify subjects or groups at most risk from the toxic effects of air pollutants and for establishing unacceptable exposure levels of these pollutants.

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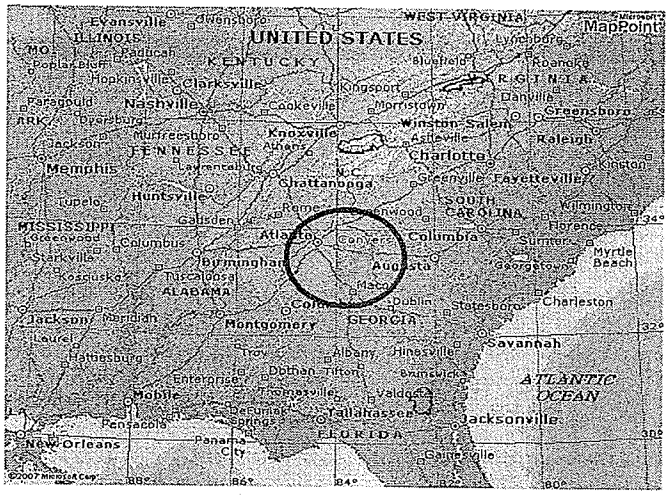
CHAPTER 1

INTRODUCTION

1.1 Preamble

A pilot study was undertaken by the Centers of Disease Control and Prevention (CDC) in Conyers, Georgia, from August 16 to 30, 2004, to examine a group of 16 practicing high school athletes. The main objective of the CDC study was to describe the magnitude of acute health effects from exposure to ambient air pollution during summer afternoon athletic practices in this sensitive subpopulation located downwind from metropolitan Atlanta (Figure 1). Health effect measures included lung function, non-invasive biomarkers obtained from exhaled breath (exhaled nitric oxide and constituents of exhaled breath condensate), and symptoms reported via a post-practice questionnaire. In addition to estimating the acute health effects from exposure to ambient air pollution, the CDC study was also to provide estimates of effect sizes in preparation for a future prospective study, and to assess the feasibility of collecting exposure data and measuring biomarkers in a field setting from adolescent study subjects.

Figure 1. Location of Conyers, Georgia



The subject of this thesis is a detailed examination of the exhaled nitric oxide (FeNO) data as a subset of the data collected in the CDC pilot study, in order to determine whether FeNO is a good biomarker of acute respiratory health effects as a result of exposure to ambient air pollution. In particular, whether FeNO is a good biomarker in a field setting, a much different and less "controlled" setting than the clinical setting where most experience with FeNO comes from, will be addressed.

1.2 Background

Air pollution is a significant problem in many large urban areas, with ozone and particulate matter (PM) being the primary air pollutants of smog. Approximately one third of summer days in the metropolitan Atlanta area are air quality alert days on which ambient ozone or PM concentrations exceed the health-based standards (Georgia Department of Natural Resources 2005a). Unlike PM, ozone concentrations follow a well-known diurnal pattern, with concentrations highest in the late afternoon and early evening (Schwartz 2004).

The health effects attributed to exposure to ozone or PM are reported to be potentially serious. Ozone can irritate lung airways and cause inflammation, wheezing, coughing, pain upon inspiration, and breathing difficulties (American Thoracic Society 1996). Even at low levels, ozone can aggravate asthma, reduce lung capacity, and increase susceptibility to illnesses like pneumonia and bronchitis. Exposure to PM has been repeatedly associated with increased mortality, although precisely how this occurs is not well understood (American Academy of Pediatrics 2004).

Students who participate in sports with outdoor summer practices typically practice in the mid- to late-afternoons, when diurnal ozone levels are highest. These student athletes are particularly susceptible to health effects of ozone and PM because their respiratory tracts are still developing and their vigorous level of physical activity increases exposure due to elevated minute ventilation and tidal volume (Schwartz 2004).

Biological markers from exhaled breath hold promise as non-invasive indicators of respiratory health effects (Bernard et al. 2005). Nitric oxide is formed in the airways, and is partly exhaled, which allows it to be captured and measured. Increased concentrations of FeNO, an early airway inflammatory indicator, have been observed in patients with asthma, upper respiratory tract infections, allergic rhinitis, bronchiectasis and atopy (Van Amsterdam et al. 2000). The purported sensitivity of FeNO to early airway inflammation preceding frank symptoms or lung function impairment, as well as the ease and non-invasive nature of FeNO collection, make it particularly appealing for use in studying the effects of air pollution.

Several studies have explored FeNO as a biomarker of respiratory morbidity from ozone exposure. In healthy subjects, it was demonstrated that FeNO increased on days characterized by high levels of outdoor air pollution (up to 20% increase), indicating that FeNO may serve as a biomarker of exposure to air pollution (Steerenberg et al. 1999; Van Amsterdam et al. 1999). Which specific air pollutant is responsible for this increase in FeNO was not determined. More recently, Nickmilder et al. (2003) found increased levels of FeNO in children exposed to higher levels of ambient ozone (an 8-hour concentration

of 135 ug/m^3 or greater). However, these children were engaged in various outdoor recreational activities but not sports or running.

1.3 Study Purpose

The purpose of this study was to test the hypothesis that FeNO is a useful biomarker of acute respiratory health effects as a result of exposure to air pollution in field epidemiology studies. To evaluate FeNO's usefulness as a biomarker, the quality of the FeNO data in this study will be examined. Since the quality of a measurement is dependant on its *validity* and *reliability* (McDowell and Newell 1996), these criteria will be applied for assessment. In addition, the *responsiveness* of the FeNO measurements to ambient air pollution (i.e., its ability to detect change) will also be considered.

1.4 Research Questions

This study will evaluate the usefulness of FeNO as a biomarker, using the criteria described above. Specific research questions that this study aims to answer are:

1. What is the reliability of the study participants' FeNO measurements?

- What is the variability in these measurements?
- What is the reproducibility of these measurements?

2. What is the validity of the study participants' FeNO measurements?

- Are baseline FeNO measurements consistent with those in the literature?
- Are baseline FeNO measurements for this sample of students, by groupings of interest (e.g. gender), different from what we would expect?

3. What is the responsiveness of the study participants' FeNO measurements to ambient air pollution?

- Is there a relationship between the most important ambient air quality parameter(s) and the participants' post-practice FeNO values, and what is the strength and nature of this relationship?

1.5 Significance of the Study

This study will examine a small sample of repeated FeNO measurements, and apply some evaluative criteria to assess the value of FeNO as a biomarker. In doing such, this study could be valuable for CDC to assess whether or not a larger scale study, using FeNO as a biomarker, might be beneficial. Further, the information obtained from this study could be important in defining and designing future field studies examining FeNO, and determining if they are warranted. As well, few studies have described the variation in FeNO measurements, and this study could make a contribution to the current literature.

Little is known about the magnitude of change that would be expected in FeNO in adolescents exposed to ambient ozone during vigorous exercise. Although this is a small study with low statistical power, any evidence to suggest (or refute) that vigorous outdoor activity in student athletes exposed to summer air pollution might induce lung inflammation would be of interest to a variety of groups. A study examining this relationship could be relevant for national regulatory agencies for consideration in modifying existing air quality standards. These results might also be important for health professionals, such as medical officers of health, who need to provide guidance to the public on the health risk of spending time outdoors on poor air quality days. School

boards could also benefit, with guidance for developing policies for outdoor summer athletic practices. Parents and caregivers of student athletes with chronic respiratory illnesses, who might be especially sensitive to ambient air pollution, might also be interested in the findings of this study.

1.6 Limitations and Delimitations

There are several limitations that will impact on the interpretation of the results of this study:

- The study was undertaken during a fixed time frame, and thus a full spectrum of air quality alert days were not captured. This may limit the power of the study to capture relationships between air quality parameters and outcome measures, such as FeNO.
- The fact that the CDC study was designed as a pilot, with a small number of subjects (n=16) presents some limitations. First, since there were relatively few participants enrolled, the generalizability of this study may be limited (i.e., study participant characteristics may not be consistent with general population of adolescents in this age group). Further, the small number of subjects may have limited statistical power to detect relationships between ambient air pollutants and post-practice FeNO.
- It was not possible to control or compensate for participants dropping out or missing practices, leaving some data gaps in a relatively small and short study.
- We did not restrict food or water consumption of the athletes before or during practice, and this may have impacted FeNO values.

- Exercise is known to decrease FeNO, and this study relied on an exercise component to test the effects of air pollution on this sensitive group of practicing adolescent athletes.
- Although there was a state monitoring site for ozone located in Conyers, the closest monitoring station for PM was approximately 14 miles away. To increase the relevance of the exposure measurement, it would have been optimal to also have PM monitored closer to the study site.
- Finally, there are a variety of additional criteria that one could have applied to evaluate FeNO as a biomarker, including its interpretability and respondent/administrative burden. Evaluation of FeNO as a biomarker by these criteria was beyond the scope of this study.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter reviews the literature pertaining to air quality, its influence on human health, and the use of FeNO to measure these respiratory health effects. The first section will briefly review air pollution. Further sections will describe the literature on FeNO, and summarize the literature to date that has examined the relationship between FeNO and air quality.

2.2 Ambient Air Pollution

This section will provide a brief overview of ambient air pollution. Because air quality alert days in Conyers, Georgia, are typically a result of exceedences of the EPA ozone or particulate standards, discussion will be limited to these two pollutants. A summary of the regulation of air quality, and its measures, will follow. Finally, a review of the health effects of ozone and particulates will be discussed.

2.2.1 Overview

Air pollution is a very complicated physical and chemical system that can be thought of as gases and particles that are dissolved or suspended in air, respectively (Yassi et al. 2001). Air pollution is derived from a variety of sources, of which the combustion of fossil-fuel products is the principal source. Pollutants directly emitted into the atmosphere are known as primary pollutants, whereas pollutants that form as a result of chemical reactions with other pollutants or atmospheric gases are known as secondary pollutants.

Ozone. Ambient ozone is a secondary pollutant formed by the action of sunlight in the presence of primary pollutants, mainly nitrogen oxides and volatile organic compounds, both of which are emitted by motor vehicles and industrial sources (Katsouyanni 2003). Ozone measurements are often expressed as ppb, but can also be expressed as $\mu g/m^3$ (1 ppb = 2 $\mu g/m^3$ at 20°C). There are several unique features of ozone which make its temporal and spatial distribution, and resulting personal exposure patterns, differ from those of other pollutants. Since it is not directly emitted from polluting sources, but produced by photochemical reactions in the atmosphere, ozone also shows strong seasonal and diurnal variations (Schwartz 2004). It is high in the summer and the afternoon and low in the night, early morning, and winter. As well, because of its generation procedure, ozone is a more important problem in areas with more prolonged sunshine (Katsouyanni 2003). In the presence of precursor primary pollutants (especially NO), ozone is 'scavenged' resulting in low concentrations occurring in busy city centers, where NO concentrations are high, and higher concentrations occurring downwind in city suburbs, where ozone is transported but where NO and other precursor concentrations are relatively low. Tropospheric (ground level) ozone pollution should be distinguished from the problem of stratospheric ozone depletion, which is linked to global warming and risks of UV radiation (Katsouyanni 2003).

Particulate Matter. Suspended particulate pollutants that are small enough to reach the lower respiratory tract, designated as ambient particulate matter (PM), are classified into three categories and expressed as a concentration ($\mu g/m^3$) (Yassi et al. 2001). Coarse PM (aerodynamic diameter, 2.5-10 μm) is derived from abraded soil, road dust (eg, brake and tire dust), construction debris, or aggregation of smaller combustion particles, whereas

fine (<2.5 μm) and ultrafine (<0.1 μm) PM is primarily formed during the combustion of fossil-fuel products and from some industrial activities. Although $PM_{2.5}$ is a PM_{10} subset, the former is separately regulated to ensure that the smaller particles, which have less mass but might be more respirable, are adequately controlled. Although a considerable amount of data implicate coarse and fine PM in adverse health effects, much less is known about the risks of ultrafine particles, which are more abundant, potentially more toxic, and not presently amenable to mass standard monitoring (Katsouyanni 2003). Smaller particles tend to be remarkably homogeneously spread over large areas, penetrate effectively indoors and consist to a larger extent of primary and secondary combustion products (containing elemental carbon and PAHs, sulphates and nitrates). The airborne particle mix in each location has different chemical and physical characteristics, and toxicity of the particle mix may vary with composition.

2.2.2 Air Quality Regulations

In the United States, the Clean Air Act, which was last amended in 1990, requires the US Environmental Protection Agency (EPA) to set National Ambient Air Quality Standards for pollutants considered harmful to public health and the environment. The EPA Office of Air Quality Planning and Standards (OAQPS) has set National Ambient Air Quality Standards for six principal pollutants, which are called "criteria" pollutants (US Environmental Protection Agency 2007) (Table 1).

Table 1. National Ambient Air Quality Standards for criteria air pollutants in 2005

Pollutant	**Primary Standards**
Ozone	
1-h average	0.12 ppm (235 $\mu g/m^3$)
8-h average	0.085 ppm (157 $\mu g/m^3$)
PM_{10}	
Annual arithmetic mean	Revoked*
24-h average	150 $\mu g/m^3$
$PM_{2.5}$	
Annual arithmetic mean	15 $\mu g/m^3$
24-h average	65 $\mu g/m^{3**}$
Sulfur dioxide	
Annual arithmetic mean	0.03 ppm (80 $\mu g/m^3$)
24-h average	0.14 ppm (365 $\mu g/m^3$)
Nitrogen dioxide	
Annual arithmetic mean	0.053 ppm (100 $\mu g/m^3$)
Carbon monoxide	
8-h average	9 ppm (10 mg/m^3)
1-h average	35 ppm (40 mg/m^3)
Lead	
Quarterly average	1.5 $\mu g/m^3$

Source: US Environmental Protection Agency

* Due to a lack of evidence linking health problems to long-term exposure to coarse particle pollution, the agency revoked the annual PM_{10} standard in 2006 (effective December 17, 2006).
** Effective December 17, 2006, this decreased to 35$\mu g/m^3$.

Despite these standards under the Clean Air Act, the air in many parts of the United States is far from clean. In 2002, approximately 146 million Americans were living in areas where monitored air failed to meet the National Ambient Air Quality Standards for at least one of the criteria air pollutants (US Environmental Protection Agency 2001).

2.2.3 Air Quality Index

The Air Quality Index (AQI) has been developed by the US EPA to provide a uniform system of measuring pollution levels for the major air pollutants regulated under the Clean Air Act (US Environmental Protection Agency 1994) (Table 2).

Table 2. US Environmental Protection Agency Air Quality Index guide

AQI Range	EPA Color Scale	EPA Descriptor	Clean Air Campaign Health Advisory
0 to 50	Green	Good	The air quality is good and you can engage in outdoor physical activity without health concerns.
51 to 100	Yellow	Moderate	At this level the air is probably safe for most people. However, some people are unusually sensitive and react to ozone in this range, especially at the higher levels (in the 80s and 90s). People with heart and lung diseases such as asthma, and children, are especially susceptible. People in these categories, or people who develop symptoms when they exercise at "yellow" ozone levels, should consider avoiding prolonged outdoor exertion during the late afternoon or early evening when the ozone is at its highest.
101 to 150	Orange	Unhealthy for Sensitive Groups	In this range the outdoor air is more likely to be unhealthy for more people. Children, people who are sensitive to ozone, and people with heart or lung disease should limit prolonged outdoor exertion during the afternoon or early evening when ozone levels are highest.
151 to 200	Red	Unhealthy	In this range even more people will be affected by ozone. Most people should restrict their outdoor exertion to morning or late evening hours when the ozone is low, to avoid high ozone exposures.
201 to 300	Purple	Very Unhealthy	Increasingly more people will be affected by ozone. Most people should restrict their outdoor exertion to morning or late evening hours when the ozone is low, to avoid high ozone exposures.
Over 300	Black	Hazardous	Everyone should avoid all outdoor exertion.

Source: US Environmental Protection Agency

Index figures are reported in all metropolitan areas of the United States with populations exceeding 200,000, and acts as a public information tool to advise the public about the general health effects associated with different pollution levels and to describe whatever precautionary steps may need to be taken if air pollution levels rise into the unhealthy range.

The EPA uses the AQI to measure five of the criteria air pollutants: PM, sulfur dioxide, carbon monoxide, nitrogen dioxide and ozone, for which it has established National Ambient Air Quality Standards under the Clean Air Act. The intervals on the AQI scale relate to the potential health effects of the daily concentrations of each of these five pollutants. Each value has built into it a margin of safety that, based on current knowledge, protects highly susceptible members of the public.

The AQI converts the measured pollutant concentration in a community's air to a number on a scale of 0 to 500. The most important number on this scale is 100, since that number corresponds to the standard established under the Clean Air Act. For example, a 0.085 ppm reading for ozone would translate to an AQI level of 100, and if ozone was the highest value of the five pollutants, 100 would be the AQI for that location on that particular day. An AQI level in excess of 100 means that a pollutant is in the 'Unhealthy for Sensitive Groups' range, or worse, on a given day; an AQI level at or below 100 means that a pollutant reading is in the satisfactory range. EPA determines the index number on a daily basis for each of the five pollutants; it then reports the highest of the five figures for each major metropolitan area, and identifies which pollutant corresponds to the figure that is reported. On days when two or more pollutants exceed the standard

(that is, have AQI values greater than 100), the pollutant with the highest index level is reported, but information on any other pollutants above 100 may also be reported.

2.2.4 Health Hazards of Air Pollution

Exposure Effects. Children and adolescents represent the largest subpopulation of those susceptible to the adverse effects of air pollution. Their organ systems are still developing and normal growth may be affected when exposed to pollutants at critical periods of development (Mathieu-Nolf 2002). In addition, their exposure to air pollution can be different from adults given the same outdoor concentrations (Schwartz 2004). This group spends more time outdoors than adults, particularly in the summer and in the late afternoon. Some of that time is spent in play and sports activities that increase ventilation rates, increasing their exposure to air pollutants compared with adults. This is particularly important for exposure to ozone since ozone has a distinct temporal pattern.

Ozone. Ozone is a powerful oxidant and respiratory tract irritant in adults and children, causing shortness of breath, chest pain when inhaling deeply, wheezing, cough and upper respiratory tract irritation (American Thoracic Society 1996). These respiratory symptoms may be associated with headache, nausea, malaise and difficulties in sustaining exercise levels (McDonnell et al. 1985). In addition, airway inflammation, increased bronchial permeability and decrements in pulmonary function have been observed (Lippmann 1989) resulting in decreases in lung function, increased respiratory tract symptoms and asthma exacerbations on days with higher levels of ambient ozone (American Thoracic Society 1996). According to the American Academy of Pediatrics Committee on Environmental Health (2004), increases in ambient ozone have been associated with

respiratory or asthma hospitalizations (Thurston et al. 1994, White et al. 1994), emergency department visits for asthma (Tolbert et al. 2000) and school absences for respiratory tract illness (Gilliland et al. 2001). For example, in Atlanta, Georgia, children's emergency department visits for asthma in summer increased 37% after 6 days when ozone levels exceeded 0.11 ppm (White et al. 1994). Further, Friedman et al. (2001) found that efforts to reduce downtown traffic congestion in Atlanta during the Olympic Games resulted in a prolonged reduction in ozone pollution, and significantly lower rates of childhood asthma events.

Ozone may be harmful at concentrations lower than 0.085 ppm, the current federal regulatory standard (8-hour average). The American Academy of Pediatrics Committee on Environmental Health (2004) reports that field studies suggest effects on children's lung function at thresholds between 0.04 and 0.08 ppm (1 hour average) (Castillejos et al. 1995, Chen et al. 1999). Vigorously exercising children exposed to 0.12 ppm ozone, the current standard (1-hour average), in a controlled chamber environment were found to have decreased lung function (McDonnell et al. 1985). These studies suggest the need to reexamine the current standards.

Long-term consequences of chronic exposure to ozone are not clearly established, but animal and epidemiological studies suggest long-term health effects (Chitano et al. 1985, Galizia and Kinney 1999). Some experimental animal and clinical toxicological evidence suggests ozone exposure acts synergistically with other pollutants and airborne allergens (Yanai et al. 1990).

$PM_{2.5}$. Numerous studies have reported an association between ambient particulate pollution and excess morbidity and mortality from cardiovascular and respiratory diseases (Dockery and Pope 1994, Schwartz 1994, American Thoracic Society 1996, Samet et al. 2000). Further, daily changes in mortality rates and numbers of people hospitalized are linked to changes in particulate air pollution (American Academy of Pediatrics 2004). The above studies have estimated that for every 10 ug/m^3 increase in $PM_{2.5}$, there is an increase in the daily mortality rate between 0.5% and 1.6%.

The precise mechanism for these associations is not fully understood. Accumulating data, though, suggest that ambient particle pollution may lead to pulmonary inflammation (Li et al. 1996, Salvi et al. 1999). This could lead to dissemination of systemic proinflammatory products (such as cytokines) that may influence vascular tone and cardiovascular function (Brook et al. 2002). The mechanism for the cardiac effects may be related to disturbances in the cardiac autonomic system, cardiac arrythmias or increased blood concentration of markers of cardiovascular risk (e.g. fibrinogen) (Dockery 2001).

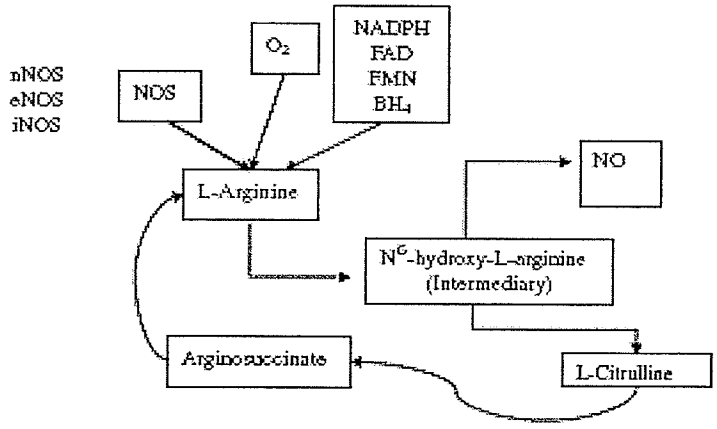
According to Mathieu-Nolf (2002), it has been reported that health effects from exposure to particulate air pollution display a time lag, with weak same day effects and stronger cumulative effects of air pollution on asthmatic children for both peak expiratory flow and symptoms (Peters et al. 1997, Peters et al. 1996, Segala et al. 1998).

2.3 Exhaled Nitric Oxide

Nitric oxide (NO) is a colorless gaseous molecule composed of a nitrogen and an oxygen atom. The molecule acts as an intracellular messenger, and is produced by several cells types in the body, including those in the upper and lower respiratory tract. It enters into the lumen by gaseous diffusion down a concentration gradient, conditioning exhaled gas with NO. It is believed to play an important role in regulatory function, including the regulation of blood flow, platelet function, neurotransmission and immunity. In the airways, NO functions in host defense against microorganisms and tumor cells, regulation of bronchial smooth muscle, production of airway secretions and ciliary function (Ricciardolo 2003).

Once formed, NO exists only for a brief time (6-10 seconds) before being converted into other substances. As depicted in Figure 2, NO is formed via the action of the enzyme nitric oxide synthetase (NOS), converting the amino acid L-arginine to the product L-citrulline with NO as a byproduct. Three NOS isoforms have been identified and classified as constitutive NOS (NOS I and III, neuronal and endothelial, respectively) and inducible NOS (NOS II). These isoforms differ in tissue distribution, regulation and activity. In the airways, constitutive NOS is basally expressed in epithelial cells and plays a regulatory role. Inducible NOS, on the other hand, is expressed in a variety of cells (airway epithelium, vascular endothelium and inflammatory cells) and appears to be involved in inflammatory diseases of the airways and in host defense against infection. The activation of the inducible form by proinflammatory cytokines is believed to be primarily responsible for the increased production of NO observed in asthma and other inflammatory disorders (Sofia et al. 2002).

Figure 2. Nitric oxide synthesis



Source: Choi et al. 2006

The presence of NO in exhaled breath of humans was first reported by Gustofsson et al. (1991), and then described in a number of publications reporting high fractional concentrations of eNO in subjects with various pulmonary diseases (Kharitonov 2004). Increased concentrations of exhaled NO have been observed in patients with asthma, upper respiratory tract infections, allergic rhinitis, bronchiectasis and atopy (Van Amsterdam et al. 2000). The level of FeNO has not been found to be consistently increased in patients with stable COPD, but a positive relationship has been established between the reversibility of airflow limitation with corticosteroid treatment and an elevated FeNO level (Landry and Eidelman 2005). In clinical medicine, the increase in FeNO in patients with certain respiratory diseases has led to its application as a biomarker for therapeutic interventions (American Thoracic Society 2005). In asthma, for example, it has been proposed to use this marker to diagnose asthma, to monitor the response to anitinflammatory medications, to verify adherence to therapy and to predict upcoming

asthma exacerbations. More recently, FeNO has been proposed as a non-invasive biomarker of pulmonary inflammation caused by air pollutants (Bernard et al. 2005).

2.3.1 Measurement of FeNO

Exhaled nitric oxide measurements have largely been performed in the research setting to date. Although there are numerous publications on FeNO, there has been a variation in published FeNO levels, much of which is attributable to the lack of a standardized technique of measurement (American Thoracic Society 2005). In order for the field of NO measurement to advance, it was felt that an international consensus on the appropriate measurement techniques was required that would lead to the collection of comparable data from normal subjects and those with disease states. The American Thoracic Society (ATS) published guidelines in 1999 on the use of FeNO as a clinical tool requiring the adoption of a standardized measurement technique (American Thoracic Society 1999). A taskforce of the European Respiratory Society (ERS) had already published European recommendations in 1997 (Kharitonov et al. 1997). In April 2005, a joint committee of the ATS and ERS updated the guidelines on the measurement of FeNO (American Thoracic Society 2005).

Levels of nitric oxide in exhaled air can be determined by direct exhalation into a NO analyzer to detect low concentrations of NO using the principle of chemoluminescence. When gaseous NO is carried by the flow of nitrogen gas via a cold trap into a chamber primed with ozone, a reaction takes place that results in the formation of nitric dioxide. Excited nitric dioxide emits light energy that can be quantified by a photomultiplier tube and continuously recorded on a writer. Both online and offline methods use this

chemoluminescence technique. *Online measurement* refers to the sampling of participant exhalations where the exhalate is continuously sampled by the NO analyzer with a real-time display of FeNO breath profiles. O*ffline testing* refers to a collection of exhalate into suitable receptacles for delayed analysis. Epidemiological studies involving FeNO measurements have traditionally used offline testing with Mylar sampling balloons to facilitate the measurement in the field setting. However, the advantage of the online method is that the test administrator can monitor the exhalation to ensure conformation to the required flow and pressure parameters and the achievement of an adequate NO plateau. Suboptimal exhalations can be immediately identified and discarded (American Thoracic Society 2005).

2.3.2 Factors Influencing FeNO Measurements

The following section provides an overview of the factors that may influence FeNO measurements, including both non-patient and patient-related factors, and is largely derived from a review of the 2005 ATS/ERS recommendations for the measurement of FeNO (American Thoracic Society 2005). This section also outlines recommendations from this document for addressing these factors. Additional studies were reviewed in some cases, and are referenced accordingly.

Non-Patient Related Factors

Sources of FeNO Contamination. Nasal NO accumulates to high concentrations relative to the lower respiratory tract, and thus exclusion of nasal NO is important when measuring FeNO. Closure of the velopharyngeal aperture during exhalation through a mouthpiece pressure of at least 5 cm H_2O is recommended to minimize nasal NO

contamination. Although gastric NO levels are very high, this does not appear to contaminate FeNO, probably due to closed upper and lower esophageal sphincters. Since environmental NO can reach high levels relative to those in exhaled breath, the inspired gas source provided should consist of NO-free air and ambient NO at the time of each test should be recorded.

Expiratory Flow Rate. The FeNO measurement varies considerably with exhalation flow rate due to variation of airway NO diffusion with transit time in the airway. Therefore, standardization of exhalation flow is critical for obtaining reproducible measurements. Low flow rates (<100 mL/s) amplify the measured NO concentrations and can aid in discriminating among participants. However, these lower flow rates result in longer exhalation times to reach an NO plateau and the prolongation of the exhalation may be uncomfortable for individuals with respiratory disease. A flow rate of 50 mL/s is recommended as a reasonable compromise between measurement sensitivity and patient comfort. In general, an exhalation is deemed adequate if the mean exhalation flow rate is 50 mL/s +/- 10% during the time of NO plateau generation and the instantaneous flow is not less than 45 mL/s or greater than 55 mL/s.

Breath-holding. Breath-holding results in NO accumulation in the nasal cavity, lower airway and probably in the oropharynx, causing NO peaks in the exhalation profiles of participants. For this reason, breath-holding should be discouraged.

Other Respiratory Maneuvers. Because spirometry has been shown to transiently reduce FeNO levels, it is recommended that NO analysis be performed before spirometry.

The same recommendation applies for other taxing respiratory maneuvers, unless these can be shown not to influence FeNO.

Patient-Related Factors

Age. There is no consistent relationship between FeNO level and age in adults. Several studies have reported no correlation of FeNO with children's age (Nadziakiewicz et al. 2006, Ekroos et al. 2000, Beraldi et al. 1999). Some reports, though, suggest that in children FeNO is related to age, younger children having lower levels (Franklin et al. 1999, Latzin et al. 2002, Buchvald et al. 2005). Buchvald et al. (2005) further concluded that there is an approximate 1 ppb increase in FeNO per year over the age ranges investigated (4-18 years). Franklin et al. (1999) hypothesized that increased lung volume and airway surface area is the main reason for the increase of FeNO with age. Latzin et al. (2002) suggested that the age dependency in children may be related to changes in airway NO diffusion coefficients, which may be dependent on surface area. It is recommended that age be recorded at the time of measurement.

Gender. There is a consensus in the literature that FeNO levels in men are higher than in women (Jilma et al. 1996; Franklin et al. 2004; Grasemann et al. 2003; Tsang et al. 2001; Kharitonov et al. 2003; Olivieri et al. 2005; Taylor et al. 2007). Taylor et al. (2007) found that FeNO levels were approximately 25% less in females. It is not clear why this difference exists but Olivieri et al. (2005) speculate that the difference in airway surface area and caliber may differently dilute NO (a decreased airway surface area and caliber in females may result in a lower NO concentration). It is recommended that gender be recorded at the time of measurement.

Ethnicity. Only a few studies have commented on the influence of ethnicity on FeNO values. Anecdotally, it has been observed that those of African-American ancestry tend to have higher baseline FeNO values (F. Holguin, personal communication, August 2004). Kovesi et al. (2007) reported that the range of FeNO concentrations in healthy Asian-Canadian school children (9 to 12 years) was significantly higher than in Caucasian school children (22.8 vs. 12.7 ppb, $p < 0.001$). FeNO values also appeared to be higher in Canadian-African children than in Caucasians, although the confidence interval was wide because of the small number of Canadian-African children sampled. As well, Buchvald et al. (2005) in his examination of FeNO in healthy subjects aged 4 to 17 years, found that non-Caucasian subjects had significantly higher mean FeNO values compared with Caucasian subjects. Togashi et al. (1997) proposed a hypothesis for this when they described significant differences in allele frequencies for the neuronal NOS gene, responsible for an enzyme involved in the endogenous NO production, for Caucasian and African-American subjects. However, these higher baseline FeNO values may also be due to differences in environmental exposures or other factors that differ across race/ethnicity. The ATS guidelines (2005) do not address ethnicity as a factor influencing FeNO.

BMI. Nadziakiewicz et al. (2006) failed to find any significant correlations between BMI and FeNO. However, Komakula et al. (2007) concluded that in adults with stable moderate to severe persistent asthma (but not in controls), increasing BMI is associated with reduced FeNO. The ATS guidelines (2005) do not address BMI as a factor influencing FeNO.

Height. Several authors have found a positive relationship between height and FeNO in adults, with increasing height being associated with increasing FeNO (Tsang et al. 2001; Olin et al. 2006; Olin et al. 2007). This relationship is also seen in children (Malmberg et al. 2006; Kovesi et al. 2007), and is consistent with the relationship described between age and FeNO in children. Malmberg et al. (2006) reported that height was found to be the best independent variable for the regression equation for FeNO, which on average showed an increase in the height range of 120-180 cm from 7 to 14 ppb. The ATS guidelines (2005) do not address height as a factor influencing FeNO.

Food and Beverages. An increase in FeNO has been found after the ingestion of nitrate- or nitrite-containing foods, such as lettuce (with a maximum effect two hours after ingestion), and drinking water and ingestion of caffeine may lead to transiently altered NO levels. Until more is known, it is advised to refrain from eating and drinking for one hour before FeNO measurement, and to question participants about recent food intake.

Circadium Rhythm. It is uncertain whether measurements need to be standardized for time of day. It is recommended that serial NO measurements be taken at the same time of the day when possible, and that the time be recorded.

Smoking. Chronically reduced levels of FeNO have been demonstrated in cigarette smokers, and similar acute effects are seen immediately after smoking. However, smokers with asthma still have elevated FeNO levels compared to non-smokers. Participants should not smoke in the hour before the study and short- and long-term active and passive smoking history should be recorded.

Infection. Upper and lower respiratory tract infections may lead to increased levels of FeNO. For this reason, FeNO measurements should be deferred until recovery if possible or the infection should be recorded.

Exercise. A few researchers have reported increases in FeNO after exercise (Iwamoto et al. 1994, Chirpaz-Oddou et al. 1997). However, these studies used highly conditioned athletes with more efficient oxygen processes. Most others have reported that FeNO decreases with exercise (St. Croix et al. 1999, Kippelen et al. 2002, Verges et al. 2005, Verges et al. 2006, Mantione et al. 2007). Mantione et al. (2005) found that the mean of FeNO was 22.8 ±4 before exercise compared to 13.0 ±2 after exercise (n=24, p=0.003). Verges et al. (2006) examined the effect of repetitive exercise (as performed in endurance sports) on FeNO, and reported a post-exercise decrement of 73.1± 2.9% of resting value 15 minutes post-exercise. The repetitiveness of prolonged exercise even every 24 hours did not result in a decrease in baseline FeNO or a greater post-exercise FeNO decrement. Mantione et al. (2007) hypothesized that the decrease in FeNO is due to greater oxygen utilization and therefore a lower partial pressure of oxygen in arterial blood immediately after exercise. The lower oxygen levels could result in diminished NOS activity of the NO-generating lung cells. While Verges et al. (2006) showed that FeNO remains decreased for several minutes after a prolonged exercise session, the precise recovery kinetics of FeNO during the following hours is unknown. It is recommended that strenuous exercise be avoided for one hour before the measurement. The European Respiratory Society Task Force (Kharitonov et al. 1997) recommends that the subject should be seated at least five minutes before actual sampling and remain seated throughout the procedure.

Medications. The potential effect of any drug on NO cannot be excluded, thus all current medication and time it was administered should be recorded. After treatment with inhaled or oral corticosteroids in asthmatic patients, FeNO falls. Leukotriene-axis modifiers also reduce FeNO. Even if a certain medication does not affect NO production, it is possible that it might affect FeNO through other mechanisms such as changes in airway caliber.

2.3.3 Normal Reference FeNO Values

Increasing use of FeNO as a measure in the diagnosis and monitoring of asthma has urged the need for reference values of FeNO measured with commercially available equipment (Buchvald et al. 2005). However, to date few studies have reported the measurement of FeNO in accordance with current ATS standards (single breath online, exhalation flow 50 ml/s) in more than 50 healthy children or adults (Table 3). Even fewer studies were designed to determine reference ranges (Buchvald et al. 2005, Olivieri et al. 2006, Travers et al. 2007). Many of the studies examining FeNO values in children were designed to explore the variability in FeNO between various age groups or the short-term repeatability of FeNO levels.

Buchvald et al. (2005) were the first to report on normal reference values of FeNO in healthy children from preschool age to adolescence performed according to ATS guidelines using a NIOX analyzer (Aerocrine, Sweden) in three European and two US centers. Geometric mean FeNO in 405 children was 9.7 ppb, and the upper 95% confidence limit was 25.2 ppb. Further, the authors defined reference values for various age groups. For adolescents 10-13 (n=105) and 14-17 (n=80) years, geometric mean

Table 3. Studies reporting FeNO among healthy subjects

Reference	Subjects	Central tendency	Range	Reproducibility	Comments
Children:					
Latzin et al. (2002)	63 healthy children 4 to 18 years (median 12.2)	Median (IQR): 11.9 (8.2 -16.8)	NR	Intra-individual CV=25.9% (range 21-51%); Inter-measurement CV=6.5% (43 subjects, 137 measurements)	Expiratory flow rate: 45 ml/s
Baraldi et al. (1999)	159 healthy children 6 to 15 years (88 girls)	Mean (95% CI): 8.7 (8.1 – 9.2)	NR; estimated 2 – 21 (from Fig 1)	NR	Expiratory flow rate: 70 ml/s
Franklin et al. (1999)	157 healthy children 7 to 13 years (mean 9.7, 77 girls)	Geometric mean (95% CI): 10.3 (9.2 – 11.5)	NR; estimated at 83 (given the coefficient of repeatability and the % of the range)	Coefficient of between-test repeatability=8.3 (9.9% of the range of FeNO)	Expiratory flow rate: 50 ml/s
Kharitonov et al. (2003)	20 control children 7 to 13 years (mean 10.7)	Mean (± SD): 15.6± 9.2	NR	Coefficient of reproducibility (pooled SD)=2.11; ICC=0.99; Mean CV within sessions=9.5±4.7%	Expiratory flow rate not recorded, but presumed 50 ml/s
Buchvald et al. (2005)	405 children 4 to 17 years (mean NR but estimated at 9.6, 214 girls)	Geometic mean (95% upper limit): 9.7 (25.2) with outliers 9.0 (19.4) without outliers 8.8 (18.5) without outliers and atopics	>34.9 was found in 16 subjects	Within-subject SD=1.6 (95% CI, 1.49-1.64)	Expiratory flow rate: 50 ml/s Reported with outliers; without outliers; and without outliers and atopics

Reference	Subjects	Central tendency	Range	Reproducibility	Comments
Adults:					
Olin et al. (2006)	2200 adults 25 to 75 years (mean NR, 1111 women)	Median (IQR): 16.0 (11.0 – 22.3)	2.4-199	NR	Expiratory flow rate: 50 ml/s General population sample
Travers et al. (2007)	193 healthy adults 26 to 76 years (mean 56.3, 100 women)	Geometric mean (90% CI): 17.9 (7.8-41.1)	NR	NR	Expiratory flow rate: 50 ml/s To establish reference range for normal subjects
Olivieri et al. (2006)	204 healthy adults 19 to59 years (mean 36.1, 102 women)	Mean (95% CI): 10.8 (3.8-19.7)	0.7-28.8	NR	Expiratory flow rate: 50 ml/s To establish reference range for normal subjects

NR= no result

FeNO (95% upper limit) was 11.2 (28.2) ppb and 13.7 (39.2) ppb, respectively. Other studies have reported mean or median FeNO values in children and adolescents ranging from 8.7 to 15.6 ppb (Table 3). Three studies have reported normal reference values for adults, with means or medians ranging from 10.8-17.9 (Table 3).

There are limitations, however, in the studies examining reproducibility of FeNO measurements. As illustrated in Table 3, past literature indicates a variability in FeNO levels for healthy control subjects, and discrepancies in these results could be due to a number of factors. Technical factors, including method of collection (online vs. offline) and expiratory flow rate, are important considerations (Malmberg 2004). Borrill et al. (2006) have recently compared the FeNO levels measured using three different commercially available analyzers and found significant differences between them, raising the important question of variability between analyzers. Muller et al. (2005) have shown that the main factors responsible for the different NO readings provided by the various analyzers are differences in calibration gases and procedures. [Of note, they found the most reproducible data was that obtained using the EcoMedics CLD88, the analyzer used in this study.] Measurement error and the natural variability of airway inflammation over time may also explain the variability in FeNO measurements (Kharitonov 2004). As well, patient-related factors, discussed previously, may play a role in these discrepancies between studies.

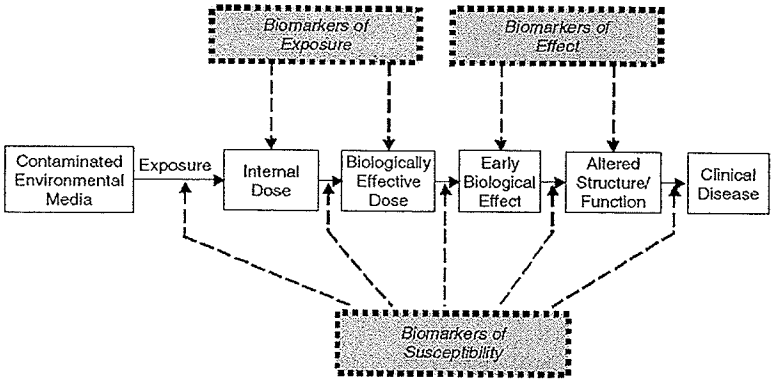
2.4 Exhaled Nitric Oxide as a Biomarker

A valuable tool in assessing human exposure to environmental contaminants is the use of biological markers. To date, airway injury or inflammation caused by air pollutants has

been evaluated mainly by analysis of bronchoalveolar lavage in adults. The assessment of respiratory risks in children and adolescents has relied on spirometry and self-reported symptoms, which are relatively late and inaccurate indicators of lung damage. However, current research in the biomarkers field is opening new opportunities for non-invasively measuring the respiratory toxicity of air pollutants (Bernard et al. 2005).

A biomarker can be broadly defined as a measurable change in a biological system that is caused by exposure to an exogenous chemical (Metcalf and Orloff 2004). Biomarkers can be divided into three categories- those pertaining to exposure, effect and susceptibility (National Research Council 1987). Exposure to a toxic chemical at a sufficient dose can initiate a sequence of events, the exposure-disease continuum, that ends with a clinically detectable disease (Figure 3).

Figure 3. Exposure-disease continuum



Source: Metcalf and Orloff 2004

Since FeNO has proven to be a reliable measure of inflammation and oxidative stress in the bronchial epithelium (Kharitonov and Barnes 2000), it can be considered a biomarker

of effect. A biomarker of effect is a measurable biochemical, physiological or other alteration in a biological system that is recognized as a known or potential health impairment or disease (Metcalf and Orloff 2004). The use of a sensitive biomarker of effect, such as FeNO, may prove to be a useful tool to identify subjects or groups at most risk from the toxic effects of air pollutants and for establishing unacceptable exposure levels of these pollutants. If no elevated exposures are observed in the segment of the population with the highest exposure potential, the likelihood for significant exposure in the rest of the population is lessened (Metcalf and Orloff 2004).

2.4.1 Characteristics of a Good Biomarker

To be useful in practice, biomarkers must meet certain criteria. Decaprio (1997) suggests that a biomarker should be *sensitive* to environmentally-relevant concentrations of the pollutant, *specific* for the pollutant of interest, *biologically relevant* to the exposure-disease continuum (Figure 3), *practical* to sample, *inexpensive* to process, and have equipment that is readily *available* for sample processing.

Bernard and Hermans (1997) state that to be useful in practice, biomarkers of early effects must meet several criteria such as *stability* in the biological sample, *specificity* with respect to the tissue or cellular targets, and *sensitivity* with respect to the exposure level. As well, importantly, sufficient *information* must be available to translate the observed changes in terms of health significance and clinical relevance. This involves localizing these changes in the sequence of events leading to toxicity and estimating the probability that they may lead to a functional deficit of the target organ. Distinguishing

adaptive from toxic effects, reversible from irreversible effects and adverse from non-adverse effects are identified as challenges.

Another way of assessing the usefulness of a biomarker would be to consider the quality of the biomarker as a measurement. McDowell and Newell (1996) identify *validity* and *reliability* as defining the quality of a measurement. They use a target as an analogy, and suggest that someone learning archery must first learn to hit the center of the target, and then learn to do this consistently. The consistency of a measurement would be represented by how close successive shots fall to each other, and validity would be represented by how close, on average, the shots come to the center of the target.

Reliability. Reliability, or consistency, is concerned with error in measurement (McDowell and Newell 1996). Traditional reliability theory views the value obtained from any measurement as a combination of the underlying true score and some degree of error. Errors are commonly grouped into two types- random errors and systematic errors or bias. Random errors include the variety of mistakes one can make in obtaining a measurement due to inattention, tiredness or mechanical inaccuracy. Random errors cancel each other out if enough observations are made, giving a good estimate of the true score. Reliability, then, refers to the extent to which a score is free of random error. More formally, the reliability of a measurement is defined as the proportion of observed variation in scores (across participants or repeated measurements) that reflects actual variation in health levels, and reaches unity when all the variance in observed scores reflects true variance. As such, two types of reliability can be distinguished- *inter-rater agreement* (whether different raters assessing a participant obtain the same result) and

test-retest reproducibility (whether the same result is obtained when the same rater makes a subsequent assessment of the participant).

Validity. Validity is commonly defined as the extent to which a test measures what it is intended to measure (McDowell and Newell 1996). There are three types of validity- content, construct and criterion. Most validation studies begin by referring to content validity. Content validity is seldom tested formally; rather, the face validity or clinical credibility of a measure is commonly inferred from the comments of experts who review its clarity, completeness and redundancy. More formal statistical procedures are used to further assess the validity of a measurement. Criterion validity considers whether the instrument correlates highly with a gold standard measure of the same theme. Sensitivity and specificity analyses commonly used to assess screening tests are a type of criterion validation. Validity testing is more challenging, and can be used when criterion validity is not possible (i.e. when a gold standard does not exist). It requires assembling multiple indicators of validity in a process known as construct validation. Construct validation begins with a conceptual definition of the topic or construct to be measured and an examination of the logical relations that should exist with other measures and/or patterns of scores across groups of individuals. When carefully applied, these comparisons build a composite picture of the adequacy of the measurement.

2.5 Relationship Between FeNO and Air Pollution

Exhaled nitric oxide has been proposed as a novel biological marker of adverse respiratory health effects attributable to air pollution (Sofia et al. 2002). Several studies have looked at the relationship between FeNO and air pollution, and some have attempted

to address which air pollutant(s) are responsible for the increase in FeNO. Most of these studies have been undertaken in Europe, have measured air pollutants in the early spring when ozone levels are low, and have not measured $PM_{2.5}$ and ultrafine particles. A very limited number of studies have specifically looked at ozone or PM in relation to FeNO, and none have explored this relationship in the exercising adolescent age group. The following review will address the literature pertaining to healthy children and adults with an emphasis on studies addressing the relationship between FeNO and ozone and/or particulate matter as a component of air pollution.

2.5.1 Ambient Air Pollution and FeNO

In an early study by Steerenberg et al. (1999), the authors aimed to assess the effect of outdoor air pollution (specifically ambient NO) on FeNO by supplying both NO-free air and unscrubbed air to 18 non-smoking participants (12 males and 6 females, aged 25-50 years) prior to FeNO sampling. Previous literature had suggested that incorrectly high values of FeNO were obtained when exhaled air is sampled on days with high environmental NO (Baraldi et al. 1998). Exhaled nitric oxide was sampled on four days with different levels of air pollution, as represented by ambient NO only (4, 30, 138 and 246 ug/m^3). On the two days with highest outdoor air pollution, FeNO was significantly increased (67-78%, $p<0.001$) above the mean baseline values assessed on four days with virtually no air pollution. The authors acknowledged that identifying the component(s) in the polluted ambient air that are responsible for the increase in FeNO would be of interest, as NO itself is unlikely to be a candidate based on the fact that smokers who are regularly exposed to high NO levels exhale lower concentrations of NO than nonsmokers.

Several studies since then have explored which air pollutant is responsible for the increase in FeNO observed in healthy subjects. Van Amsterdam et al. (1999) sampled FeNO once daily during a three week period in 16 nonsmoking subjects (5 females and 11 males, mean ages 34±4.5 years and 36±2.7 years, respectively) who were exposed regularly to varying outdoor air pollution levels. For each individual, the authors expressed daily levels of FeNO as a percentage of his/her baseline FeNO value (mean of measurements on four study days with the lowest ambient NO and CO levels). The daily level of FeNO was significantly correlated with ambient CO and NO (r=0.85 and 0.81, respectively). A poor linear correlation was observed between FeNO and ambient PM_{10} (r=0.52) and NO_2 (r=0.49). The concentrations of O_3 and SO_2 remained very low and showed virtually no variation during the study. Exposure during the morning hours to high levels of NO and CO was associated with a 50% increase in FeNO (significant compared with previous day) which persisted five hours later (32% increase in FeNO, not significant). The authors speculated that air pollutants other than NO and CO may be responsible for the increase in FeNO. They acknowledged that they could not assess what contribution SO_2 and O_3 may have had to the observed increase, and unmeasured pollutants such as $PM_{2.5}$ and ultrafine particles may be contributors (Adamkiewicz et al. 2004).

In a study examining how traffic-related air pollution affects peak expiratory flow, FeNO and inflammatory nasal markers, Steerenberg et al. (2001) further explored this point by investigating lag effects within their study design, including mean air pollutant levels recorded on (1) the same day (sampling time), (2) the previous day (lag 1), (3) the most recent three day period (lag 3) and (4) the most recent one week period (week). The

authors compared short term health effects of children aged 8 to 13 years from either an urban area or a suburban area. Urban children were found to have higher FeNO values in response to increased air pollution (3-28 ppb increase in FeNO per ug/m^3 pollutant) than did suburban children. Ozone and SO_2 levels remained very low during the study, and were not used in any data analyses. A significant increase in FeNO in urban children was noted following increased exposure to (1) PM_{10} (sampling time, lag 1, lag 3 and week), (2) black smoke (sampling time, lag 1, lag 3 and week), (3) NO_2 (lag 1, lag 3 and week) and (4) ambient NO (sampling time, lag 1, lag 3 and week). In suburban children, positive associations were noted only between FeNO and PM_{10} (sampling time), black smoke (sampling time and week) and ambient NO (sampling time and week).

In a similar study, Fischer et al. (2002) examined 68 children (10-11 years) living in an urban environment. For seven weeks respiratory complaints were diarized daily, and lung function measures and FeNO levels were measured once a week on days with various levels of air pollution. A variety of air pollutants were examined, but not ozone because the measurements were performed in the winter season when ozone levels are known to be negligible. Since all measurements were performed during the morning, the concentrations from the previous one (lag 1) and two days (lag 2) were used as the exposure variables. Levels of PM_{10}, black smoke and NO of the previous day (lag 1) were significantly ($p<0.05$) associated with FeNO, as were levels of NO_2, CO and NO of two days before (lag 2). The level of FeNO significantly increased by 3% to 31% per unit in air pollution level. The prevalence of respiratory symptoms such as sore throat, runny nose, 'having a cold' and 'sick at home', but not cough, were significantly and positively associated ($p<0.05$) with the level of FeNO measured in the following week.

2.5.2 *Ozone and FeNO*

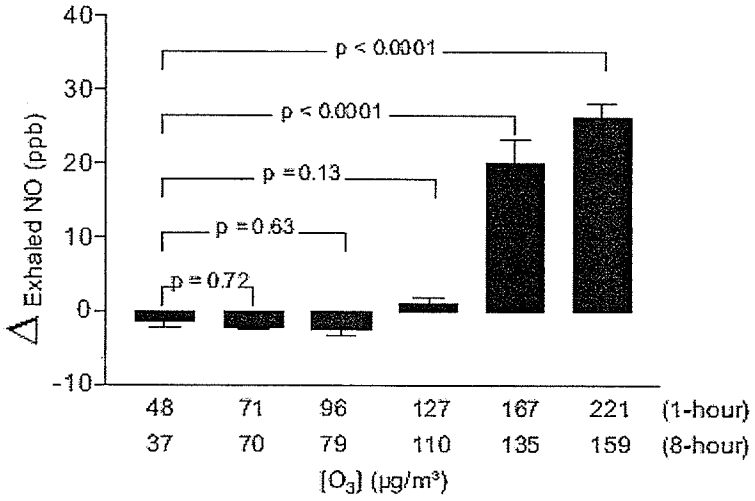
Olin et al. (1999) examined FeNO among Swedish pulp mill workers reporting gassing incidents involving the bleaching agents ozone and chlorine dioxide, and found significantly increased FeNO levels of those workers exposed to high peak levels of ozone (1-2 ppm) compared to coworkers not reporting such incidents. In this study, the peak ozone exposure preceded the NO measurements by months, possibly reflecting chronic inflammatory changes in the airways. Olin et al. (2004) repeated the study with a larger population of bleachery workers from three Swedish pulp mills using ozone as a bleaching agent, including the previously investigated workers. There was no significant difference in the median concentration of FeNO between bleachery workers and unexposed controls; however, workers in the highest exposure class (i.e. those reporting four or more gassings involving ozone) had a higher median concentration of FeNO in comparison with those who reported no such gassings (19.2 vs. 15.7 ppb, $p=0.04$). The increase was modest, FeNO being only 22% higher, suggesting that lower exposures (i.e. fewer than four gassings) will have limited effects on the respiratory system. Among the exposed subjects there was an exposure-response relationship for FeNO to increase with an increasing number of years with ozone gassings ($p=0.02$).

These results run contrary to those from experimental studies of acute ozone exposure in humans, including one from the above authors' group. Olin et al. (2001) also investigated whether FeNO measurement could be a useful biomarker for monitoring the effects of ozone at ambient levels on the respiratory tract. Eleven healthy non-smoking adults (mean age 24 years, range 20-29) were exposed to 0.2 ppm ozone and filtered air for two hours on two separate occasions. Exhaled NO and nasal NO were measured before and on

five occasions following the exposures (up to 24 hours). There was a slight but non-significant decrease in FeNO directly after the ozone exposure. One hour after the ozone exposure, FeNO levels were normalized and remained so for the rest of the follow-up period. In a similar study by Nightingale et al. (1999), no increase of FeNO was also found after acute exposure to the same concentration of ozone. Olin et al. (2001) speculated that the airway inflammation after this relatively low exposure level might be mild and/or due to the fact that an increased production of NO occurs in the most distal airways and is undetectable. Also, the nature of the exposure might differ (Olin et al. 2004). The workers have been exposed previously to repeated high peaks of ozone, whereas in the experimental study the subjects were exposed immediately and only once to a maximal ozone level of 0.2 ppm. The authors also considered that NO formed in the airways as a result of acute exposure might have been scavenged by other radicals and formed peroxynitrate, and thus would not be detected in exhaled air.

More recently, Nickmilder et al. (2007) measured FeNO twice daily in 72 healthy children, aged 6.5 to 15 years (mean ages 9.6-11.3 for each of the camps), that were attending one of six summer camps in rural southern Belgium. Children were exposed to various concentrations of ambient ozone in the various summer camps (48-221 ug/m^3 or 0.024 to 0.110 ppm); the concentrations of other pollutants were low and stable, or even decreased during the study days. Although the children remained outdoors during the day, they did not do sports or running. While a small evening decrease in levels of FeNO was observed in children exposed to ozone concentrations less than 100 ug/m^3 (0.05 ppm), those exposed to the highest ozone concentrations demonstrated a marked and significant increase of their evening levels (compared to the morning levels) (Figure 4).

Figure 4. Change in FeNO in children exposed to increasing concentrations of ambient ozone.



Source: Nickmilder et al. 2007

The increased level of FeNO was pronounced at the two sites with an 8-hour concentration of 135 ug/m3 (0.068 ppm) or greater. These two sites had maximum 1-hour concentrations of 167 (0.084 ppm) and 221 ug/m3 (0.11 ppm), which do not quite exceed the EPA standard (0.12 ppm). Of note, at this higher ozone concentration, the increase in FeNO was not accompanied by lung function decrements, reinforcing the idea that FeNO is an early marker of airway inflammation as a result of exposure to ambient ozone.

2.5.3 $PM_{2.5}$ and FeNO

As with ozone, few studies have investigated associations between exposure to $PM_{2.5}$ and FeNO. Several of these studies have taken place in Seattle, one of the most traffic-congested cities in the United States, as part of an intensive exposure assessment and health effects panel study of susceptible subpopulations from 1999 through 2002 (Koenig et al. 2003, Mar et al. 2005).

Koenig et al. (2003) examined offline FeNO for 10 days in winter and/or spring in 19 children (6 to 13 years of age) with asthma exposed to ambient $PM_{2.5}$ in Seattle. The authors found consistent associations between same-day (no lag) ambient $PM_{2.5}$ (24-hour average) and FeNO, and observed an approximately 4 ppb average increase in FeNO for a 10 ug/m^3 increase in $PM_{2.5}$. The authors did not state whether associations were present at other lag periods. Of note, $PM_{2.5}$ values were considerably higher during winter (IQR=9.8 ug/m^3 in winter vs. 5.3 ug/m^3 in spring) when fine particles from wood stoves predominate. In a follow-up report of this study, Koenig et al. (2005) found that the estimated ambient-generated fraction of the personal PM exposure was positively associated with FeNO, but not the estimated indoor-generated fraction.

Most studies of relationships between $PM_{2.5}$ air pollution and health are based on 24-hour average $PM_{2.5}$ measurements, and these studies do not allow investigators to ask questions about very short term (hourly) lags between health outcomes and $PM_{2.5}$ exposure. Mar et al. (2005) examined the associations between short-term (hourly) exposures to $PM_{2.5}$ and FeNO in the same 19 asthmatic children in Seattle, and compared them to the results of Koenig et al. (2003). Using a polynomial distributed lag model for $PM_{2.5}$ up to 48 hours after exposure, the authors found that FeNO was associated with hourly averaged $PM_{2.5}$ exposure up to 10 to 12 hours before the health measurement in subjects. There was also some suggestion of an increase in FeNO between 38 and 41 hours after exposure. The overall effect of a prolonged exposure to $PM_{2.5}$ (48 hours) was 7.0 ppm FeNO per 10 ug/m^3 increase in $PM_{2.5}$, and was obtained by summing up the estimated effects at each time lag.

Adamkiewicz et al. (2004) also aimed to evaluate the potential association between $PM_{2.5}$ and FeNO in Steubenville, Ohio. Air pollution in Steubenville has been dominated historically by industrial sources. The authors examined FeNO for 29 nonsmoking elderly subjects (27 female, median age 70.7 years) weekly over a three month period. A significant association was observed between FeNO an ambient $PM_{2.5}$ (as well as NO) across various exposure windows during the previous day's exposure (1-day lag). An increase in the mean $PM_{2.5}$ concentration (24 hour average) of 17.7 ug/m^3 was associated with a 1.45 ppb increase in FeNO. Two-pollutant models suggested that other pollutants did not confound the $PM_{2.5}$ effect. Of note, the authors also observed negative associations (non-significant) between ozone exposures and FeNO. The authors note that ambient ozone concentrations are typically inversely correlated with NO concentrations in areas with local sources, since NO reacts rapidly with ozone.

Delfino et al. (2006) examined the relationship between FeNO and ambient air pollution ($PM_{2.5}$ and NO_2) in a panel of 45 schoolchildren (9-18 years old) with persistent asthma living in southern California with both personal and central monitors for $PM_{2.5}$. The strongest positive associations were between FeNO and the $PM_{2.5}$ moving average for the 48 hours preceding the FeNO measurement, although the estimates of effect were small for all of the lag models (<2.5 ppb FeNO per 24 ug/m^3 $PM_{2.5}$). Exhaled NO in all subjects was associated with $PM_{2.5}$ exposure in the 5 hours preceding measurement. Beyond 24 hours, no significant associations between PM and FeNO were found. Of note, ambient ozone was not associated with FeNO.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Overview and Design

The CDC study examined a small group (n=16) of adolescent student athletes who trained in long-distance running. The location of the study was downwind from metropolitan Atlanta, and the study took place during the last two weeks of August, 2004- historically considered the peak "smog season". Before and after training for each study day, a questionnaire was self-administered (to gather information about respiratory symptoms), spirometry (EasyOne spirometer, ndd, Andover, MA) was performed, exhaled nitric oxide (DFENOX 88, EcoMedics, MI) was measured, and exhaled breath condensate (RTube®, Respiratory Research, Charlottesville, VA) was collected on each participant. Same-day hourly ambient air concentrations of ozone and particulate matter $\leq$2.5 microns in diameter ($PM_{2.5}$) were obtained from the Georgia Ambient Air Monitoring System.

This was a prospective observational study with a repeated measures design in which ambient air concentrations of ozone and $PM_{2.5}$ were the independent variables of interest, and post-practice exhaled nitric oxide (FeNO) was the primary outcome of interest. As a result of the small sample size, the power of this study is low, and analyses were largely exploratory in nature.

3.2 Sample, Population and Study Participants

Participants in the CDC study were recruited from the cross-country running team at a high school in Conyers, Georgia (Rockdale County), where the participants trained outdoors between 4 and 5 pm. This location was selected because it generally experiences the highest ozone concentrations in metropolitan Atlanta. In partnership with the coach of the cross-country team, the study investigators invited all potential study participants (approximately 50) and their parents/legal guardians to an informational meeting before the study began. If athletes were interested in participating in the study, they were asked to return a completed consent form (by parents), assent form (by athletes), and baseline questionnaire to their coach.

The first 16 athletes who enrolled and were eligible for the study were selected, representing a convenience sample of the athletic team. The study was limited to 16 participants for several reasons, including the fact that this was a feasibility study whose objectives were not based on a required sample size. As well, the study was limited by the number of testing devices and staff available, and study coordinators were sensitive to the time commitment of the student volunteers. Exclusionary criteria were (1) student athletes younger than 12 and older than 18, (2) those with a history of upper or lower respiratory infection within the four weeks prior to the beginning of the study, and (3) those with a latex allergy.

3.3 Study Procedures

Measurements were taken on the 16 participants for all weekdays within the 15-day study period (August 16-30, 2004), except Wednesday, August 25, when the team was at a meet. A total of ten study days were included in this study. Due to some technical difficulties with the machine that measured FeNO, no measurements for FeNO were obtained for Day 1, and only post-practice measurements were obtained for Day 2, for a maximum of 8 pre-practice and 9 post-practice FeNO measurements for each participant. Participants had a range of 5 to 9 valid pre-practice or post-practice FeNO values, except Participant #10, who dropped out of the study on Day 4.

Study participants were asked to commit approximately 20 minutes before and after practice on each study day. Before and after training, study coordinators completed the sequence below for each participant:

- collection of exhaled breath condensate (EBC) and administration of pre-practice or post-practice questionnaire (ten minutes);
- measurement of exhaled nitric oxide (five minutes); and
- spirometry (five minutes).

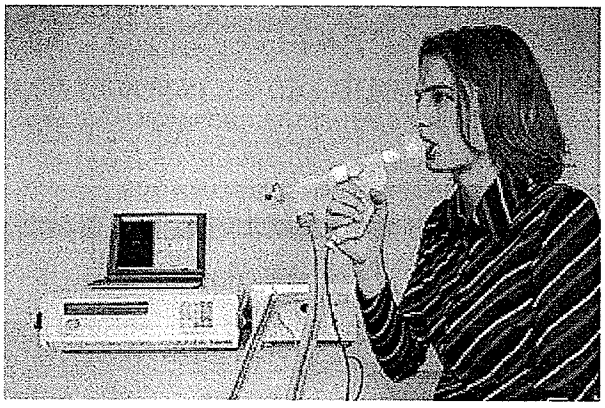
Multiple stations were set up to do spirometry and collect EBC so participants could rotate through the various stations in a timely manner. However, there was only one FeNO station, thus it took approximately one hour to process all participants both before and after practice.

3.4 Study Measures

3.4.1 Clinical Measures

Since this thesis research is focused on the FeNO component of the CDC study, only the methods for collection of FeNO will be discussed. The methods for exhaled breath collection and pulmonary function testing will not be further described. Results from the exhaled breath collection will be reported in detail separately.

Exhaled Nitric Oxide. Prior to spirometry, participants underwent a determination of FeNO following standardized American Thoracic Society guidelines available at the time of the study (American Thoracic Society 1999). Exhaled nitric oxide measurements were collected for each participant twice daily during the study period, before and after athletic practices. An online continuous chemoluminescence nitric oxide (NO)-analyzer with integrated ultrasonic flowmeter (DFENOX 88, EcoMedics, MI) was used to measure NO (shown in photograph below).



Study subjects inhaled NO-free air supplied by a unit that contains a filter to generate NO-free air, and a blower to supply a continuous flow of NO-free air. Subjects were

asked to insert the mouthpiece and inhale over 2 to 3 seconds through the mouth, and exhale immediately (to avoid elevations in NO due to breath-holding) for approximately ten seconds. Subjects were required to exhale against an expiratory resistance (restricted exhalation method) and maintain a positive mouthpiece pressure (between 10-20 cm H_20) to ensure that the soft palate was closed against the nasal cavity, thus avoiding contamination of NO derived from nasal and paranasal regions. They were guided to adjust their exhalation force by viewing a target area on the online screen representing the target mouthpiece pressure. The DFENOX 88 unit allowed for a relatively constant expiratory flow of 50 mL/s. The analyzer required at least a 3-sec NO plateau at a mean flow rate of 50 mL/s ($\pm$ 10%) and a total 6-sec exhalation time. Exhalation maneuvers were repeated three times, and then a summary sheet was printed for each subject. To assure quality control of measurements, the analyzer was calibrated to zero NO gas daily by passing NO-free air through the NO analyzer with the activation of the blower mode on the DFENOX 88 unit. Calibrations to a known NO ppb concentration was completed just prior to the start of the study.

Using this procedure, participants exhale directly into the tubing of the NO analyzer and the NO level is analyzed continuously during exhalation. This analysis results in a NO profile versus time or exhaled volume, together with other exhalation variables (airway flow and pressure) displayed in real time. In short, the participant should be seated comfortably and asked to inhale NO-free air through a mouthpiece to total lung capacity over two to three seconds, and then exhale immediately. A constant expiration flow can be achieved through displaying the flow to the patient on a computer screen with the target range.

3.4.2 Survey Instruments

The survey instruments included (1) a baseline questionnaire administered once at the beginning of the study; (2) a questionnaire administered prior to practice on each study day ("pre-practice" questionnaire); and (3) a questionnaire administered after practice on each study day ("post-practice" questionnaire).

Baseline Questionnaire. This written questionnaire was administered once to the student, with assistance from the parent/guardian if needed (Appendix A). A separate short questionnaire enquiring about smoking or exposure to environmental tobacco smoke (ETS) was administered to the student only (Appendix B). The objective of this questionnaire was to obtain information needed to interpret the results of the clinical measurements and to confirm that the student met eligibility requirements. Information collected on this questionnaire included demographics, history of respiratory and allergic symptoms and diagnoses, and information about factors that affect the clinical measurements such as exposure to tobacco smoke from active or passive smoking. Participant eligibility was determined based on this questionnaire.

Pre-Practice Questionnaire. The objective of the pre-practice questionnaire was to obtain information on activities within the previous hours that may influence the results of the clinical measures and to assess respiratory symptoms (if any) prior to exposure to ambient air pollution during exercise. There are two versions of the pre-practice questionnaire. A slightly longer version was used on the first study day (Day 1 version) to elicit information about smoking and ETS exposure in the last month. A similar but

shorter version (Day 2-10 version) was used on study days two through ten. These questionnaires, shown in Appendix C, include questions about recent outdoor activities, smoking and ETS exposure, use of vitamins and anti-inflammatory drugs, and respiratory symptoms such as cough and chest tightness. The pre-practice questionnaire was completed independently by each participant during the collection of exhaled breath condensate.

Post-Practice Questionnaire. The objective of the post-practice questionnaire was to obtain information on respiratory symptoms (if any) after exposure to ambient air pollution during exercise, including questions about chest tightness, shortness of breath, wheezing, and other respiratory symptoms (see Appendix D). This questionnaire was also completed independently by each participant during the collection of exhaled breath condensate.

3.5 Exposure Assessment

Georgia's Department of Natural Resources measures and records air quality data hourly from a variety of monitoring sites across the state. For each day within the 15-day study period, measures of air quality index (AQI) and ambient concentrations of ozone and $PM_{2.5}$ were obtained from the Georgia Department of Natural Resources' Ambient Monitoring Program database (Georgia Department of Natural Resources 2005a). Data were extracted from the nearest monitoring station for the ambient air quality parameters listed in Table 4. The nearest monitoring station was <1 mile from the study site for ozone, and approximately 14 miles for $PM_{2.5}$.

Table 4. Ambient air quality parameters from the Georgia Ambient Air Monitoring System (GAAMS) used in this study

Ambient Air Quality Parameter	Time Period Averaged	Monitoring Station
Maximum ozone (ppm)	1 hour	Conyers
Maximum ozone (ppm)	8 hour	Conyers
1700 h ozone (ppm)	1 hour	Conyers
1700 h ozone (ppm)	8 hour	Conyers
Maximum $PM_{2.5}$ (ug/m^3)	1 hour	South Dekalb
1700 $PM_{2.5}$ (ug/m^3)	1 hour	South Dekalb
Maximum AQI (primary pollutant)	none	Highest concentration at a metropolitan Atlanta station
1700 h AQI (primary pollutant)	none	Highest concentration at a metropolitan Atlanta station
EPA Color/Descriptor	none	Highest concentration at a metropolitan Atlanta station

In addition to the data that was obtained from the nearest Georgia Ambient Air Monitoring System (GAAMS) monitoring station, ozone and particulate monitoring, as well as ambient air temperature and relative humidity, were conducted on site with stationary monitors for the period that the investigators were there. Personal monitoring was also used to measure ozone exposure, but was exploratory in nature to test various new measurements methods in a field setting. Since the GAAMS data is the most reliable measure of air quality in the study area, this is the only air quality data that will be used in the data analyses.

3.6 Statistical Methods

Analyses were conducted in Microsoft Excel 2003 and SAS version 9.1 (SAS Corporation, Carey, NC) to generate descriptive and analytic results. The data set was anonymous, with each study participant coded by their participant number. Excel was

used to generate descriptive statistics and graphs for the participants using information from the baseline questionnaire. The following describe the analyses undertaken to answer the research questions, and are organized by the content of the research questions- *reliability, validity* and *responsiveness*.

3.6.1 Reliability

This study aims to examine the test-retest reproducibility of the pre-practice (baseline) FeNO measurements, one of the two types of reliability defined. Test-retest reproducibility is the degree to which an instrument yields stable scores over time among participants who are assumed not to have changed on the domains being assessed (Medical Outcomes Trust 1995). To evaluate the reliability, the variability of the baseline FeNO values were explored, and specific measures were calculated to evaluate the reproducibility of baseline FeNO.

Variability. Excel was used to conduct a thorough univariate analysis of the pre-practice FeNO values. The purpose of this analysis was to examine the variability of the pre-practice FeNO values collected both between and within individuals, an area not fully understood in the literature. Histograms were used to examine the distribution of the pre-practice FeNO data. Data were examined aggregately (all FeNO values pooled), as well as by subject (study days pooled) and by study day (subjects pooled) to explore within-subject and between-subject variation. Descriptive statistics, including mean, median, minimum, maximum, range, quartiles, interquartile range, standard deviation, standard error and coefficient of variation were generated, and graphs were used to further describe the data. Finally, a partition of the total variation in baseline FeNO was estimated from

the model generated from the SAS GLM procedure, where SSid and SStotal were used to calculate the proportion of variance that was due to between-subject variation.

Reproducibility. A key measure of reliability is the reproducibility or stability of a measurement over time. Reproducibility of the FeNO measurements was assessed in three different ways: 1) by the intraclass correlation coefficient (ICC); 2) by the within-participant coefficient of variation; 3) by the pooled SD. The ICC is a dimensionless statistic bounded by 0 and 1 that describes the reproducibility of repeated measurements in the same population. Calculated measures of reproducibility were compared to other studies or reference values, where available. In a stable population ICC values in excess of 0.6 are thought to be clinically significant, and those less than 0.6 are probably not (Faul et al. 1999). A desirable CV for the purposes of this research will be <20%, and an undesirable CV will be >30%. These criteria are based on Reed et al. (2002), who translated the CV into a probability that two measurements on the same person differ by a factor of k. According to their nomogram, with a CV of 30% there is a probability of 0.10 that two samples on the same person differ by a factor of two or more, whereas with a CV of 20% this same probability drops to only 0.013.

3.6.2 Validity

The validity of a test is the degree to which the test measures what it is supposed to measure (McDowell and Newell 1996). An assessment of construct validity was undertaken, through a comparison of group differences in the baseline FeNO study data to those described in the literature. This step of the analysis was also important in discerning

which participant characteristics were important control variables in a regression model. Groupings of participants was by age, gender, race, BMI, home ETS exposure, asthma or allergy or hayfever, number of symptoms in the past 24 hours, and those who have had wheeze or cough in the last month. The MIXED procedure in SAS was employed to account for repeated measurements on the same individual. The LSMEANS statement computed adjusted means and standard errors for each category of the groups of interest, and determined if the groups were significantly different. These adjusted means were compared to those computed by the GLM procedure in SAS (that does not take into account the repeated measures). Excel was utilized to graph these results.

3.6.3 Responsiveness

The criterion of responsiveness requires asking whether the measure can detect differences in outcomes that are important, even if those differences are small. Multiple regression analysis was used to examine the responsiveness of FeNO to air quality parameters, and the following hypothesis was tested:

H_0: In this adolescent age group, there is no change in FeNO, after adjusting for the control variables of interest, with an increasing exposure to ambient ozone ($PM_{2.5}$) during vigorous exercise in the late afternoon.

H_1: In this adolescent age group, there is a change in FeNO, after adjusting for the control variables of interest, with an increasing exposure to ambient ozone ($PM_{2.5}$) during vigorous exercise in the late afternoon.

The following variables were considered for inclusion in the regression model:

Predictor Variables: Ambient ozone concentration measure
Ambient $PM_{2.5}$ concentration measure

Control Variables:	Pre-practice FeNO Participant characteristics (gender, age, BMI, race) Exposure to ETS Respiratory morbidity (asthma, allergies, hayfever) Exercise intensity, distance and duration (**rival hypothesis variables**)
Outcome Variable:	Post-practice FeNO

A series of analytic steps were undertaken to determine if there is a relationship between post-practice FeNO and the most important air quality parameters, and the strength and nature of this relationship. First, univariate analyses of the post-practice FeNO (outcome variable) and extracted GAAMS air quality parameters (ozone, $PM_{2.5}$ and AQI measures) (potential predictor variables) that were gathered nearest the study site were conducted. Distributions of all these variables were examined, to determine if they were normal. Transformations were undertaken if applicable. Bivariate scatterplots and correlations between each of the air quality parameters and post-practice FeNO were then examined, and the air quality measures most highly correlated with post-practice FeNO (i.e., ozone and PM) were selected for inclusion in a regression model. The effect of lagging the ambient air quality by one and two days prior to the post-practice FeNO measure was examined using all GAAMS data available over the study period (including weekends and non-study days).

Control variables were then considered. Since pre-practice FeNO was highly correlated with post-practice FeNO ($r = 0.94$), it was included as a control variable. Other control variables were selected based on the results of the exploratory analyses examining groups of interest. If significant differences ($p<0.10$) in the mean pre-practice FeNO values between groups were found using the mixed linear model (as described in the previous

section), the variable was used as a control. A mixed linear baseline control model was built to explore the relationship between post-practice FeNO (outcome variable) and these primary control predictors. Rival hypothesis variables were added to see how well they could explain the outcome.

Finally, a series of regression models were built to examine the association between post-practice FeNO and ambient air pollutants, after controlling statistically for all other effects. Models in which the ozone and $PM_{2.5}$ concentrations were lagged by one and two days were also examined, as were models with both raw and natural log-transformed post-practice FeNO. Given the nature of the FeNO data, the MIXED procedure in SAS was utilized to generate a mixed linear model that accounted for correlation within subjects.

3.7 Variable Definitions

"Number of symptoms in past 24 hours" is defined as the number of positive responses to questions asking if the subject experienced wheeze, cough, shortness of breath, chest tightness, chest pain, watery eyes, runny nose, itchy or scratchy throat, sneezing or headache within 24 hours prior to the start of practice. Each day, athletes rated their perceived "exertion" during running on a scale from 1 (least vigorous) to 10 (most vigorous).

3.8 Ethical Considerations

There were several components of the CDC study that required some consideration regarding ethics. First was the ethical dilemma of going ahead with the study with the knowledge from the literature that air pollution causes respiratory health effects. The high school involved in this study was contacted and it was confirmed that there was no school policy regarding canceling practices on air pollution alert days. It was also decided that a CDC physician would be present during the study practices to further evaluate symptoms disclosed and take action, if necessary.

Other ethical considerations were made regarding the recruiting and retaining of participants. First, how to choose participants if there was an overwhelming interest in participating in the study? It was decided that a lottery system would be undertaken if this were the case. There was also consideration around the administration of sensitive questions on the baseline questionnaire that a parent might have access to, such as whether the athlete smoked. A separate supplementary questionnaire that enquired about these more sensitive questions was designed for the athlete to fill in privately once they handed in the baseline questionnaire. The provision of an incentive for study completion was also considered, and it was decided that we would supply one ticket to a local amusement park per participant, whether they completed the study or not. This was agreeable with the CDC Institutional Review Board.

Finally, the team considered under what circumstances parents would be notified of a test result that deviated from what might be expected. It was decided that since FeNO and EBC were newer proposed biomarkers without much literature regarding what might be

considered an 'abnormal' result, that these measurements would not be shared with parents. However, abnormal spirometry test results (i.e. results that suggested a diagnosis of asthma or other airway disease) were to be reported to parents immediately, and all participants received a letter at the completion of the study indicating whether they had normal or abnormal spirometry test results.

The CDC study was approved by both the CDC and Emory University Institutional Review Boards. An anonymous dataset was provided by CDC for the purposes of this research. This thesis research, as a sub-study of the CDC study, was also reviewed and approved by the University of Manitoba Faculty of Medicine Research Ethics Board.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Overview

This chapter examines the results of this study, organized by research question, and discusses the significance of these results. The following sections describe the study participant descriptive statistics, then go on to examine the reliability, validity and responsiveness of the study participants' FeNO measurements, respectively. Discussion of these results is threaded throughout the sections below.

4.2 Study Participant Descriptive Statistics

Mean age of participants was 14.9 years, 56% were male, and 69% were white (Table 5). Of the non-white study participants, four were black (25%) and one was Asian (6%). Two of 16 (13%) reported having asthma, one of whom only participated in the study for the first three days. There was overlap in those study participants reporting asthma, allergy or hayfever, with five of the 16 (31%) participants reporting one or more of these conditions. None were smokers.

Table 5. Selected characteristics of study participants (n=16 subjects)

Variable	Value
Age, yr	
Mean (SD)	14.9 (0.9)
Range	14 -17

Variable	Value
Sex, N (%)	
Male	9 (56)
Female	7 (44)
Race, N (%)	
White	11 (69)
Non-White	5 (31)
Black	4 (25)
Asian	1 (6)
Body mass index, kg/m^2	
Mean (SD)	19.8 (1.7)
Range	17.5 – 23.5
Height, cm	
Mean (SD)	166 (6.4)
Range	157 – 178
Self-reported asthma diagnosis, N (%)	
Yes	2 (13)
No	14 (88)
Self-reported allergy diagnosis, N (%)	
Yes	4 (25)
No	12 (75)
Self-reported hayfever diagnosis, N (%)	
Yes	1 (6)
No	15 (94)
Wheeze or cough in past month, N (%)	
Yes	4 (25)
No	12 (75)
Home ETS exposure, N (%)	
Yes	2 (12)
No	14 (88)

4.3 Reliability

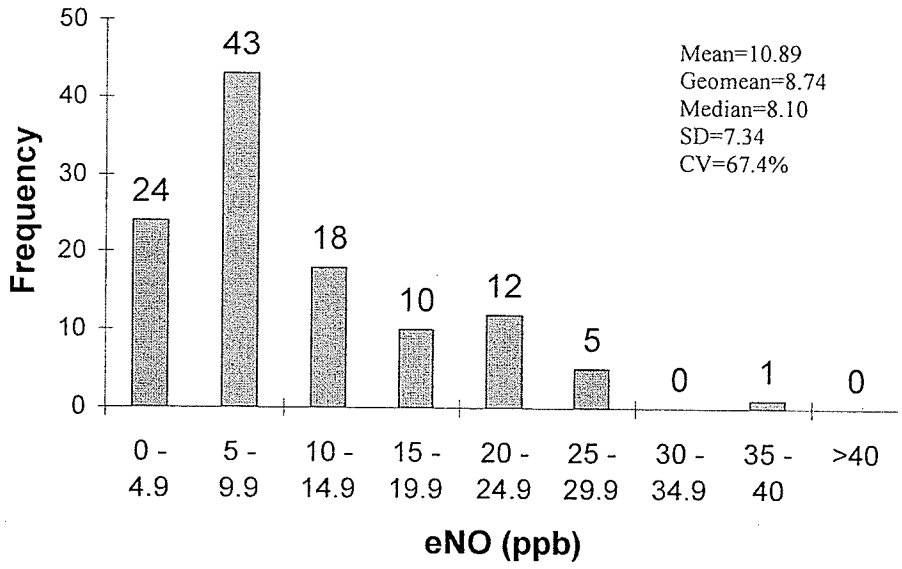
This section examines the variability in, and reproducibility of, the pre-practice FeNO values in the study participants. Data will be presented aggregately (all FeNO values pooled), as well as by subject (study days pooled) and by study day (subjects pooled) to explore both between-subject and within-subject variability.

4.3.1 Variability

A total of 113 pre-practice FeNO samples were obtained from 16 subjects over 10 study days. It should be noted that these 113 samples are repeated measures on the 16 study subjects, not independent observations. Repeated observations on each study subject are inherently correlated to one another, and this correlation was taken into account in deriving various statistics.

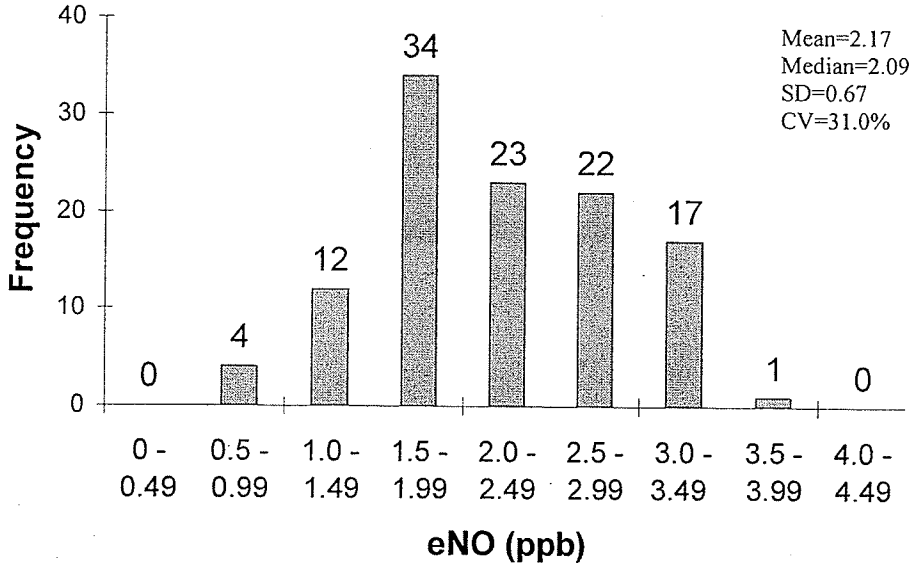
Overall. A histogram of the pre-practice FeNO values reveals that these are not normally distributed (Figure 5). The distribution is unimodal, but asymmetric and skewed to the left. The majority of the samples appear to have values in the lower ranges, suggesting that most individuals (85/113 or 75%) had baseline FeNO values from 0 to 15 ppb.

Figure 5. Histogram of pre-practice FeNO, subjects and study days pooled



A lognormal transformation of the data produces a histogram of pre-practice FeNO values that are now more normally distributed (Figure 6).

Figure 6. Histogram of pre-practice FeNO log-normally transformed, subjects and study days pooled



The raw mean (geometric) pre-practice FeNO was 8.7; median was 8.1 (Table 6). These values are similar, suggesting that a lognormal transformation of the data was appropriate. The standard deviation is quite large compared to the geometric mean (SD = 7.3), and the other measures of variation for these data were also high (range = 2.1 – 35.2; IQR = 5.3 – 14.5; CV=67.4%). These values are from the pooled data, and do not take into account the correlation within subject.

Table 6. Pre-practice FeNO, subjects and study days pooled

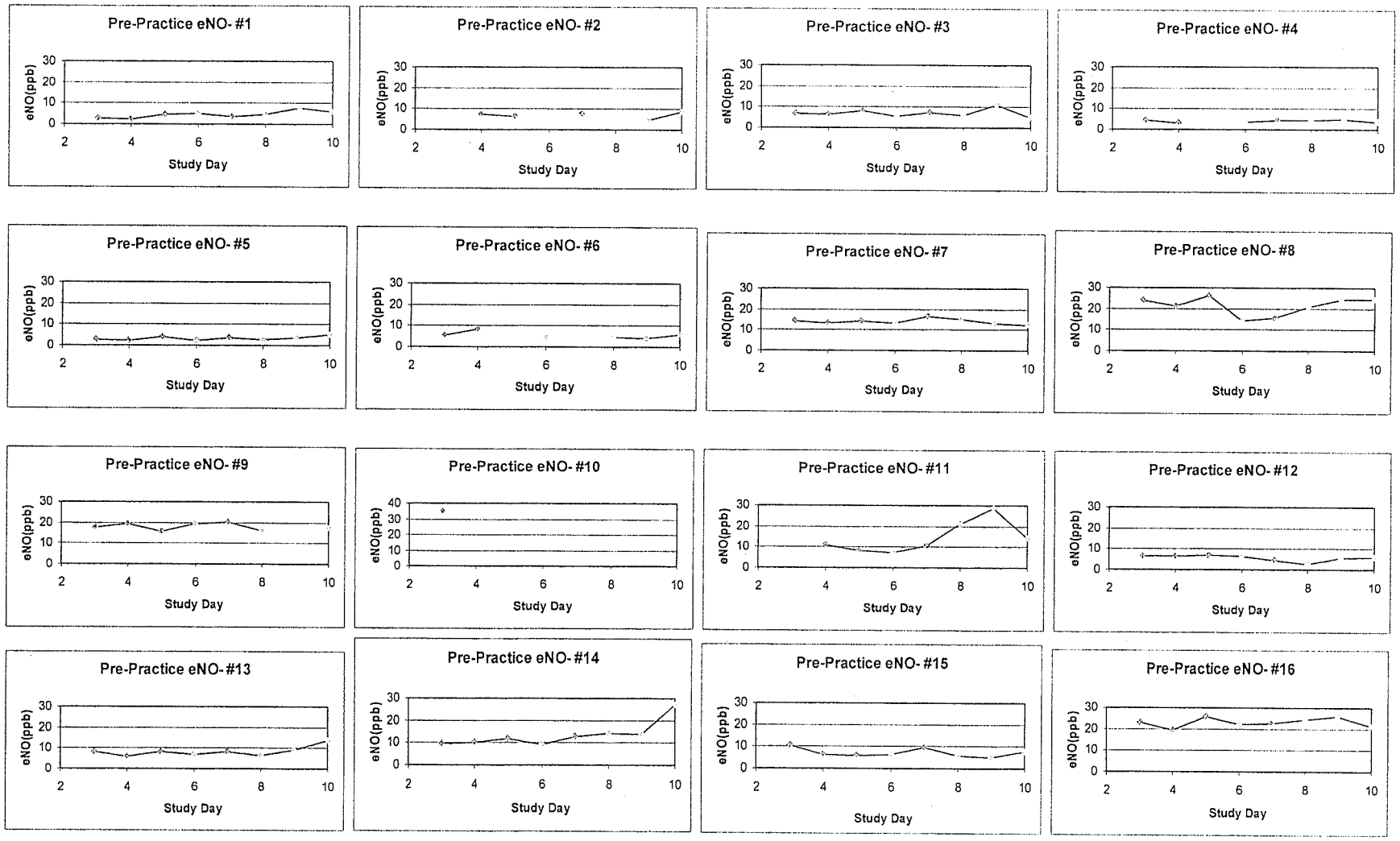
N	**113**
MEAN (ARITHMATIC)	**10.9**
MEAN (GEOMETRIC)	**8.7**
MEDIAN	**8.1**
MIN	**2.1**
MAX	**35.2**
RANGE	**33.1**
SD	**7.3**
CV (%)	**67.4**
IQ-1	**5.3**
IQ-3	**14.5**
IQR	**9.2**

The mean (geometric) pre-practice FeNO in this sample (8.7 ppb) is lower than what is reported in the literature. Buchvald et al. (2005) reported a mean (geometric) FeNO (95% upper limit) of 13.7 (39.2) ppb for those 14-17 (n=80) years. Other studies have reported mean or median FeNO values in children and adolescents ranging from 8.7 to 15.6 ppb, but none of these samples differentiated FeNO values for children and adolescents (Table 3). It is unknown why the values in this study were at the very low end of this range. As discussed previously, a variety of factors can cause discrepancies in the results between these studies. However, in this study, the small sample size could provide an explanation for the mean pre-practice FeNO deviating from that reported in the literature. Although

there are limited reports of measures of distribution of baseline FeNO values, the range of FeNO values found in this study is consistent with the literature (Table 3).

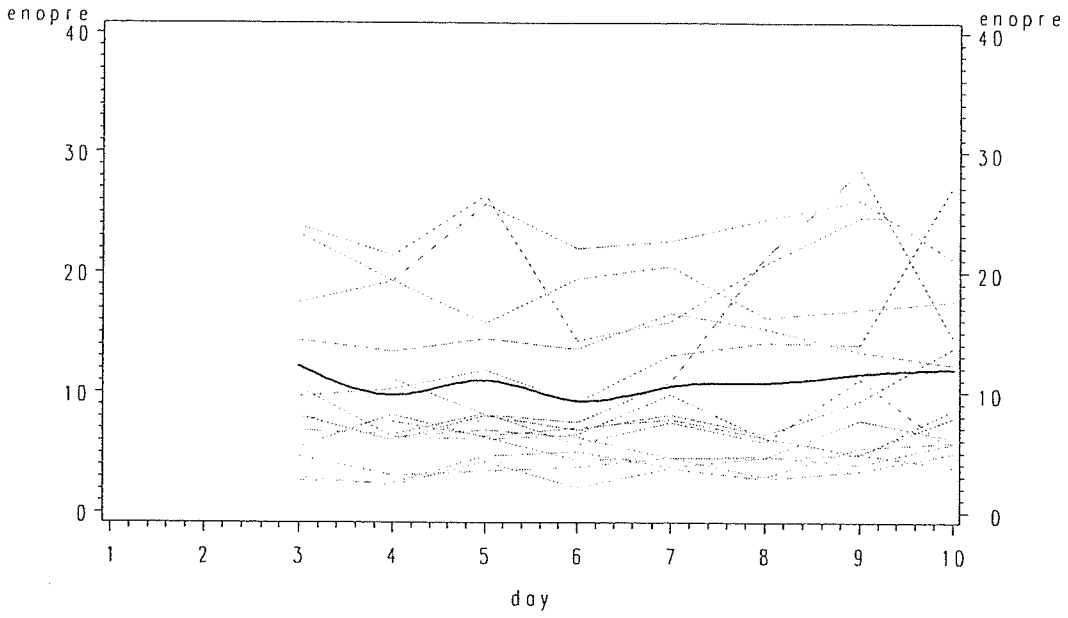
Figure 7 illustrates the graphs of each subject's pre-practice FeNO values by study day. Each value is color coded to depict which color alert was present on a particular study day. Upon general inspection, many subjects appear to have FeNO values in the lower ranges (0-10 ppb) with little variation between measurements (i.e. subjects are fluctuating around their mean fairly tightly). However, there appear to be several individuals who have higher baseline FeNO values (e.g. Subjects 8 and 16), suggesting a high between-subject variation. These subjects appear to have higher within-subject variation compared to the subjects with lower baseline FeNO values. There does not appear to be any pattern with respect to the various alert days. For example, as a general pattern, we do not see higher FeNO values the day after an orange alert day.

Figure 7. Pre-practice FeNO by subject (Yellow and Orange Alert days indicated)



Upon merging the individual graphs represented in Figure 7, a spline suggests that there is no upward or downward trend among the measurements (Figure 8). The pattern of individual measurements is relatively consistent across study days (i.e. individuals with relatively high FeNO measurements continue to have relatively high FeNO measurements and vice versa). The increased variability in measurements between subjects with higher baseline FeNO values compared to those with lower values is evident.

Figure 8. Pre-practice FeNO by subject with spline



By Subject. On average, each subject provided 7.1 pre-practice FeNO samples over 8 study days (Table 7). Individual mean (geometric) pre-practice FeNO ranged from 3.2 to 22.9 ppb. These means are quite variable between individuals (Figure 9), and suggest a high between-subject variation, confirming our previous observation in the data. The variation in pre-practice FeNO values also differed considerably between subjects over the study days (SE = 0.24 - 1.54; range = 1.8 - 21.0) (Figures 9 and 10), suggesting that some students had quite consistent baseline FeNO values (e.g. Subject 5) and others had

Table 7. Pre-practice FeNO descriptive statistics by subject, study days pooled

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Mean
n	8	5	8	7	8	6	8	8	7	1	7	8	8	8	8	8	**7.1**
MEAN	4.7	7.0	7.2	4.2	3.3	5.4	14.2	21.5	18.0		14.6	5.6	8.4	13.7	7.3	23.0	**10.5**
GEO-MEAN	4.4	6.9	7.0	4.2	3.2	5.3	14.1	21.0	18.0		13.1	5.4	8.1	12.9	7.1	22.9	**10.2**
MEDIAN	4.7	7.6	6.7	4.5	3.2	5.1	14.0	22.7	17.6		11.1	6.1	8.1	12.5	6.4	22.9	**10.2**
MIN	2.5	4.8	5.3	3.1	2.1	4.0	12.3	14.3	15.8	35.2	7.5	3.0	6.0	9.1	5.0	19.3	
MAX	7.7	8.6	11.1	4.9	5.0	8.2	16.6	26.4	20.5	35.2	28.5	6.9	13.7	27.2	10.4	26.0	
RANGE	5.2	3.8	5.8	1.8	2.7	4.2	4.3	12.1	4.7		21.0	3.9	7.7	18.1	5.4	6.7	**7.2**
SD	1.7	1.5	1.9	0.65	1.0	1.5	1.3	4.4	1.8		7.7	1.3	2.4	5.8	1.9	2.3	**2.5**
SE	0.61	0.66	0.66	0.24	0.35	0.62	0.47	1.54	0.67		2.91	0.44	0.85	2.05	0.68	0.81	**0.85**
CV (%)	37.2	21.0	26.2	15.4	29.9	27.9	9.4	20.2	9.8		52.9	22.5	28.9	42.3	26.4	9.9	**25.3**
IQ-1	3.5	6.4	5.9	3.8	2.7	4.5	13.4	19.5	16.9		9.5	5.2	6.8	10.2	6.2	21.8	
IQ-3	5.4	7.8	7.7	4.7	3.9	5.7	14.7	24.5	19.4		17.9	6.3	8.5	14.1	8.4	24.7	
IQR	1.9	1.4	1.9	0.85	1.2	1.3	1.3	5.1	2.6		8.4	1.1	1.7	3.9	2.2	3.0	**2.5**

Figure 9. Mean pre-practice FeNO by subject

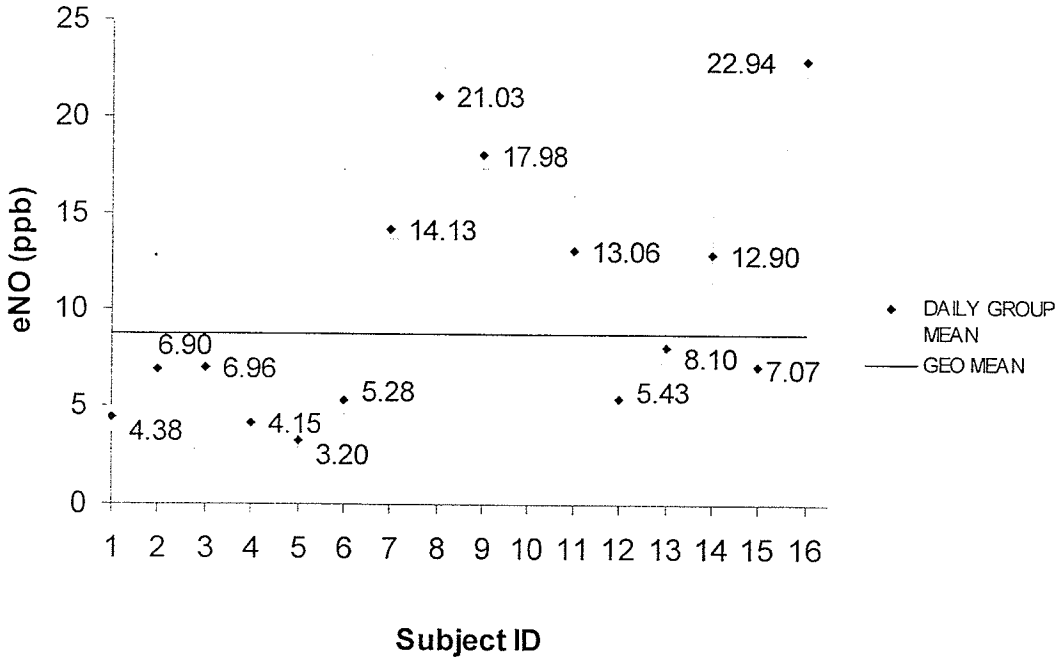
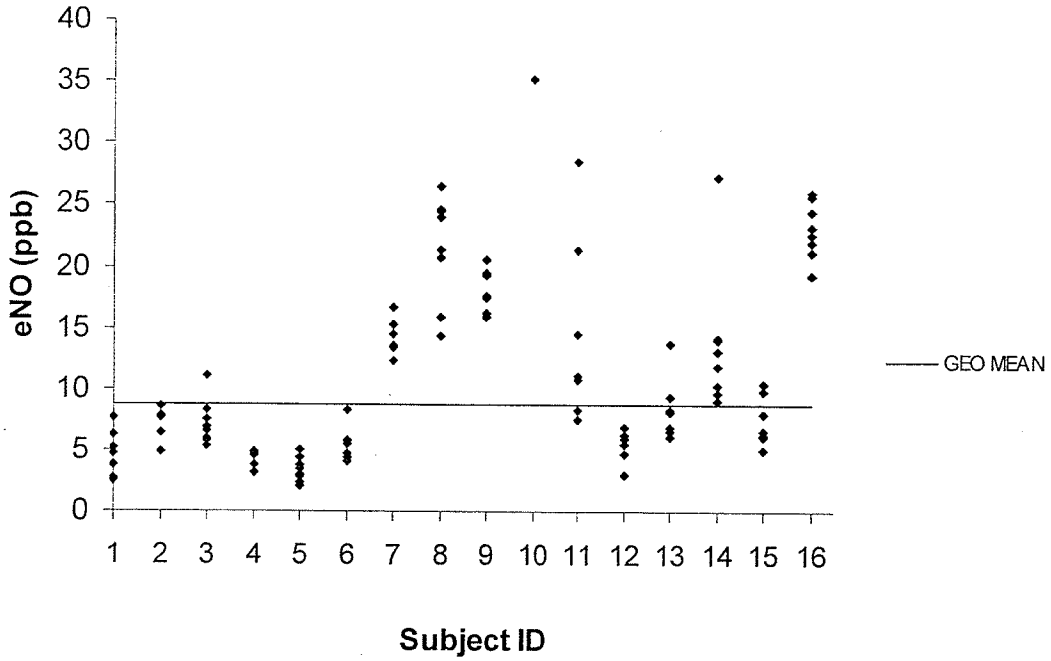


Figure 10. Pre-practice FeNO by subject



quite variable baseline FeNO values (e.g. Subject 11). Most subjects, though, had pre-practice FeNO values that were reasonably tightly distributed (Figure 10). This suggests an overall relatively low within-subject variation (range = 7.2; SD = 2.5; CV = 25.3%), and is expected with a series of repeated baseline FeNO measurements on a particular individual. These measures of distribution are similar to what is reported in the literature. Latzin et al. (2002) reported an intra-individual (or within-subject) CV of 25.9%, and Buchvald et al. (2005) reported a within-subject SD of 1.6.

By Study Day. On average, we obtained 14.1 pre-practice FeNO samples per day from 16 study subjects (Table 8). The mean (geometric) pre-practice FeNO value ranged from 7.46 to 9.91 ppb. As Figure 11 illustrates, these means are relatively consistent for each study day. Since these are pre-practice values (i.e. no exposure), we would expect these means to be similar each day despite the variable air quality (low 'between-day' variation). The variation was also quite consistent among days, but large (Figures 11 and 12) (SE = 2.0; range = 22.8), which is also what we would expect as the same individuals with variable baseline FeNO values are being measured on each study day (high 'within-day' variation). In contrast, there appears to be substantially less variation in within-subject FeNO (Figure 10). In other words, the large variation in this data set appears to be largely contributed by the between-subject variation, reflecting a variable baseline FeNO value between individuals. The estimation that 88% of the variation in the pre-practice FeNO dataset is explained by between-subject variation, through the SAS GLM procedure, further validates this observation. This substantial variability between subjects may limit FeNO's function as a biomarker since baseline values would have to be known before exposure effects could be determined.

Table 8. Pre-practice FeNO descriptive statistics by study day, subjects pooled

Study Day	n	MEAN (ARITHMATIC)	MEAN (GEOMETRIC)	MEDIAN	MIN	MAX	RANGE	SD	SE	CV (%)	IQ-1	IQ-3	IQR
3	14	12.2	9.3	8.9	2.7	35.2	32.5	9.6	2.6	78.1	5.7	16.7	11.0
4	15	9.6	7.7	7.6	2.3	21.4	19.1	6.2	1.6	64.9	6.2	12.3	6.1
5	13	11.3	9.6	8.2	4.3	26.4	22.1	7.4	2.1	65.4	6.4	14.5	8.1
6	14	9.1	7.5	6.7	2.1	22.0	19.9	6.0	1.6	66.4	5.3	12.5	7.2
7	14	10.7	9.0	9.0	3.7	22.6	18.9	6.2	1.7	58.2	5.3	15.2	9.9
8	14	10.7	8.3	6.4	2.8	24.4	21.6	7.6	2.0	71.1	4.6	16.0	11.4
9	14	11.6	9.0	8.5	3.5	28.5	25.0	8.7	2.3	75.3	4.9	13.9	8.9
10	15	12.0	9.9	8.6	3.8	27.2	23.4	7.6	2.0	63.6	5.9	16.1	10.2
MEAN	**14.1**	**10.9**	**8.8**	**8.0**			**22.8**	**7.4**	**2.0**	**67.9**			**9.1**

Figure 11. Mean pre-practice FeNO by study day

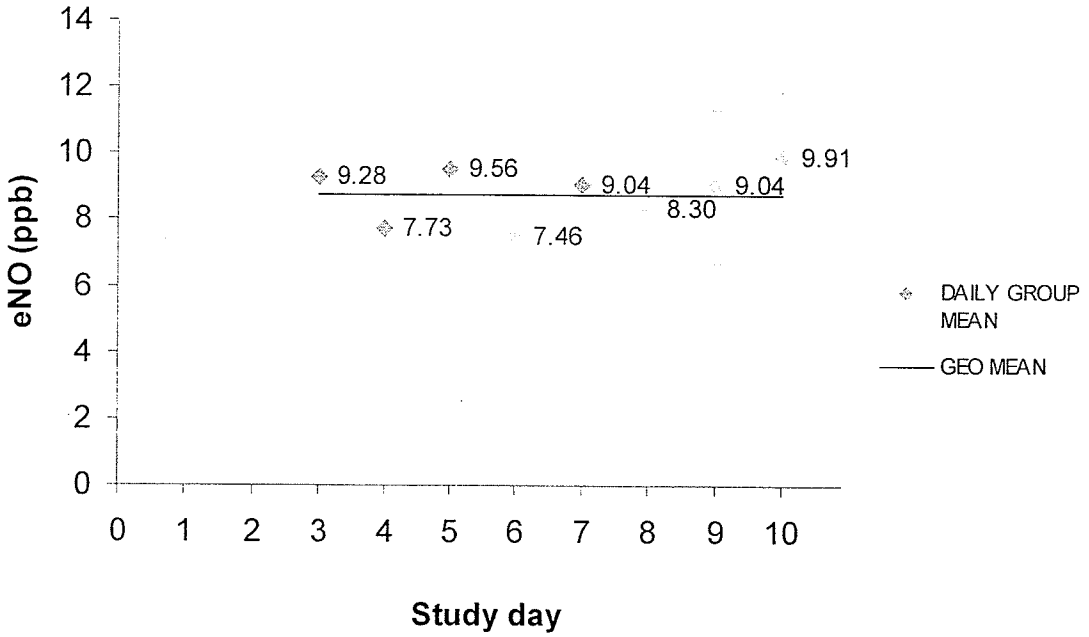
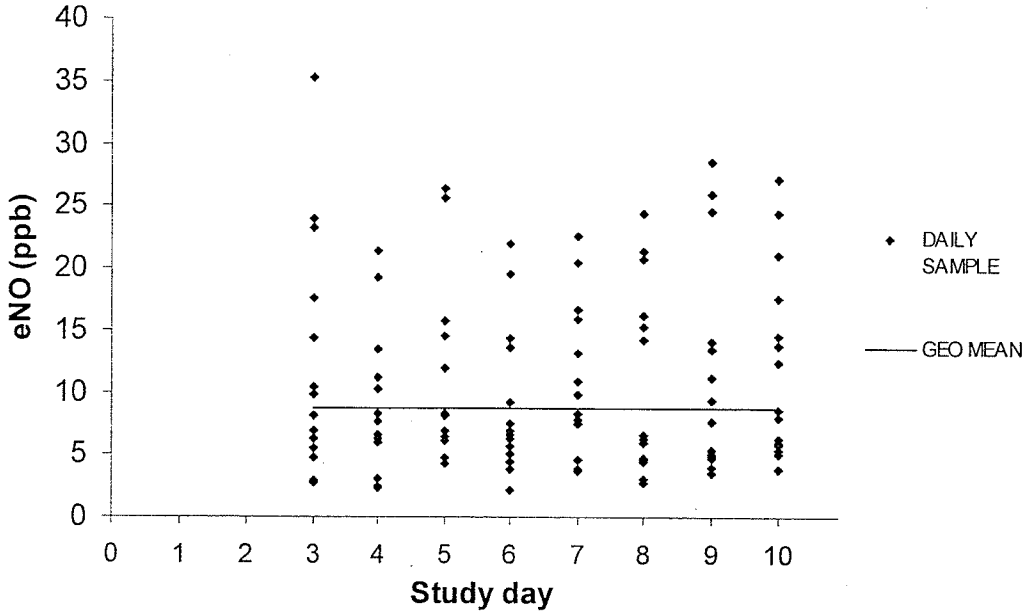


Figure 12. Pre-practice FeNO by study day



4.3.2 Reproducibility

The intraclass correlation coefficient (ICC) (95% CI) was 0.87 (0.75 – 0.96), and suggests that FeNO meets the criterion of acceptable reproducibility of ICC $\geq$ 0.6 (Faul et al. 1999). The within-subject coefficient of variation (CV) ranged from 9.4 - 52.9% (Table 7), and is similar to the range reported by Latzin et al. (2002). Although there were two participants with relatively large variation in their FeNO measurements (Subjects 11 and 14), the majority had FeNO measurements that were quite consistent. Although the average within-subject CV of 25% was similar to that reported by Latzin et al. (2002), it was higher than the CV of other lung function measurements in the CDC study (within-subject CV of pre-practice FEV and EBC being 4.2% and 16%, respectively- J. Ferdinands, personal communication, January 2005), and is considered a borderline desirable CV (CV desirable- <20%; CV undesirable>30%). The coefficient of reproducibility (expressed as the mean pooled SD) was 7.3. This is quite a bit higher than a larger study that reported a coefficient of reproducibility of 2.11 ppb (n=59, 675 estimations) for a sample that included both children and adults (Kharitonov et al. 2003). The discrepency may have been due, in part, to the fact that the CDC study was conducted in a field setting, in contrast to the above studies that were undertaken in a more "controlled" setting, where one might expect less variation.

4.4 Validity

Selected sample characteristics were examined to explore any differences in pre-practice FeNO values between groups (Table 9, Figure 13). Mean pre-practice FeNO was significantly different by age group (p=0.012) and in those of a non-white race compared to a white race (p=0.08; p<0.10 was considered significant due to the small sample size)

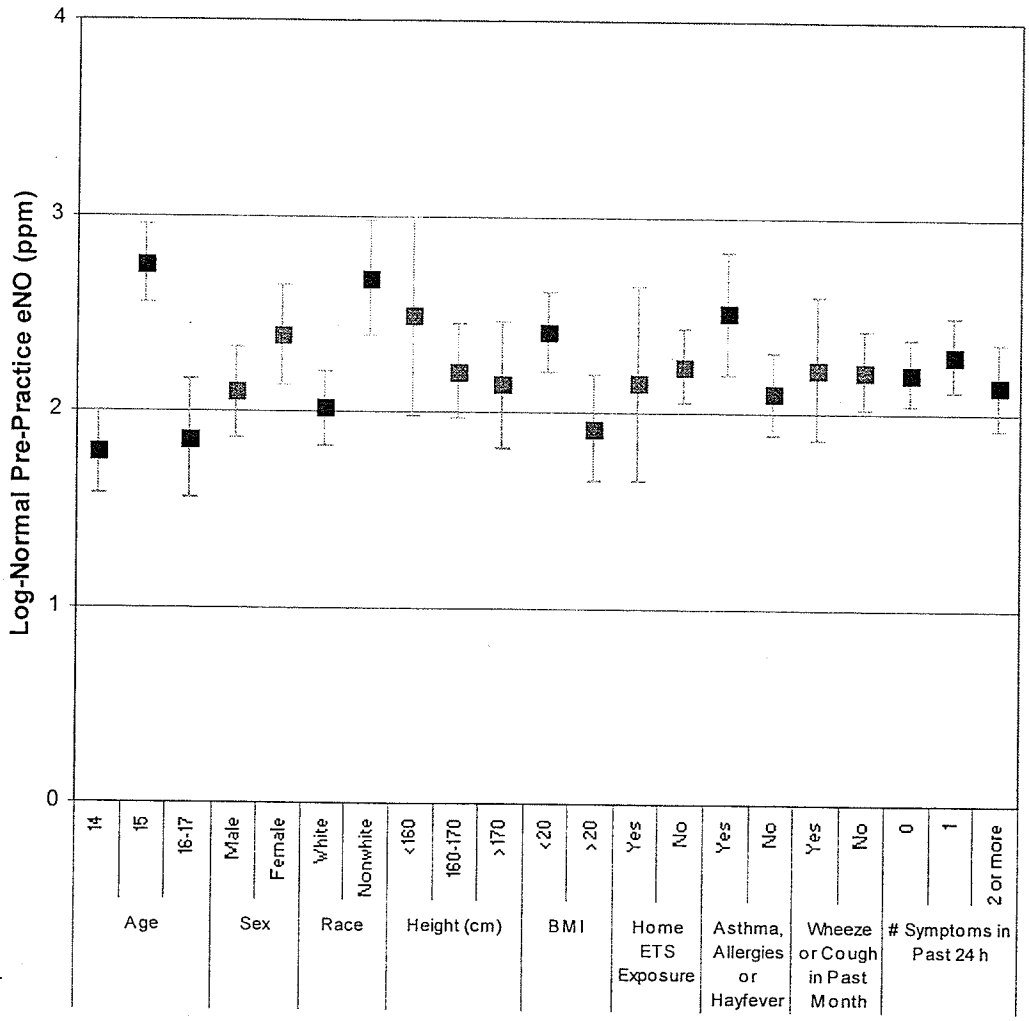
Table 9. Mean and standard error of pre-practice FeNO values by mixed or general linear models for selected sample characteristics

Variable	Number of Subjects	Obs (n)	MIXED Mean (SE) (*p value*)	GLM Mean (SE) (*p value*)
Age, yr				
14	6	47	1.79 (0.21)	1.80 (0.08)
15	7	45	2.75 (0.20)	2.70 (0.08)
16-17	3	21	1.86 (0.30)	1.85 (0.11)
			(0.012)	*(<0.0001)*
Sex				
Male	9	68	2.10 (0.23)	2.11 (0.08)
Female	7	45	2.39 (0.26)	2.26 (0.10)
			(0.42)	*(0.26)*
Race				
White	11	81	2.02 (0.19)	2.03 (0.07)
Nonwhite	5	32	2.68 (0.29)	2.52 (0.11)
			(0.08)	*(0.0004)*
Height, cm				
<160	2	16	2.49 (0.51)	2.49 (0.17)
160-170	9	63	2.21 (0.24)	2.06 (0.08)
>170	5	34	2.14 (0.32)	2.21 (0.11)
			(0.85)	*(0.066)*
BMI, kg/m^2				
<20	10	70	2.41 (0.21)	2.32 (0.08)
≥20	6	43	1.92 (0.27)	1.91 (0.09)
			(0.17)	*(0.0014)*
Asthma or Allergy or Hayfever				
Yes	5	33	2.51 (0.31)	2.32 (0.12)
No	11	80	2.10 (0.21)	2.11 (0.07)
			(0.28)	*(0.13)*
Wheeze or cough in past month				
Yes	4	25	2.23 (0.36)	1.91 (0.13)
No	12	88	2.22 (0.20)	2.24 (0.07)
			(0.98)	*(0.03)*
Number of symptoms in past 24 hours*				
0	13	92	2.21 (0.17)	2.18 (0.07)
1	2	16	2.30 (0.19)	2.18 (0.16)
2 or more	1	5	2.14 (0.22)	1.92 (0.30)
			(0.58)	*(0.71)*

* Symptoms included wheeze, cough, scratchy or itchy throat, runny nose, sneezing, and watery eyes

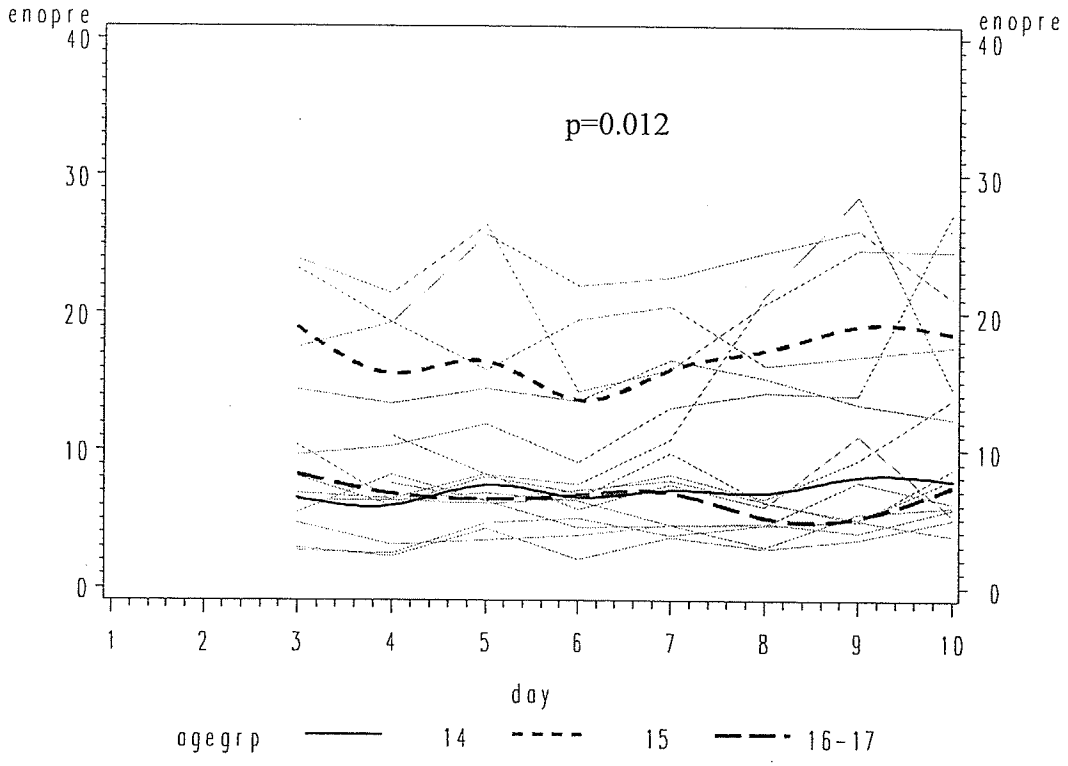
Variable	Number of Subjects	Obs (n)	MIXED Mean (SE) (*p value*)	GLM Mean (SE) (*p value*)
Home ETS exposure				
Yes	2	16	2.15 (0.50)	2.15 (0.17)
No	14	97	2.24 (0.19)	2.17 (0.07)
			(*0.87*)	(*0.89*)

Figure 13. Mean pre-practice FeNO by selected sample characteristics



by the MIXED procedure in SAS. The significant difference between these means becomes more pronounced with the GLM procedure (standard linear model), due to the fact that this procedure counts each observation as an independent measurement (i.e. does not account for correlation between measurements from the same individual). The very significant difference found between age groups was only somewhat consistent with the literature. There is some suggestion that in children, FeNO increases with age (Franklin et al. 1999, Latzin et al. 2002, Buchvald et al. 2005). If one ignores the 15-year group, this general pattern can be seen. However, the significant difference between these groups is largely contributed by individuals with higher than average FeNO values in the 15-year group, and is likely a product of analyzing such a small sample (Figure 14).

Figure 14. Pre-practice FeNO by study day with splines for age group



Although there is a paucity of literature regarding whether or not one would expect a difference in baseline FeNO values by race, what literature exists suggests that those of African and Asian descent have substantially higher FeNO values (Kovesi et al. 2007; Buchvald et al. 2005), and a reasonable hypothesis explaining why those of African-American ancestry might have higher baseline FeNO values has been put forth (Togashi et al. 1997). These observations are consistent with the data from this study (Figure 15 and 16). However, when the nonwhite race is further broken down to include those of Asian descent (n=1), the difference between the groups no longer becomes significant (p=0.16) (Figure 16). These data should be interpreted with caution given the small sample size overall and within each race.

Figure 15. Pre-practice FeNO by study day with splines for race (2 categories)

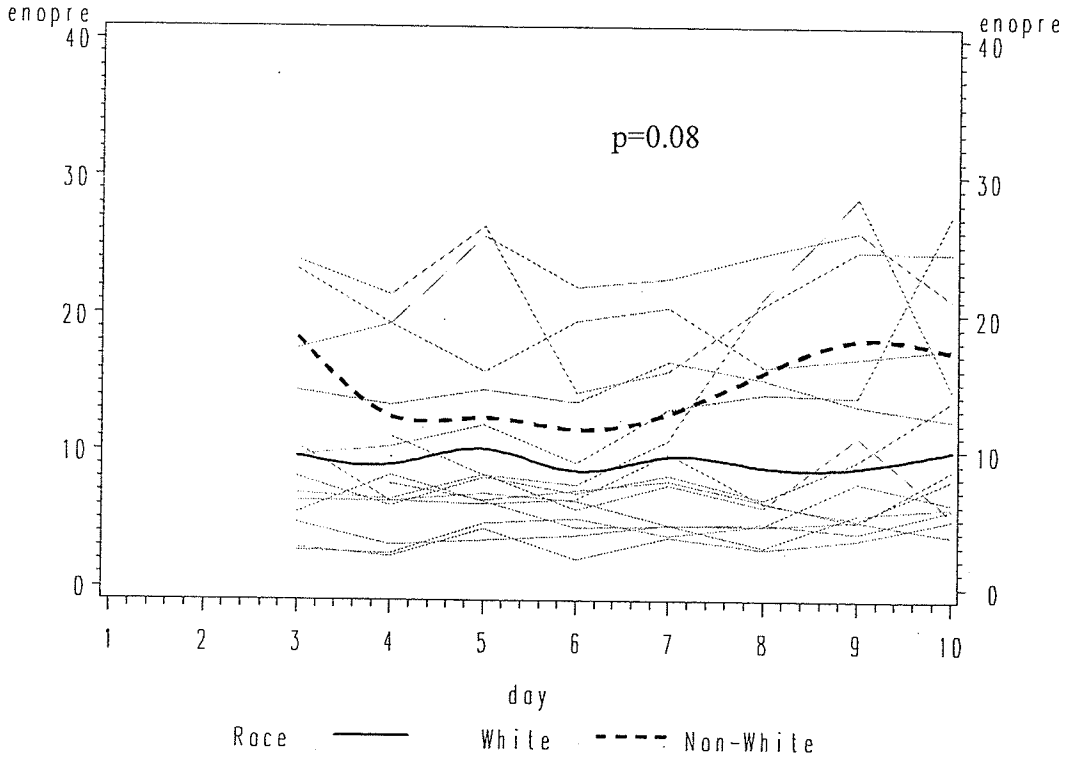
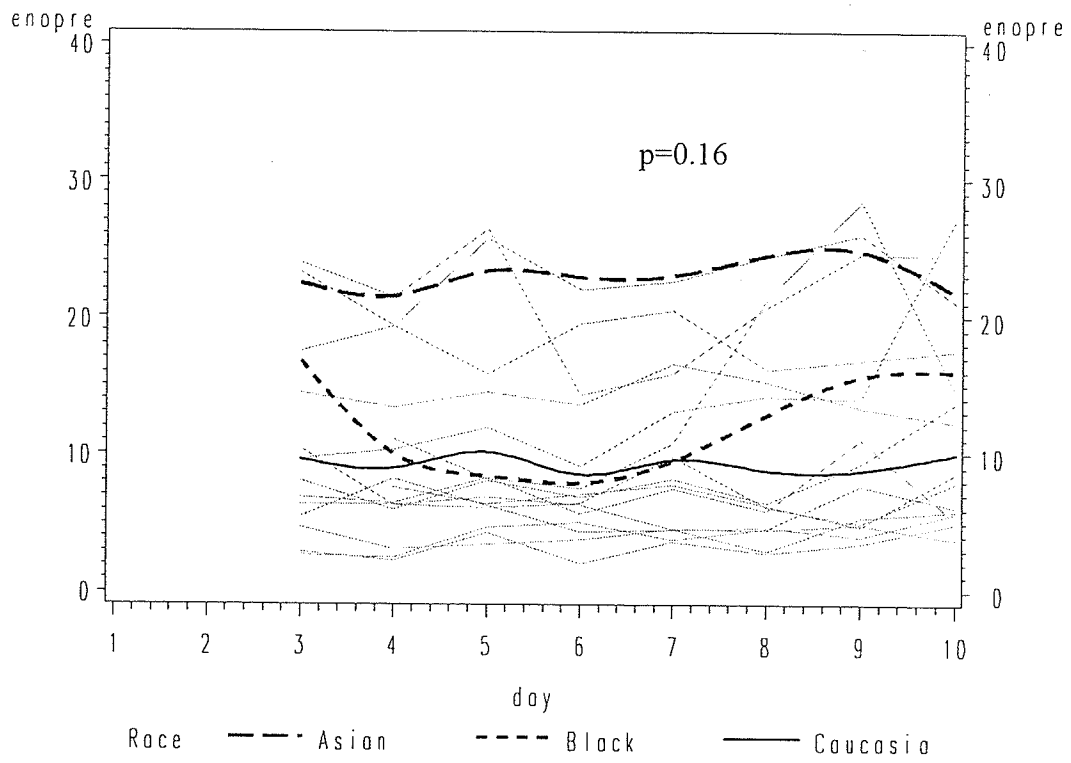


Figure 16. Pre-practice FeNO by study day with splines for race (3 categories)



Although not found to be significant, there were several sample characteristics that showed patterns that were consistent with the literature. Mean pre-practice FeNO was higher in those with a BMI<20 compared to those with a BMI>20; lower with those exposed to home environmental tobacco smoke (ETS) compared to those who weren't; higher in those with asthma, allergies or hayfever compared to those without these conditions; and higher in those with a wheeze or cough in the past month compared to those without these symptoms. Other characteristics had a difference in mean pre-practice FeNO values that were in a direction contrary to what would be expected. For example, mean pre-practice FeNO was expected to be higher in males compared to females, but instead the opposite effect was seen. As well, FeNO decreased with height in our sample of athletes, and was expected to increase. Both of these contradictory trends could be at least partially attributable to a significant proportion of asthmatics and Afrrican-American

athletes in our study sample (who have higher baseline FeNO values) who were female and shorter than average. Similarly, it was thought that those with more symptoms in the past 24 hours (suggesting some underlying inflammation of the respiratory tract) might have higher FeNO values; however, this pattern was not seen. Appendix E illustrates these non-significant spline graphs.

4.5 Responsiveness

The following section outlines the results of the multiple regression analysis that was undertaken, starting with univariate analyses of the outcome and predictor variables. The selection of control variables will then be described. Finally, the results of the linear mixed models that were built will be reviewed.

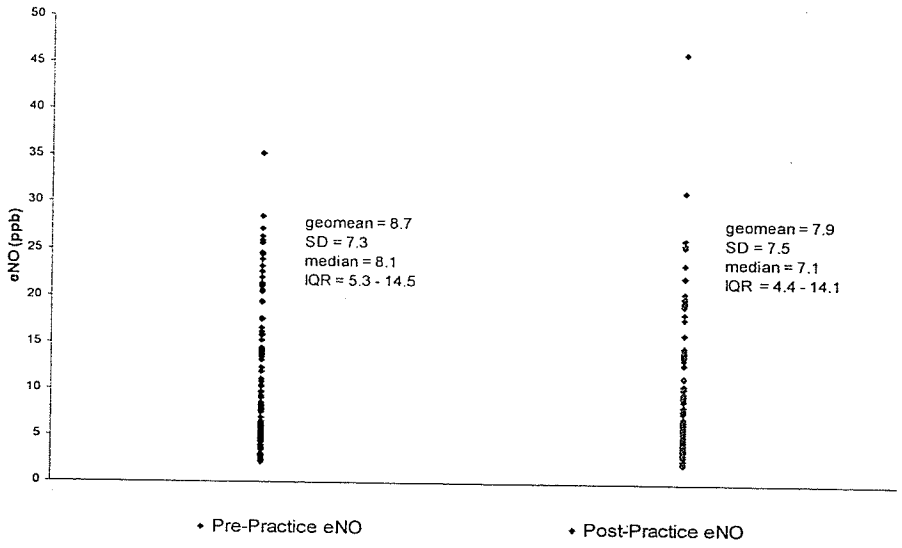
4.5.1 Outcome Variable

Univariate analyses for post-practice FeNO are presented in Appendix F. Post-practice FeNO is log-normally distributed (Figures 1 and 2, Appendix F). When individual graphs of participants' post-practice FeNO are examined, with yellow and orange alert days indicated, no apparent relationship to the level of air pollution can be discerned (Figure 3, Appendix F). Upon merging the individual graphs represented in Figure 3, a spline suggests that there is no trend corresponding with the orange alert days (Figure 4, Appendix F).

Mean (geometric) post-practice FeNO was 7.9; range was 2.1 – 46.2; IQR was 4.4 – 14.1 (Table 1, Appendix F). Exhaled nitric oxide decreased significantly after exercise ($p<0.001$ by paired *t*-test) (Figure 17). This finding was not unexpected (St. Croix et al.

1999, Kippelen et al. 2002, Verges et al. 2005, Verges et al. 2006, Mantione et al. 2007); however, it was hypothesized that the effect of exposure to ambient air pollution might cause FeNO to increase, or at least remain relatively unchanged with the known exercise effect. Since FeNO decreased significantly, it is presumed that this exercise effect is dominant.

Figure 17. Pre-practice vs. post-practice FeNO



Compared to pre-practice FeNO, the overall variation in post-practice FeNO increased slightly (SD = 7.3, CV = 67.4% for pre-practice FeNO; SD = 7.5, CV = 73.9% for post-practice FeNO). This increased variation in post-practice FeNO is reflected both in the average within-subject variation (SD = 3.1, CV = 30.5% for post-practice FeNO; SD = 2.5; CV = 25.3% for pre-practice FeNO) (Table 2, Figures 5-6, Appendix F), as well as the average within-day variation (SD = 7.6; CV = 74.7% for post-practice FeNO; SD = 7.4, CV = 67.9% for pre-practice FeNO) (Table 3, Figures 7-8, Appendix F). These observations suggest that the variation in air quality days (only reflected in the post-practice FeNO measurements) may have had a contribution to this increased variation.

4.5.2 *Predictor Variables*

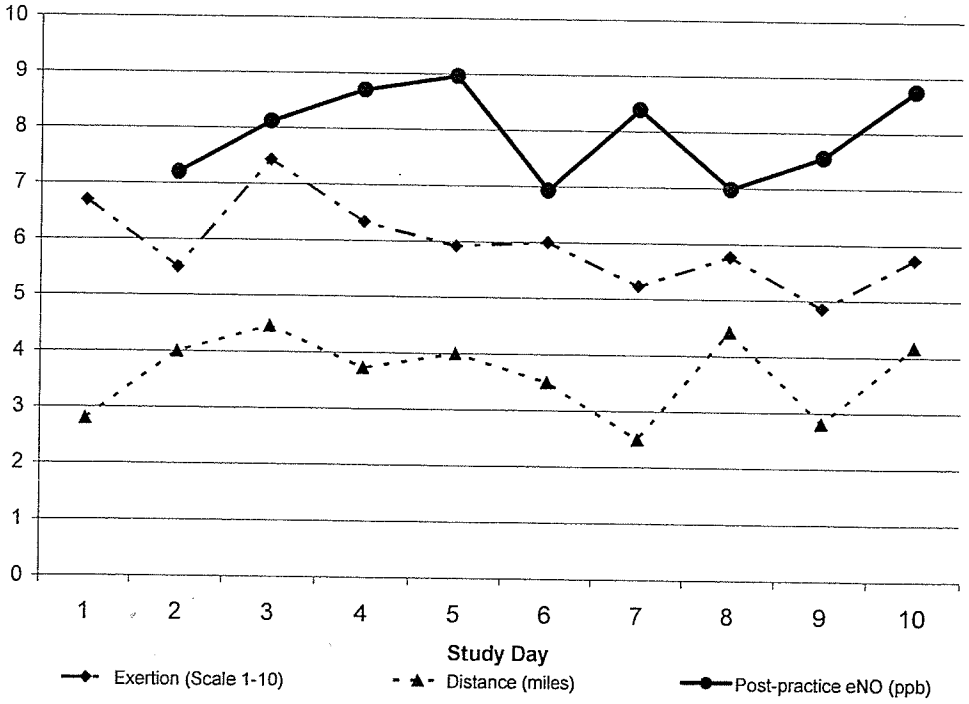
The air quality data extracted from the Georgia Department of Natural Resources' Ambient Monitoring Program database are listed in Appendix G. Four of 10 study days were air quality (orange) alert days, with two days each triggered by high ozone and high PM levels, respectively (Table 3, Appendix G).

Univariate analyses for air quality measures are presented in Appendix H. Graphs of the extracted air quality measures by study day are presented in Figures 1-7 (Appendix H), and their descriptive statistics are shown in Table 1 (Appendix H). Figure 8 (Appendix H) illustrates the relationship between ozone, PM and post-practice FeNO. Based on the calculated Pearson correlation coefficients (Table 1, Appendix H), it was determined that ambient maximum ozone concentration (1-hour average) and PM measured at 5 pm were the air quality measures most correlated to post-practice FeNO, and these variables were used in the regression models. Mean (±SD) ambient maximum ozone concentration (1-hour average) was 0.071 (0.019) ppm, and median (interquartile range, IQR) was 0.066 (0.058-0.073) ppm. Mean (±SD) $PM_{2.5}$ measured at 5 pm was 28.2 (9.7) $\mu g/m^3$, and median (IQR) was 24.1 (23.0 – 35.8) $\mu g/m^3$. Pearson correlation coefficients were also calculated for ambient maximum ozone concentration (1-hour average) and PM measured at 5 pm lagged by one or two days (Table 2, Appendix H). These measures without a lag had the highest correlation coefficients ($r = 0.49$ and 0.58 for ozone and PM, respectively). Since scatterplots (Figures 9-12, Appendix H) revealed that log-normal transformation of these data was appropriate, log-normal transformed Pearson correlation coefficients were also calculated ($r = 0.51$ and 0.59 for ozone and PM, respectively) (Table 3, Appendix H).

4.5.3 Control Variables

Since pre-practice FeNO was highly correlated with post-practice FeNO (r = 0.94), it was included as a control variable in the regression models. Based on the results of the exploratory analyses examining groups of interest, only gender was considered significant (p=0.08), and thus was also included as a control variable. Potential rival hypotheses, including self-reported distance, duration and exertion were not found to be highly correlated with post-practice FeNO (r = −0.18, −0.14, 0.10, respectively) (Figure 18). Self-reported exertion, the most proximate potential predictor of post-practice FeNO, was added to the baseline control model as a potential rival hypothesis variable to see how well the outcome could be explained. However, it was not found to be a significant predictor of post-practice FeNO (p=0.18), and was not included in the final regression models.

Figure 18. Relationship between mean self-reported exertion, mean distance run, mean number of symptoms in the past 24 hours and mean post-practice FeNO by study day



4.5.4 Regression Models

In choosing a correlation structure for the mixed linear regression models, a first-order autoregressive structure was thought to be most appropriate since post-practice FeNO measures would likely be decreasingly correlated the further apart the measures were. Further, several correlation structures were examined in building the mixed linear regression models. However, results differed little between structures. No random effects were assumed. Same-day maximum ozone (1-hr avg) (ppb) and $PM_{2.5}$ at 5pm (ug/m^3) were the primary predictors of interest, and were log-transformed for statistical analysis. Since ozone and $PM_{2.5}$ concentrations were highly correlated ($r = 0.85$), separate models were run for ozone and $PM_{2.5}$.

A statistically significant association between post-practice FeNO (natural log-transformed) and 1-day lagged maximum ozone (1-hr avg.) concentration (natural log-transformed) was observed ($p<0.01$), controlling for race and pre-practice FeNO (Model 3, Table 10). Similarly, a statistically significant association between post-practice FeNO (natural log-transformed) and 1-day lagged $PM_{2.5}$ at 5pm (natural log-transformed) was observed, controlling for the same factors (Model 3, Table 11). In other words, the post-practice FeNO measurements on a particular day appear to be influenced by the ozone or particulate concentrations on the previous day.

Results from the literature also suggest that the effects of air pollution on FeNO might not be immediate. Other studies have pointed to possible cumulative and lag effects on FeNO in non-smoking elderly adults (Adamkiewicz et al. 2004), healthy schoolchildren (Fischer et al. 2002, Steerenberg et al. 2001), and more immediate effects (previous several hours)

Table 10. Estimated regression coefficients from mixed linear models† with post-practice FeNO as outcome and 1-hour ozone concentration as ambient air pollutant predictor

	Model 1	**Model 2**	**Model 3**	**Model 4**
Outcome variable	Raw post-practice FeNO (ppb)	Ln post-practice FeNO (ln ppb)	Ln post-practice FeNO (ln ppb)	Ln post-practice FeNO (ln ppb)
Lag time	Same day	Same day	1-day lag	2-day lag
AIC criterion	503.9	27.8	22.2	28.9
Estimated regression coefficient, β (standard error):				
Race				
Nonwhite	-0.79 (0.48)	-0.11 (0.06)*	-0.10 (0.06)*	-0.11 (0.06)*
White	Ref	Ref	Ref	
Raw pre-practice FeNO (ppb)	0.86(0.03)****	----	----	----
Ln pre-practice FeNO (ln ppb)	----	0.91(0.04)****	0.91(0.04)****	0.91(0.04)****
Same-day ln max ozone (ln ppb)	1.26 (0.90)	0.16 (0.11)	----	----
1-day lagged ln max ozone (ln ppb)	----	----	0.26 (0.09)***	----
2-day lagged ln max ozone (ln ppb)	----	----	----	0.11 (0.10)

† All models use a first-order autoregressive correlation structure

* p<0.10

** p<0.05

*** p<0.01

**** p<0.001

Table 11. Estimated regression coefficients from mixed linear models† with post-practice FeNO as outcome and $PM_{2.5}$ concentration as ambient air pollutant predictor

	Model 1	**Model 2**	**Model 4**	**Model 6**
Outcome variable	Raw post-practice FeNO (ppb)	Ln post-practice FeNO (ln ppb)	Ln post-practice FeNO (ln ppb)	Ln post-practice FeNO (ln ppb)
Lag time	Same day	Same day	1 day lag	2 day lag
AIC criterion	505.0	28.8	19.9	30.1
Estimated regression coefficient, β (standard error):				
Race				
Nonwhite	-0.81 (0.48)	-0.11 (0.06)*	-0.10 (0.06)*	-0.11 (0.06)*
White	Ref	Ref	Ref	Ref
Raw pre-practice FeNO (ppb)	0.85(0.03)****	----	----	----
Ln pre-practice FeNO (ln ppb)	----	0.91(0.04)****	0.92(0.04)****	0.91(0.04)****
Same-day ln $PM_{2.5}$ at 5pm (ln $\mu g/m^3$)	0.91 (0.73)	0.11 (0.08)	----	----
1-day lagged ln $PM_{2.5}$ at 5pm (ln $\mu g/m^3$)	----	----	0.27 (0.08)***	----
2-day lagged ln $PM_{2.5}$ at 5pm (ln $\mu g/m^3$)	----	----	----	0.06 (0.06)

† All models use a first-order autoregressive correlation structure

* $p<0.10$

** $p<0.05$

*** $p<0.01$

**** $p<0.001$

on FeNO in healthy adults (Van Amsterdam et al. 1999) and asthmatic schoolchildren (Mar et al. 2005, Delfino et al. 2006). These observations may reflect acute-phase and late-phase responses (Hamid et al. 2003). Acute-phase inflammation, from an early release of mediators by mast and other cells, could signal proinflammatory cytokines (e.g.IL-6) that control a cascade of events, including the production of acute phase proteins as well as the induction of nitric oxide synthetase (NOS) (Gabay and Kushner 1999). This could be followed by a late-phase response peaking a few hours later and characterized by lymphocyte activation and infiltration that may remain raised for hours to days (Hamid et al. 2003).

Although the estimates of effect were small (<2.8 ppb increase in FeNO per 10 unit increase of pollutant) (Table 12), inasmuch as FeNO is a marker of airway inflammation, this would suggest that air pollution increases inflammation. Further, it is possible that these estimates of effect size were dampened by the exercise effect on post-practice FeNO. It is impossible to know whether these estimates of effect are clinically relevant or not. However, these small effect sizes were similar to that found in the literature. Koenig et al. (2003) reported an approximately 4 ppb increase in FeNO per 10 ug/m^3 ambient $PM_{2.5}$; Mar et al. (2005) reported the overall effect of a prolonged exposure to $PM_{2.5}$ (48 hours) was 7 ppm increase per 10 ug/m^3 $PM_{2.5}$. Delfino et al. (2006) reported effect sizes < 2.5 ppb per 24 ug/m^3 ambient $PM_{2.5}$. Similar unit comparisons for ozone are not available, but Nickmilder et al. (2007) also reported a small average increase in FeNO (estimated as <2 ppb, Figure 4) in his study population for ozone concentrations approximately 63 ppb (the average ozone concentration in this study was 71 ppb). Interestingly, Nickmilder et al. (2007) found that at approximately 84 ppb, the average

Table 12. Association between post-practice FeNO and air pollutant, controlling for pre-practice FeNO and race

Exposure	Change in FeNO (ppb) (95% CI) per 10 unit increase in pollutant	p-value
Maximum Ozone (ppb)		
Lag 0	1.63 (-0.43 to 3.69)	0.13
Lag 1	2.64 (0.85 to 4.43)	0.0048
Lag 2	1.11 (-0.93 to 3.15)	0.29
$PM_{2.5}$ at 5 pm (ug/m^3)		
Lag 0	1.12 (-0.51 to 2.75)	0.18
Lag 1	2.74 (1.12 to 4.36)	0.0013
Lag 2	0.60 (-0.58 to 1.78)	0.32

increase in FeNO jumps to about 20 ppb, suggesting that a threshold between these values where airway inflammation becomes more pronounced.

Other air quality variables (ozone at 5 pm and maximum $PM_{2.5}$) were substituted in the models to see if the results differed. For ozone, the levels of significance remained the same and effect sizes did not substantially vary. However, for PM results did vary somewhat. Interestingly, significance was reached with a Lag 0 model, as well as with a Lag 1 model (Table 13).

Table 13. Association between post-practice FeNO and maximum $PM_{2.5}$, controlling for pre-practice FeNO and race

Exposure	Change in FeNO (ppb) (95% CI) per 10 unit increase in pollutant	p-value
Maximum $PM_{2.5}$ (ug/m^3)		
Lag 0	2.38 (2.22 to 2.54)	0.0049
Lag 1	2.18 (2.01 to 2.35)	0.015
Lag 2	0.31 (0.20 to 0.42)	0.59

No other significant associations were observed with same-day maximum ozone (1-hr avg.) or same-day $PM_{2.5}$ at 5pm controlling for race and pre-practice FeNO, or when ozone and $PM_{2.5}$ concentrations were lagged by 2 days (Tables 10 and 11, respectively). Adjusted post-practice FeNO was lower among nonwhite subjects compared to white subjects ($p<0.10$ in both ozone and particulate models using the natural log-transformed post-practice FeNO only). As expected, post-practice FeNO was positively associated with pre-practice FeNO ($p<0.001$ in all models).

CHAPTER 5

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.1 Summary

In this study, the utility of FeNO as a biomarker was evaluated. In doing so, the *reliability*, *validity* and *responsiveness* of FeNO were examined. The reliability of the FeNO measurements was evaluated through examining the variability in the FeNO measurements, as well as their reproducibility. There was substantial variability in the FeNO data, with most of this variation (88%) explained by between-subject variation. Some measures of reproducibility were quite acceptable. The intraclass correlation coefficient (ICC) was 0.87, and suggests that FeNO meets the criterion of acceptable reproducibility of ICC$\geq$ 0.6. The average within-subject coefficient of variation (CV) of 25%, though, is only considered a borderline desirable CV. As well, the coefficient of reproducibility (expressed as the mean pooled SD) of 7.3 was much higher than that reported in the literature.

Next, an assessment of construct validity was undertaken, through 1) a comparison of baseline FeNO measurements in this study; and 2) a comparison of group differences in the baseline FeNO study data to those described in the literature. The mean pre-practice FeNO in this sample is somewhat lower that what is reported in the literature (8.3 ppb vs. 13.7 ppb in one study examining the same age group). A variety of factors are known to cause discrepancies in the results between studies, including the use of different types of analyzers; however, in this study it could likely be attributed to, at least in part, the small sample size. Mean pre-practice FeNO was significantly different by age group and those

of a non-white race compared to a white race, and these observations are consistent with the literature. Although not found to be statistically significant, body mass index, those with asthma, allergies, hayfever, wheeze or cough in the last month, and exposure to home environmental tobacco smoke all showed patterns that were consistent with the literature. A few characteristics, including gender, height and those experiencing symptoms in the past 24 hours, showed a difference in mean pre-practice FeNO values that were in a direction contrary to what would be expected.

Finally, the responsiveness of FeNO was evaluated by examining the association of FeNO with ambient ozone and $PM_{2.5}$ concentrations among the 16 participants exposed to these air pollutants during vigorous outdoor exercise. Of note, FeNO decreased significantly with exercise ($p<0.001$), an effect which has been reported previously in the literature. It is likely that the exercise effect is larger than the effects that air pollutants might have had on FeNO. However, a statistically significant positive association between post-practice FeNO (natural log-transformed) and 1-day lagged maximum ozone (1-hr avg.) concentration (natural log-transformed) was observed ($p<0.01$), controlling for race and pre-practice FeNO. Similarly, a statistically significant positive association between post-practice FeNO (natural log-transformed) and 1-day lagged $PM_{2.5}$ at 5pm (natural log-transformed) was observed, controlling for the same factors. These results suggest that the post-practice FeNO measurements on a particular day appear to be influenced by the ozone and particulate concentrations on the previous day, and this observation is consistent with results found in the literature. When other air quality variables (ozone at 5 pm and maximum $PM_{2.5}$) were substituted in the models, same-day and 1-day lagged maximum $PM_{2.5}$ were also found to be a significant predictors of post-

practice FeNO. No other significant associations were observed with same-day maximum ozone (1-hr avg.) or same-day $PM_{2.5}$ at 5pm controlling for race and pre-practice FeNO, or when ozone and $PM_{2.5}$ concentrations were lagged by 2 days. Although the estimates of effect were small (<2.8 ppb FeNO per 10 unit increase of pollutant), they were similar to that found in the literature.

5.2 Conclusions

In the evaluation of FeNO as a biomarker of effect, several conclusions can be drawn. There are some limitations to the use of FeNO as a biomarker of effect. First, the substantial variability in FeNO between subjects may limit FeNO's function as a biomarker since baseline values would have to be known before exposure effects could be determined. One measure of reproducibility, the average within-subject coefficient of variation, was considered only borderline desirable and was higher than those of other lung inflammation/function measurements in the CDC study. Similarly, the coefficient of reproducibility was lower than that referenced in a similar study. As well, in the assessment of construct validity of the FeNO measurements, a comparison of baseline FeNO values to those in the literature suggested that the mean baseline FeNO value in this study was lower than that reported in the literature. This could be at least partially attributed to the small sample size in this study, but may also highlight the fact that FeNO measured with different analyzers could produce different results (and may continue to cause some confusion with respect to reference values for FeNO).

However, several other observations from this study indicate that FeNO could be quite useful as a biomarker in the field setting. The high intraclass correlation coefficient

suggests that FeNO meets the criterion of acceptable reproducibility. Further, in another assessment of the construct validity of FeNO as a measurement, a comparison of group differences in the baseline FeNO study data was compared to those group differences in the literature. The results of this assessment were favorable with most group differences in this study being consistent with the literature. Finally, as an assessment of the responsiveness of FeNO to ambient air pollutants, multiple regression analysis was undertaken. The results of this analysis revealed that, in this adolescent group of practicing athletes, exposure to ambient ozone and $PM_{2.5}$ is associated with an increase in FeNO, a marker of pulmonary inflammation. There appears to be a 1–day lag effect to this relationship. Although the estimates of effect are small, the criterion of responsiveness requires asking whether the measure can detect differences in outcomes that are important, even if those differences are small. Caution is needed in attempting to generalize these results, though, as this was a small convenience sample of healthy student athletes with low power.

In conclusion, although there are some limitations to using FeNO as a biomarker of effect, this study found evidence to suggest that FeNO has potential as a reasonably reliable, valid and responsive measure that can detect pulmonary inflammation as a result of exposure to ambient air pollution. The use of a sensitive biomarker of effect, such as FeNO, may prove to be a useful tool to identify subjects or groups at most risk from the toxic effects of air pollutants and for establishing unacceptable exposure levels of these pollutants.

5.3 Recommendations

Further field research with a larger sample size is needed to confirm FeNO's utility as a biomarker, as well as to explore the findings from this study suggesting that air pollution increases lung inflammation. To attempt to 'tease' out the individual effects of ozone and PM, this research should consider the time of year and location. This study somewhat selected for ozone effects, being more amenable to public health intervention than PM, but PM was also relatively high in this location. A larger CDC study to further examine FeNO's utility as a biomarker of respiratory health effects as a result of ozone exposure should consider a suburban location, such as Conyers, where ozone is known to be high; however, a location where PM is known to be relatively low would be optimal. The merits of offline FeNO sampling should be explored, considering the poor portability of the online FeNO analyzer.

Further, although not addressed by the American Thoracic Society guidelines (2005) at this time, this study suggests that ethnicity should be recorded at the time of FeNO measurement.

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Appendix A

Baseline Questionnaire

Label

Baseline Questionnaire

Participant # _____
(Leave blank)

Date (mm/dd/yyyy) ___/___/____

Thank you for volunteering to participate in our study. Please tell us some basic information about yourself. This information will help us determine if we can include you in our study. If you need help answering a question, please ask your parent or legal guardian to help you.

1. What is your month and year of birth? _____ (month) _____ (year)

2. What is your gender?
   - ☐ Male
   - ☐ Female

3. What is your height without shoes? _____ feet and _____ inches.

4. What is your weight? _____ pounds.

5. Are you of Hispanic or Latino ancestry?
   - ☐ Yes
   - ☐ No
   - ☐ Don't know

6. What is your race (check all that apply)?
   - ☐ Asian
   - ☐ Black or African American
   - ☐ White
   - ☐ Native Hawaiian/Other Pacific Islander
   - ☐ American Indian/Alaskan Native
   - ☐ Other _____

7. Does any one who lives in your home smoke tobacco in your home?
   - ☐ Yes
   - ☐ No
   - ☐ Don't know

Baseline Health Status

Please tell us some information about your health and medical history. (If you are the parent or legal guardian of the child who is participating in our study, please provide the information for the child if he/she is unable to answer some of the questions).

8. Has a doctor ever told you that you had any of the following health conditions, and if so, do you still have it?

	Doctor told you?			Do you still have it?		
	No	Yes		No	Yes	Don't know
Asthma	☐	☐	→	☐	☐	☐
Hay fever	☐	☐	→	☐	☐	☐
Eczema	☐	☐	→	☐	☐	☐
Allergy to latex rubber	☐	☐	→	☐	☐	☐
Allergies to other things	☐	☐	→	☐	☐	☐

If other, allergies to what? _____

11. Have you ever had shortness of breath when you are near animals?

☐Yes
☐No
☐Don't know

12. Have you ever had an itchy or stuffy nose or sneezing when you are near animals?

☐Yes
☐No
☐Don't know

13. *During the past month (30 days)*, how often did you have the following symptoms:

a. Cough
- ☐ Not at any time
- ☐ Less than once a week
- ☐ Once or twice a week
- ☐ More than two times a week, but not everyday
- ☐ Everyday

b. Wheezing or whistling sound in the chest
- ☐ Not at any time
- ☐ Less than once a week
- ☐ Once or twice a week
- ☐ More than two times a week, but not everyday
- ☐ Everyday

c. Shortness of breath
- ☐ Not at any time
- ☐ Less than once a week
- ☐ Once or twice a week
- ☐ More than two times a week, but not everyday
- ☐ Everyday

d. Chest tightness or chest pain
- ☐ Not at any time
- ☐ Less than once a week
- ☐ Once or twice a week
- ☐ More than two times a week, but not everyday
- ☐ Everyday

14. *During the past month (30 days)*, have you had a cold or other respiratory infection?

- ☐ Yes
- ☐ No (Please skip ahead to "Thank you")
- ☐ Don't know (Please skip ahead to "Thank you")

If yes, please tell us

a. About how long did the cold/respiratory infection last? _____ days

b. About how long ago did the cold/respiratory infection end?_____ days ago

c. Did you have a cough?

- ☐ Yes
- ☐ No
- ☐ Don't know

d. Did you have a runny nose?

- ☐ Yes
- ☐ No
- ☐ Don't know

e. Did you see a doctor or other health professional because of this?

- ☐ Yes
- ☐ No

15. Practice is optional for you on Saturday, August 21st and Saturday, August 28th. Do you think you will come to those practice sessions?

- ☐ Will probably come to at least one
- ☐ Will probably come to both
- ☐ Probably won't come to either one
- ☐ Don't know yet

Thank you for providing this information. Please put it in the envelope provided and give it to a study investigator or bring it to your next practice.

Appendix B

Supplementary Short Questionnaire

For those athletes that are interested in this study and returned completed study packages, please take a few minutes to answer a few additional questions.

All of your answers are confidential. We will not share your information with your parents, teachers, coaches, or anyone else.

Name _____ Date (mm/dd/yyyy) ____/____/_____

1. How old are you? _____ years

2. Are you a smoker?

☐Yes
☐No

If yes, and you are interested in being in this study, would you be willing to not smoke from when school ends until the end of the second set of tests after practice (about 2-3 hours) during the two week study period?

☐Yes

☐No

3. Have you smoked cigarettes in the past 30 days?

☐Yes
☐No

If yes, about how many cigarettes have you smoked *in the past 30 days*?_____

About how many cigarettes have you smoked *in the past 24 hours?* _____

Have you smoked within the last hour? ☐Yes ☐No

4. How many 4:00 pm practices do you plan on going to per week over the next two weeks? _____

Thank you. Please return this completed form to a study investigator.

Appendix C

Pre-Practice Questionnaire (Day 1 version)
Pre-Practice Questionnaire (Days 2 through 10 version)

Pre-practice Questionnaire (Day 1 version)

Participant # _____ Date (mm/dd/yyyy) ____/____/_____

All of your answers are confidential. We will not share your information with your parents, teachers, coaches, or anyone else.

1. Have you smoked cigarettes *in the past 30 days*?
   - ☐ Yes
   - ☐ No
   - ☐ Don't know

   If yes, about how many cigarettes have you smoked *in the past 30 days*?_____

   About how many cigarettes have you smoked *in the past 24 hours?* _____

   Have you smoked within the last hour? ☐ Yes ☐ No

2. Have you been exposed to cigarette smoke <u>outside</u> your home *in the past 30 days*?
   - ☐ Yes
   - ☐ No
   - ☐ Don't know

   If yes, how often would you say you have been exposed?
   - ☐ Daily
   - ☐ Several times a week
   - ☐ Once a week
   - ☐ A few times only

Now please think only about the <u>past 24 hours.</u>

3. About how many hours did you spend in a place where you could tell you were breathing smoke from somebody else's cigarettes, cigars or pipes (*in the past 24 hours*)? _____ hours

4. About how many hours did you spend outdoors between 12noon and 8pm? ____ hours

   4a. Where were you (city, state)? _____

4b. For how many hours during this time (between 12noon and 8pm) were you doing something (like exercise) where your heart rate was faster than normal? _____ hours

5. Have you taken any prescription or non-prescription medications (*in the past 24 hours*)?

☐Yes
☐No
☐Don't know

If yes, did you take:

Aspirin?	☐Yes	☐No	☐Don't know
Acetaminophen (TylFeNOl)?	☐Yes	☐No	☐Don't know
Ibuprofen (Advil, Motrin)?	☐Yes	☐No	☐Don't know
Other (oral) pain reliever or anti-inflammatory medicine (e.g. Aleve)?	☐Yes	☐No	☐Don't know

5b. If you have asthma, did you take any medications for asthma?

☐Yes
☐No
☐Don't know

If yes, which medicines did you take (please list all of the asthma medication that you took in the past 24 hours) and at what time did you last take them?

Medicine name:	Time that you last took it:
_____	_____am/ pm
_____	_____am/ pm
_____	_____am/ pm
_____	_____am/ pm

6. In the past 24 hours, have you taken vitamin C, vitamin E, or a multivitamin?

☐Yes
☐No
☐Don't know

7. Have you had any of the following symptoms in the past 24 hours? *If yes*, how severe was it?

	Had symptom?			How severe was it?		
	No	**Yes**		**Mild**	**Moderate**	**Severe**
Cough	☐	☐	→	☐	☐	☐
Wheezing or whistling sound in the chest	☐	☐	→	☐	☐	☐
Shortness of breath	☐	☐	→	☐	☐	☐
Chest tightness	☐	☐	→	☐	☐	☐
Chest pain	☐	☐	→	☐	☐	☐
Runny nose	☐	☐	→	☐	☐	☐
Sneezing	☐	☐	→	☐	☐	☐
Itchy or scratchy throat	☐	☐	→	☐	☐	☐
Headache	☐	☐	→	☐	☐	☐
Watery eyes	☐	☐	→	☐	☐	☐
Other cold symptoms (What?_____)	☐	☐	→	☐	☐	☐

Mild symptom – you had the symptom but it did not interfere with your activities

Moderate symptom – you had the symptom and it interfered slightly with your activities

Severe symptom – you had the symptom and it interfered with your usual activities like walking

Thank you. Please return this completed form to a study investigator.

Pre-practice Questionnaire (Day 2-10 version)

Participant # _____ Date (mm/dd/yyyy) ____/____/_____

All of your answers are confidential. We will not share your information with your parents, teachers, coaches, or anyone else.

Please think only about the past 24 hours.

1. About how many cigarettes have you smoked *in the past 24 hours?* _____

Have you smoked within the last hour? ☐Yes ☐No

2. About how many hours did you spend in a place where you could tell you were breathing smoke from somebody else's cigarettes, cigars or pipes (*in the past 24 hours*)? _____ hours

3. About how many hours did you spend outdoors between 12noon and 8pm? ____ hours

3a. Where were you (city, state)? _____

3b. For how many hours during this time (between 12noon and 8pm) were you doing something (like exercise) where your heart rate was faster than normal? _____ hours

4. Have you taken any prescription or non-prescription medications (*in the past 24 hours*)?

☐Yes
☐No
☐Don't know

If yes, did you take:

Aspirin?	☐Yes	☐No	☐Don't know
Acetaminophen (TylFeNOl)?	☐Yes	☐No	☐Don't know
Ibuprofen (Advil, Motrin)?	☐Yes	☐No	☐Don't know
Other (oral) pain reliever or anti-inflammatory medicine (e.g. Aleve)?	☐Yes	☐No	☐Don't know

4b. If you have asthma, did you take any medications for asthma?

☐Yes
☐No
☐Don't know

If yes, which medicines did you take (please list all of the asthma medication that you took in the past 24 hours) and at what time did you last take them?

Medicine name:	Time that you last took it:
_____	_____am/ pm
_____	_____am/ pm
_____	_____am/ pm
_____	_____am/ pm

5. In the past 24 hours, have you taken vitamin C, vitamin E, or a multivitamin?

☐Yes
☐No
☐Don't know

PLEASE GO ON TO NEXT PAGE

6. Have you had any of the following symptoms today? If yes, how severe was it?

	Had symptom?			How severe was it?		
	No	Yes		Mild	Moderate	Severe
Cough	☐	☐	→	☐	☐	☐
Wheezing or whistling sound in the chest	☐	☐	→	☐	☐	☐
Shortness of breath	☐	☐	→	☐	☐	☐
Chest tightness	☐	☐	→	☐	☐	☐
Chest pain	☐	☐	→	☐	☐	☐
Runny nose	☐	☐	→	☐	☐	☐
Sneezing	☐	☐	→	☐	☐	☐
Itchy or scratchy throat	☐	☐	→	☐	☐	☐
Headache	☐	☐	→	☐	☐	☐
Watery eyes	☐	☐	→	☐	☐	☐
Other cold symptoms (What?_____)	☐	☐	→	☐	☐	☐

Mild symptom – you had the symptom but it did not interfere with your activities

Moderate symptom – you had the symptom and it interfered slightly with your activities

Severe symptom – you had the symptom and it interfered with your usual activities like walking

Thank you. Please return this completed form to a study investigator.

Appendix D

Post-Practice Questionnaire

Post-Practice Questionnaire

Participant # _____ Date (mm/dd/yyyy) ___/___/_____

1. Please tell us how far you ran today during practice and what your time was.

Distance _____ Time _____

2. Please circle the number that best matches your level of physical exertion during practice today, where 1 is the easiest and 10 is the hardest:

1 2 3 4 5 6 7 8 9 10
(Easy) (Hard)

3. Indicate if you experienced each symptom below during today's practice. For each "yes" answer, indicate how it interfered with your practice.

	Had symptom during practice?			**How did it interfere?**		
	No	**Yes**		**Continued to practice**	**Stopped practice for short time**	**Stopped practice for the day**
Cough	☐	☐	→	☐	☐	☐
Wheezing or whistling sound in the chest	☐	☐	→	☐	☐	☐
Shortness of breath	☐	☐	→	☐	☐	☐
Chest tightness	☐	☐	→	☐	☐	☐
Chest pain	☐	☐	→	☐	☐	☐
Runny nose	☐	☐	→	☐	☐	☐
Sneezing	☐	☐	→	☐	☐	☐
Itchy or scratchy throat	☐	☐	→	☐	☐	☐
Headache	☐	☐	→	☐	☐	☐
Watery eyes	☐	☐	→	☐	☐	☐
Other cold symptoms (What?_____)	☐	☐	→	☐	☐	☐

Thank you. Please return this completed form to a study investigator.

Appendix E

Non-Significant Spline Graphs

Figure 1. Pre-practice FeNO by study day with splines for gender

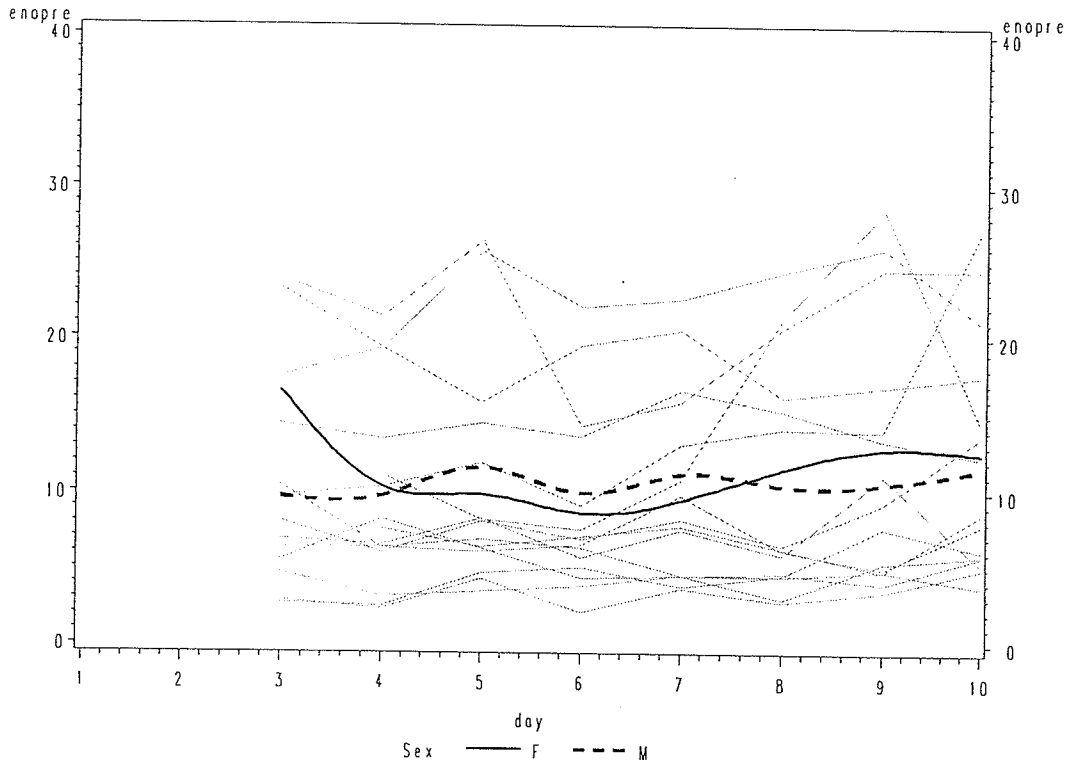


Figure 2. Pre-practice FeNO by study day with splines for height groups

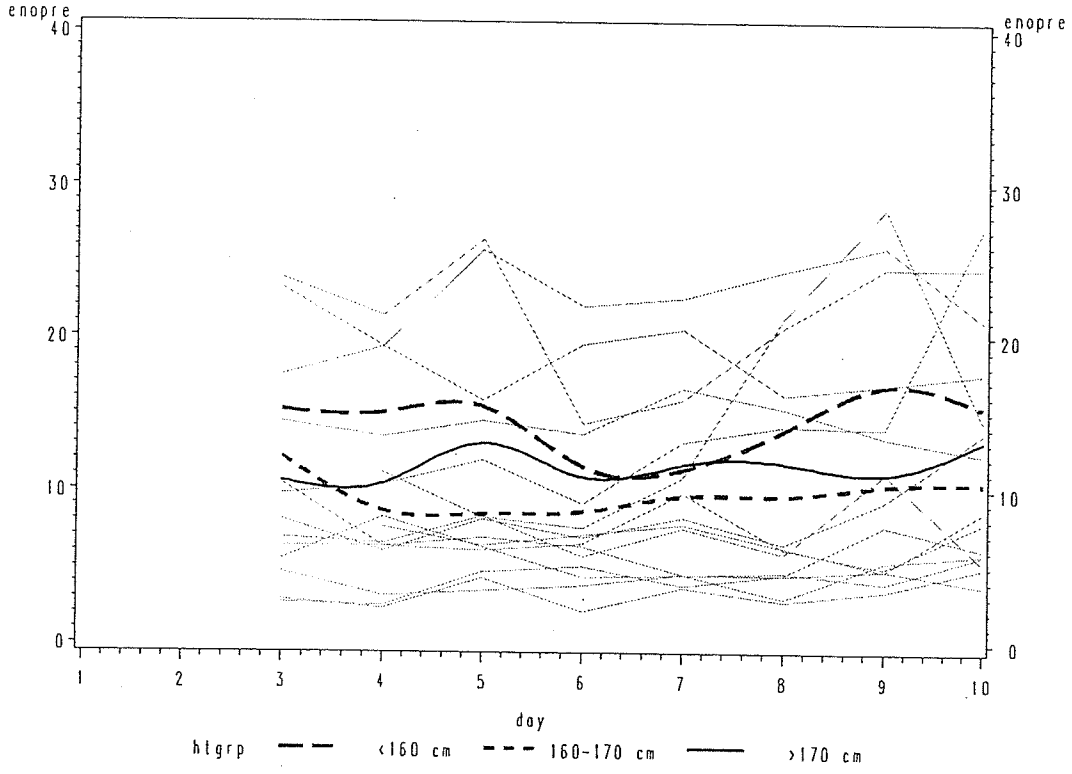


Figure 3. Pre-practice FeNO by study day with splines for body mass index groups

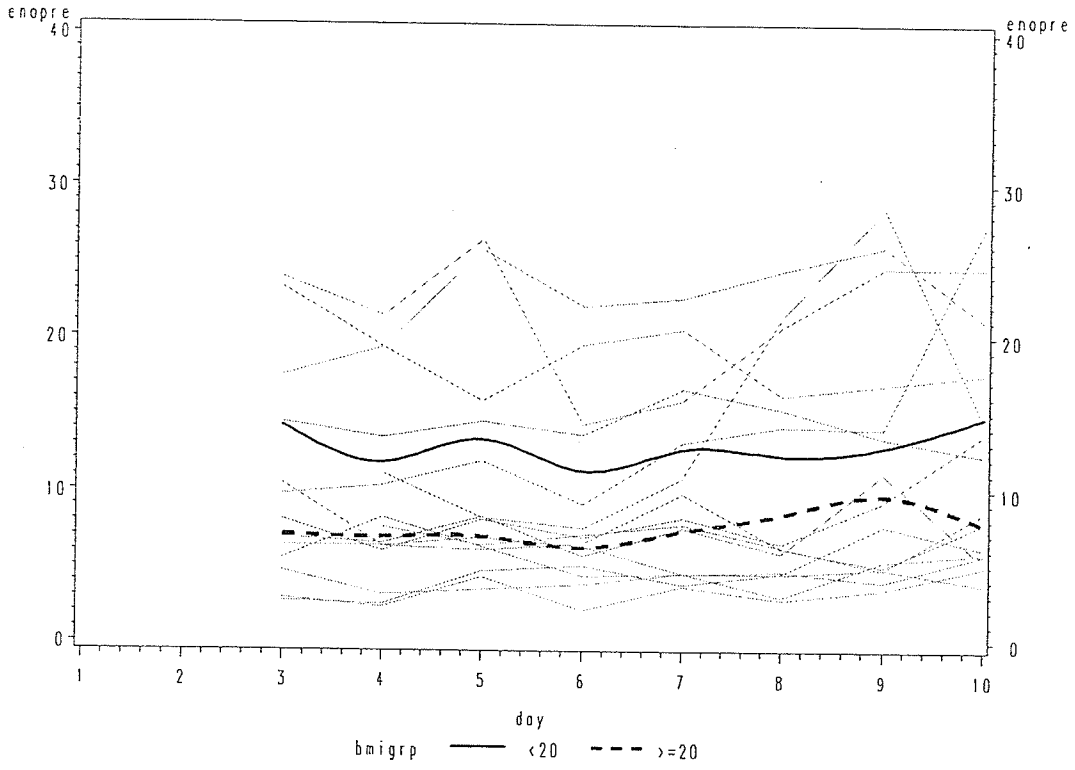


Figure 4. Pre-practice FeNO by study day with splines for asthma diagnosis

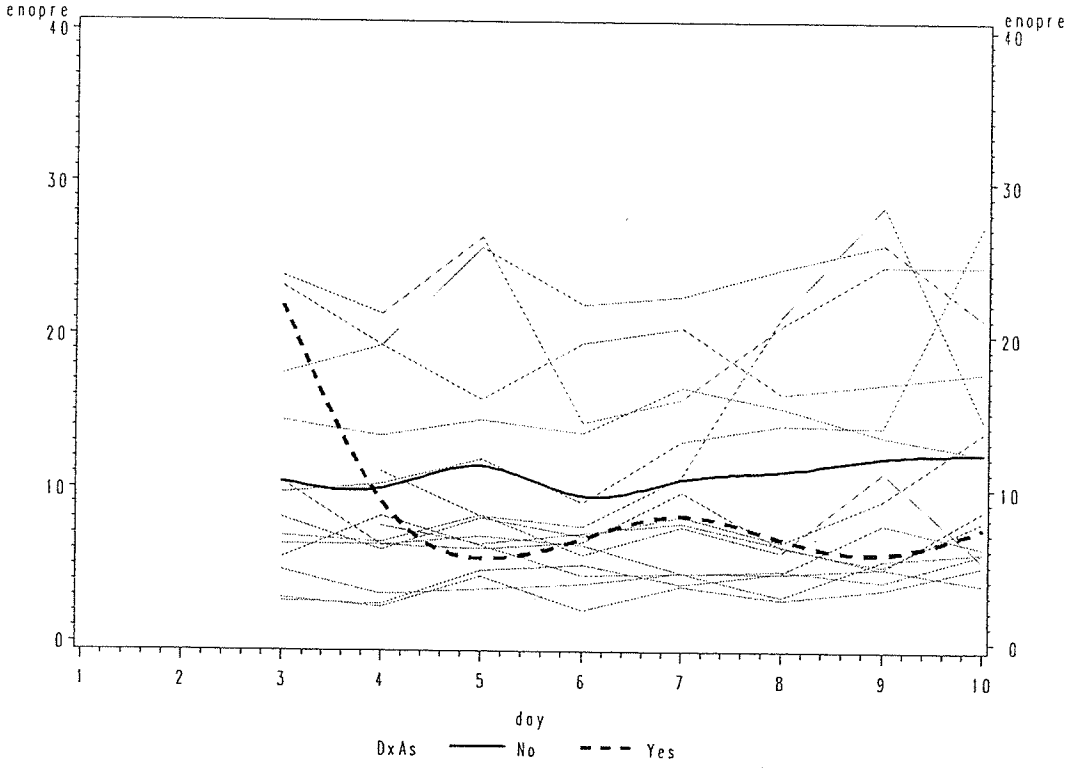


Figure 5. Pre-practice FeNO by study day with splines for allergy diagnosis

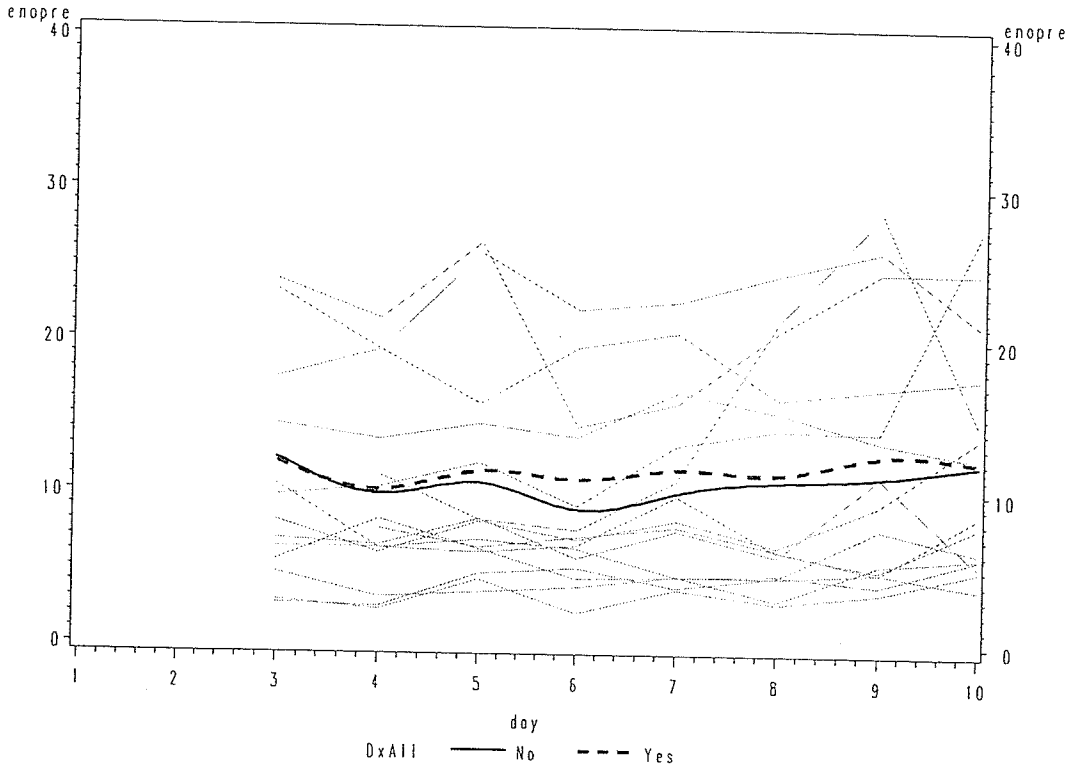


Figure 6. Pre-practice FeNO by study day with splines for asthma or allergy or hayfever diagnosis

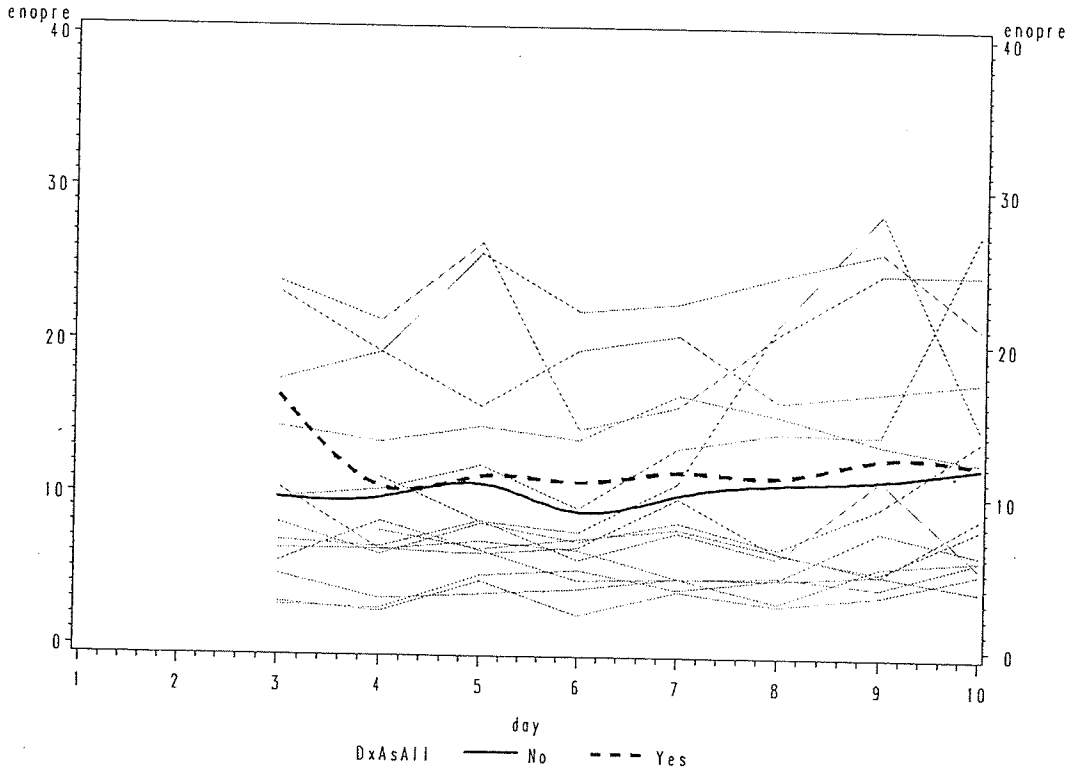


Figure 7. Pre-practice FeNO by study day with splines for "wheeze or cough in the past month" groups

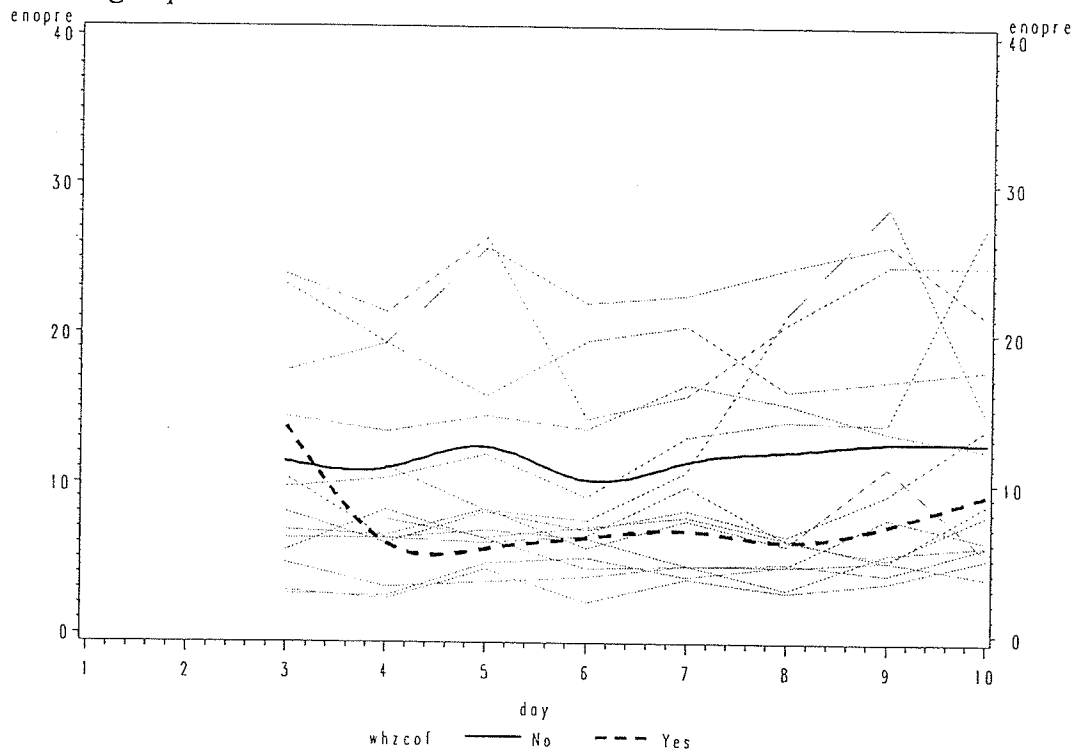


Figure 8. Pre-practice FeNO by study day with splines for "number of symptoms in the past 24 hours" groups

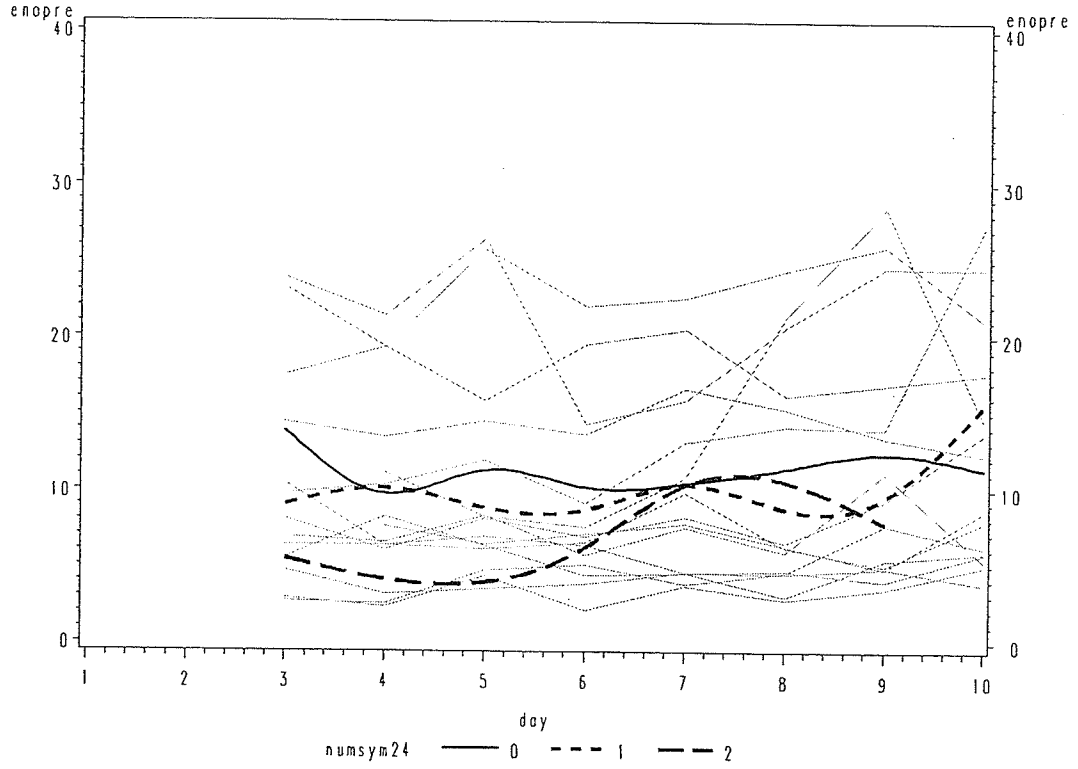
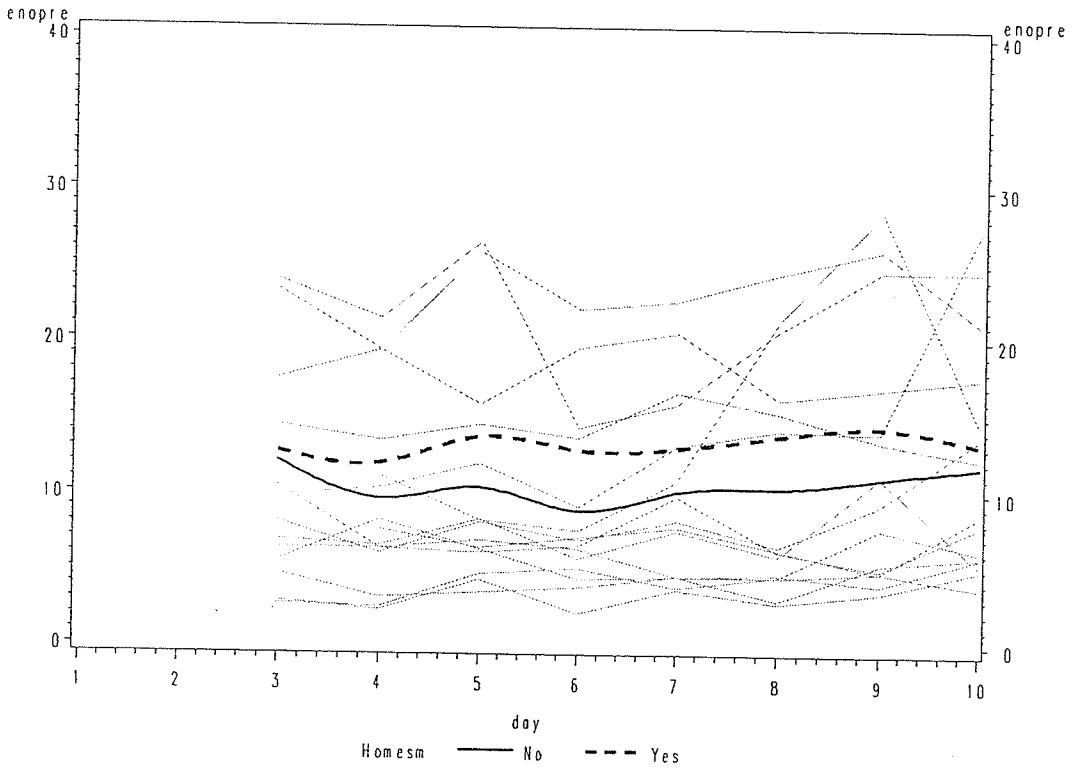


Figure 9. Pre-practice FeNO by study day with splines for home ETS exposure



APPENDIX F

Univariate Analysis of Post-Practice FeNO

We obtained a total of 120 post-practice samples from 16 subjects.

Figure 1. Histogram of post-practice FeNO, subjects and study days pooled

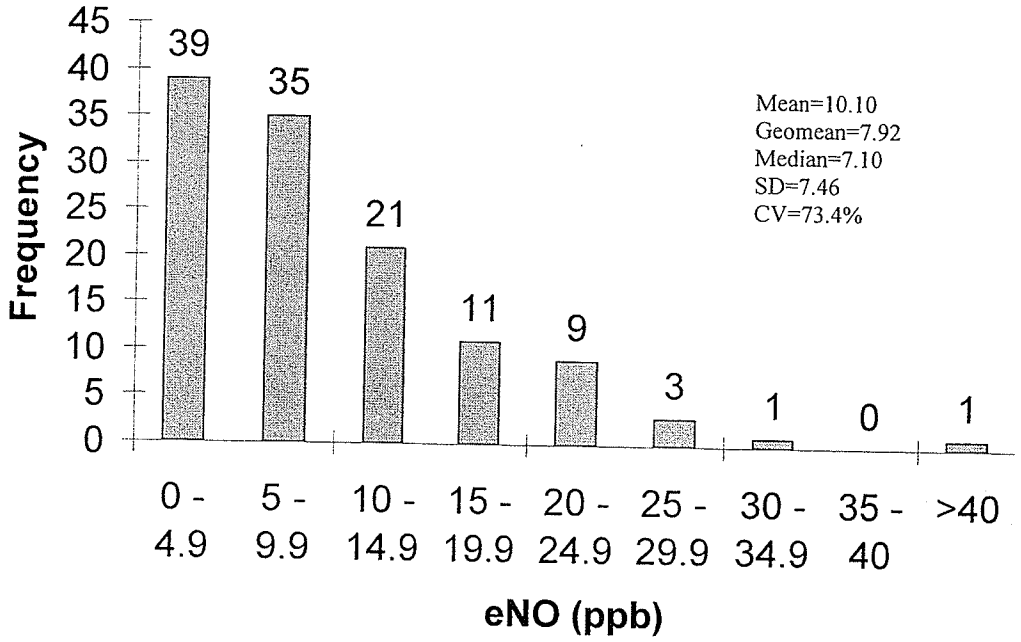


Figure 2. Histogram of post-practice FeNO log-normally transformed, subjects and study days pooled

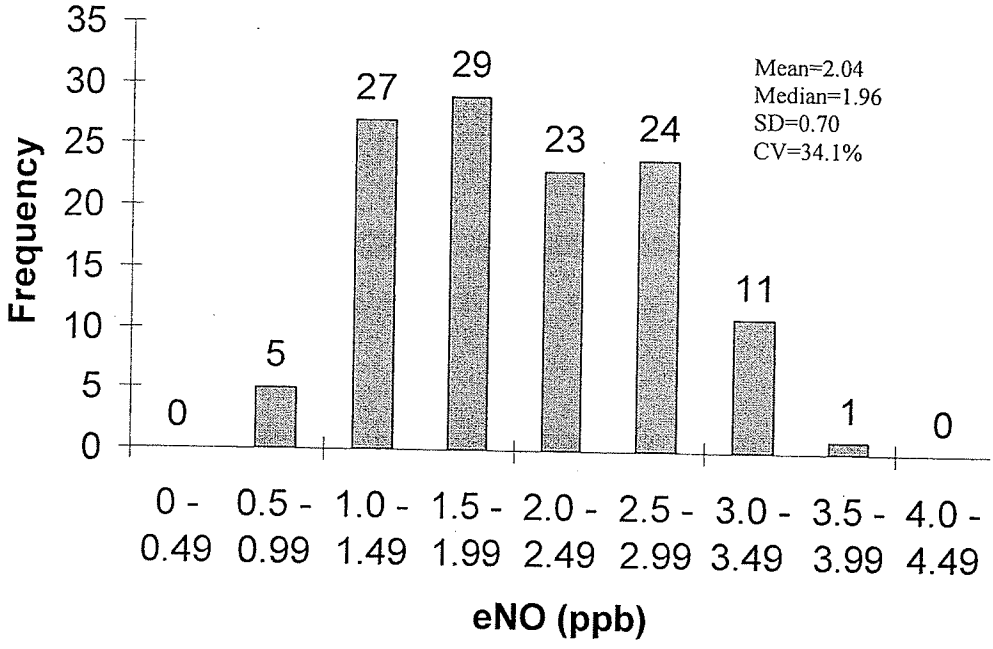


Figure 3. Post-Practice FeNO by subject (Yellow and Orange Alert days indicated)

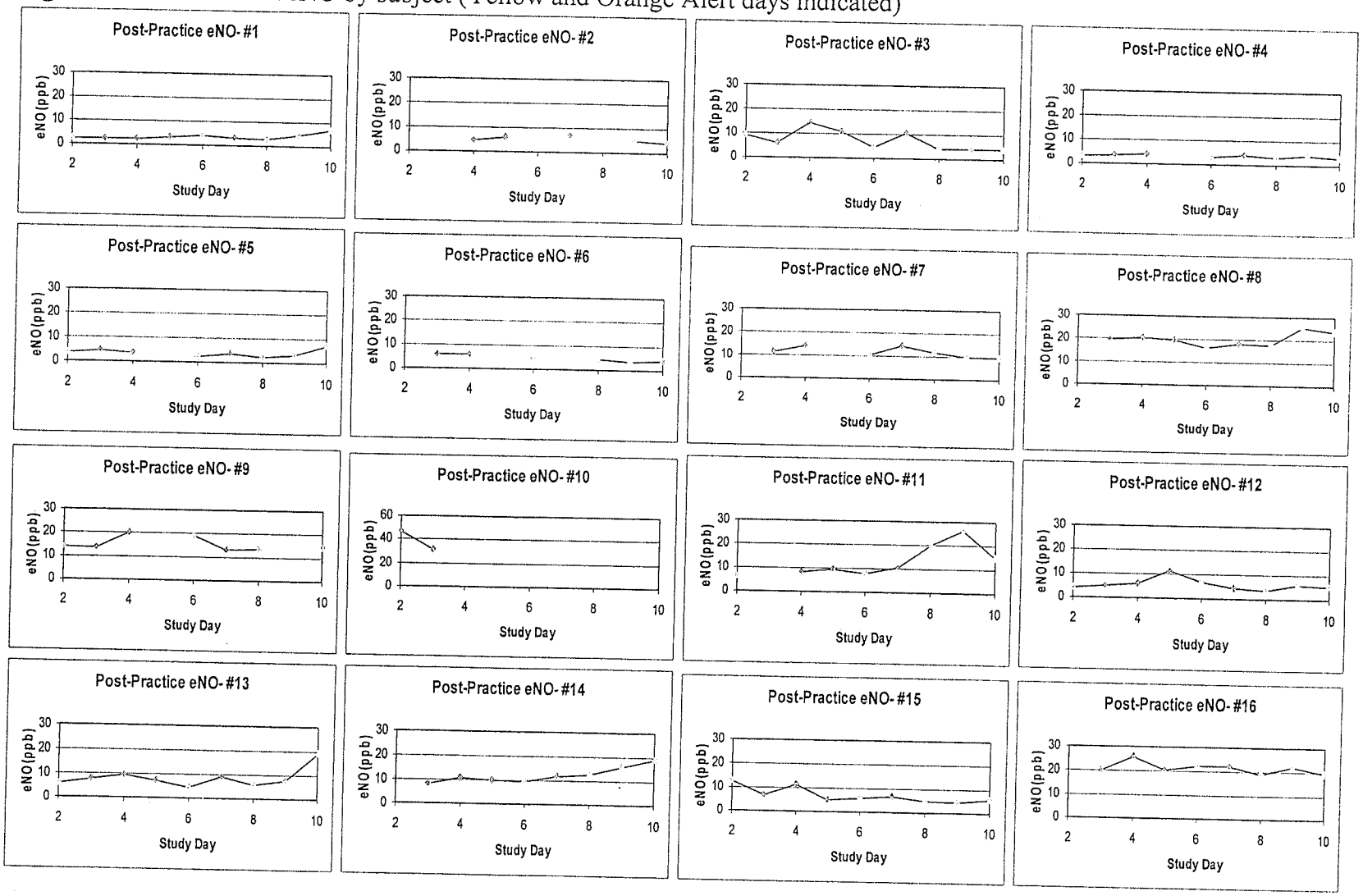


Figure 4. Post-practice FeNO by subject

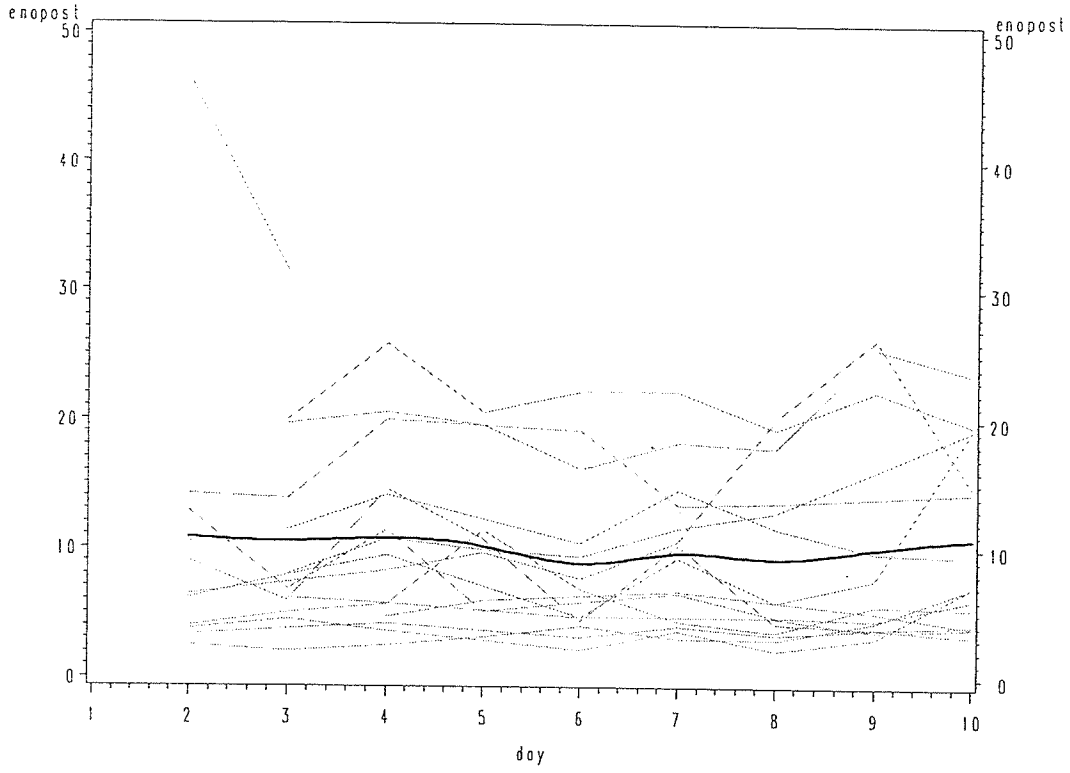


Table 1. Post-practice FeNO, subjects and study days pooled

N	**120**
MEAN (ARITHMATIC)	**10.1**
MEAN (GEOMETRIC)	**7.9**
MEDIAN	**7.1**
MIN	**2.1**
MAX	**46.2**
RANGE	**44.1**
SD	**7.5**
CV (%)	**73.9**
IQ-1	**4.4**
IQ-3	**14.1**
IQR	**9.7**

Table 2. Post-practice FeNO descriptive statistics by subject, study days pooled

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Mean
n	9	5	9	8	8	6	7	8	7	2	8	9	9	8	9	8	**7.5**
MEAN	3.6	5.3	7.5	3.7	3.8	4.8	11.6	20.2	15.5	38.8	13.0	5.8	8.6	12.2	7.1	21.5	**11.4**
GEO-MEAN	3.3	5.3	6.7	3.6	3.6	4.8	11.5	20.0	15.3	38.1	11.6	5.5	7.9	11.8	6.7	21.4	**11.1**
MEDIAN	3.1	5.2	5.9	3.6	3.7	4.7	11.4	19.7	14.2	38.8	10.2	5.5	7.7	11.2	6.1	21.4	**10.8**
MIN	2.1	4.1	3.9	3.2	2.2	3.7	9.5	16.2	13.4	31.4	6.4	3.6	4.7	8.0	4.6	19.3	
MAX	7.1	6.7	14.6	4.2	7.1	6.1	14.6	25.6	20.0	46.2	26.3	11.4	19.3	19.3	12.9	25.8	
RANGE	5.0	2.6	10.7	1.0	4.9	2.4	5.1	9.4	6.6	14.8	19.9	7.8	14.6	11.3	8.3	6.5	**8.2**
SD	1.5	1.0	3.8	0.40	1.6	0.95	2.0	3.1	2.8	10.5	7.0	2.3	4.3	3.8	3.0	2.1	**3.1**
SE	0.51	0.46	1.3	0.14	0.55	0.39	0.76	1.1	1.1	7.4	2.5	0.78	1.4	1.3	0.99	0.75	**1.3**
CV (%)	42.8	19.3	51.1	10.9	41.0	19.7	17.3	15.3	18.2	27.0	53.8	40.1	50.	30.8	41.4	9.8	**30.5**
IQ-1	2.5	4.7	4.3	3.3	3.0	4.2	10.1	18.2	13.7	35.1	8.2	4.4	6.1	9.8	5.2	19.9	
IQ-3	4.1	6.0	10.3	4.0	4.0	5.5	12.9	21.4	16.8	42.5	16.2	5.7	9.3	13.7	6.8	22.2	
IQR	1.6	1.3	6.0	0.65	1.0	1.4	2.8	3.2	3.1	7.4	8.0	1.3	3.2	3.9	1.6	2.3	**3.0**

Figure 5. Mean post-practice FeNO by subject

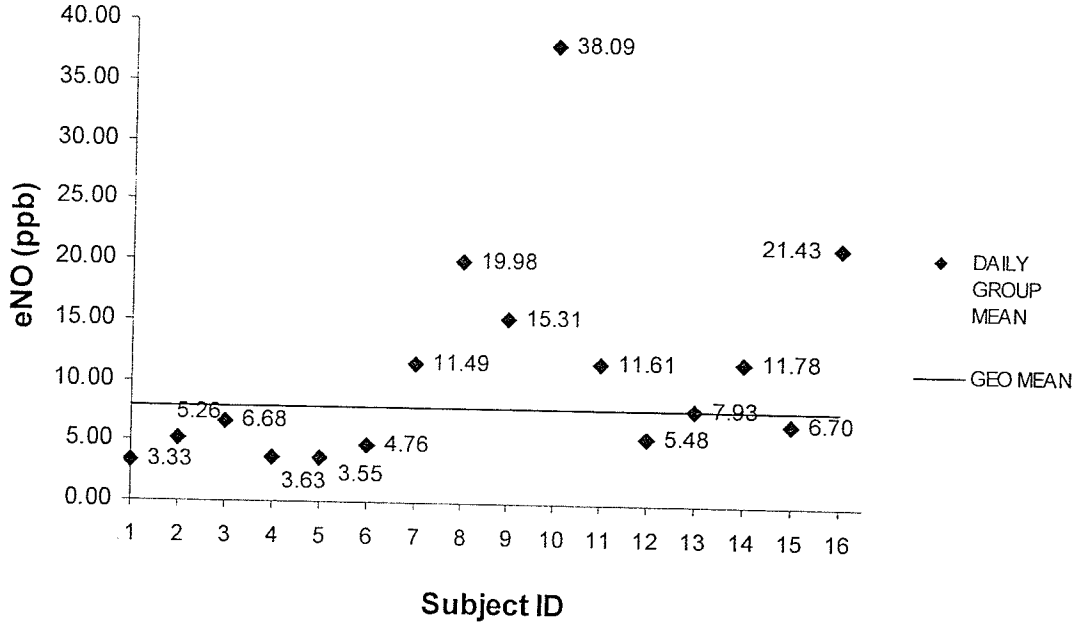


Figure 6. Post-practice FeNO by subject

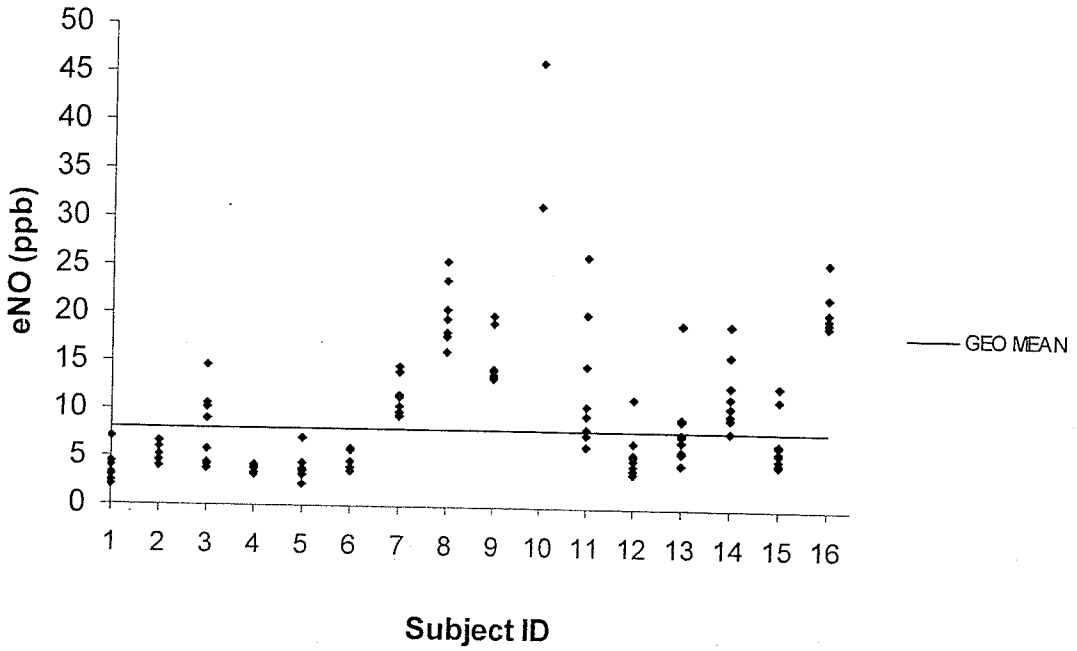


Table 3. Post-practice FeNO descriptive statistics by study day, subjects pooled

STUDY DAY	n	MEAN (ARITHMETIC)	MEAN (GEOMETRIC)	MEDIAN	MIN	MAX	RANGE	SD	SE	CV (%)	IQ-1	IQ-3	IQR
2	10	10.8	7.2	6.3	2.5	46.2	43.7	13.1	4.1	120	3.9	11.9	8.1
3	14	10.5	8.1	7.4	2.1	31.4	29.3	8.2	2.2	78.3	5.2	13.2	8.0
4	15	10.8	8.7	9.5	2.5	25.8	23.3	7.0	1.8	65.3	5.2	14.4	9.2
5	10	10.3	9.0	9.8	3.2	20.6	17.4	5.8	1.8	55.8	6.3	11.2	5.0
6	14	8.7	6.9	6.4	2.2	22.2	20	6.3	1.7	72.2	4.5	10.2	5.7
7	14	9.9	8.4	9.8	3.1	22.2	19.1	5.8	1.5	58.0	5.0	13.0	8.0
8	14	9.1	7.0	5.4	2.2	20.2	18	6.6	1.8	72.4	3.8	13.4	9.7
9	14	10.2	7.6	5.5	3.2	26.3	23.1	8.6	2.3	84.7	4.0	14.5	10.5
10	15	10.8	8.8	7.1	3.3	23.6	20.3	7.0	1.8	64.9	4.9	17.1	12.2
Mean	**13.3**	**10.1**	**8.0**	**7.4**			**23.8**	**7.6**	**2.1**	**74.7**			**8.5**

Figure 7. Mean post-practice FeNO by study day

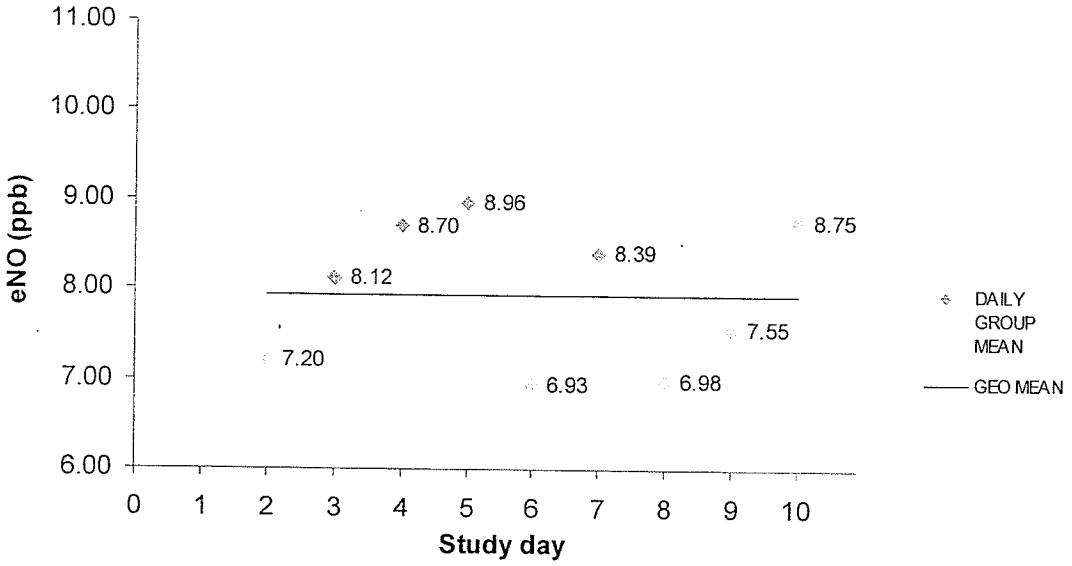
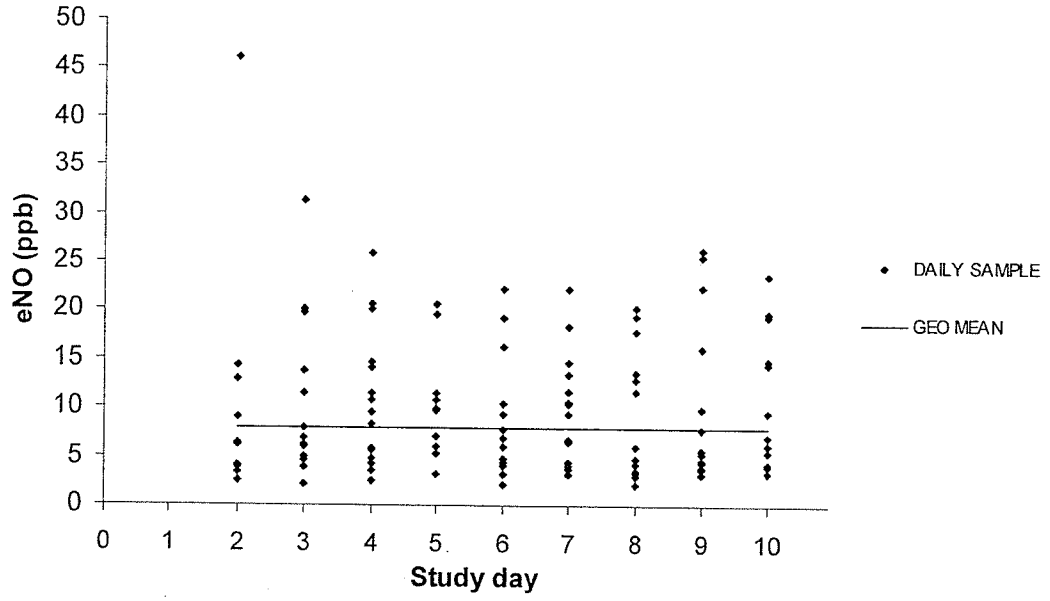


Figure 8. Post-practice FeNO by study day



Appendix G

Air Quality Data

Table 1. Ozone (ppm) at EPA Conyers Station

Study Day	Max (1hr avg)	Time (h)	Max (8hr avg)	Time (h)	1700 h (1hr avg)	1700 h (8hr avg)
1	0.052	1700	0.042	1900, 2000	0.052	0.036
2	0.061	1800	0.044	1900	0.054	0.037
3	0.106	1800	0.081	2000, 2100	0.098	0.060
4	0.103	1800	0.087	1900, 2000	0.098	0.071
5	0.073	1300	0.058	1800	0.050	0.053
6	0.057	1700	0.047	1900	0.057	0.039
7	0.055	1300	0.051	1900	0.054	0.051
8	0.064	1700	0.058	1900, 2000	0.064	0.052
9	0.067	1800	0.062	2000	0.064	0.060
10	0.072	1500	0.063	1900	0.067	0.050

Table 2. $PM_{2.5}$ (ug/m^3) at EPA South Dekalb Station

Study Day	Max (1 hr avg)	Time (h)	1700 h (1 hr avg)
1	17.7	1900	16.5
2	32.1	2100	25.6
3	48.4	1800	44.8
4	63.5	2300	39.2
5	55.2	0100	24.1
6	30.4	2300	24.0
7	39.4	1000	22.9
8	30.9	1000	20.6
9	26.6	0100	23.2
10	40.6	1700	40.6

Table 3. Air Quality Index at EPA Atlanta Station

Study Day	Alert	Max AQI	Time (h)	Pollutant	AQI-1700	Pollutant
1	Yellow	69	0500-1000	$PM_{2.5}$	65	$PM_{2.5}$
2	Yellow	63	2400	$PM_{2.5}$	60	$PM_{2.5}$
3	Orange (ozone)	116	2100, 2400	Ozone (8hr), $PM_{2.5}$	89	$PM_{2.5}$
4	Orange (ozone)	129	2000, 2100	Ozone (8hr)	108	$PM_{2.5}$
5	Orange (PM)	114	1400-1900	$PM_{2.5}$	114	$PM_{2.5}$
6	Yellow	70	2400	$PM_{2.5}$	69	$PM_{2.5}$
7	Orange (PM)	114	2400	$PM_{2.5}$	94	$PM_{2.5}$
8	Yellow	97	2100	Ozone (8hr)	74	$PM_{2.5}$
9	Yellow	95	2000	Ozone (8hr)	80	$PM_{2.5}$
10	Yellow	70	1500, 1600, 1900, 2300, 2400	$PM_{2.5}$	69	$PM_{2.5}$

APPENDIX H

Univariate Analyses of Air Quality Data

Table 1. Air quality descriptive statistics

	Ozone (ppm)				$PM_{2.5}$ (ug/m^3)		AQI	
	Max (1 hr avg.)	**Max (8 hr avg.)**	**1700 h (1 hr avg.)**	**1700 h (8 hr avg.)**	**Max (1 hr avg.)**	**1700 h (1 hr avg.)**	**Max**	**1700 h**
n	10	10	10	10	10	10	10	10
MEAN (Arithmetic)	0.071	0.059	0.067	0.051	38.4	28.2	93.7	82.2
MEAN (Geometric)	0.069	0.058	0.065	0.050	36.1	26.8	90.8	80.4
MEDIAN	0.066	0.058	0.062	0.052	35.8	24.1	96.0	77.0
MIN	0.052	0.042	0.052	0.036	17.7	16.5	63.0	60.0
MAX	0.106	0.087	0.098	0.071	63.5	44.8	129	114
RANGE	0.054	0.045	0.046	0.035	45.8	28.3	66.0	54.0
SD	0.019	0.015	0.017	0.011	14.0	9.7	24.2	18.5
CV (%)	26.7	25.2	25.7	22.0	36.3	34.3	25.8	22.5
IQ-1	0.058	0.048	0.055	0.042	30.5	23.0	70.0	69.0
IQ-3	0.073	0.063	0.066	0.058	46.5	35.8	114	92.8
IQR	0.015	0.015	0.012	0.017	15.9	12.8	44.0	23.8
r*	0.49	0.42	0.37	0.35	0.50	0.58	0.16	0.23

* Correlation with post-practice FeNO

Figure 1. Maximum ozone concentration (1 hr avg.) by study day

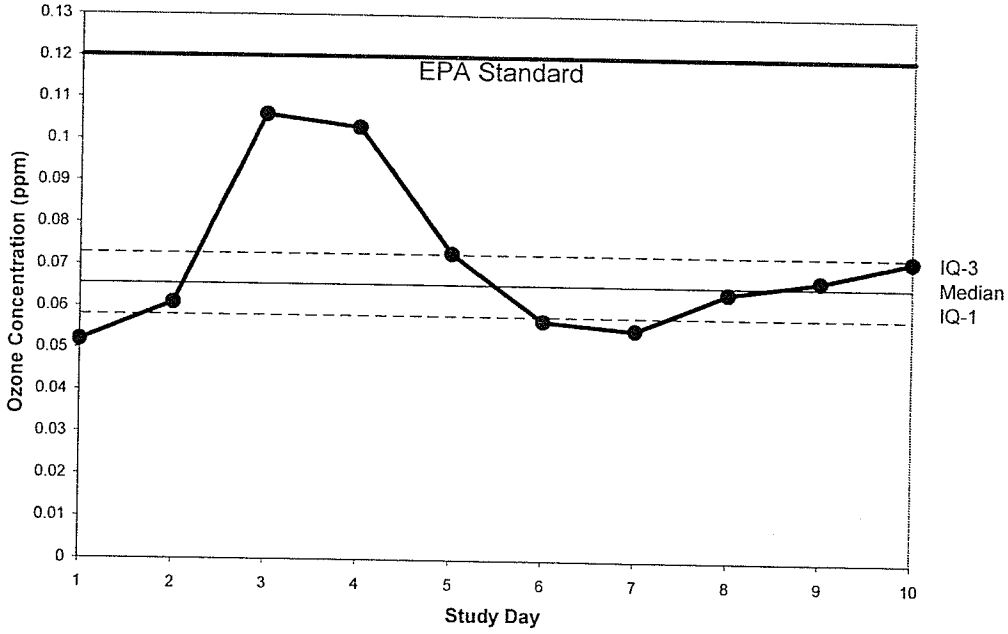


Figure 2. Maximum ozone concentration (8 hr avg.) by study day

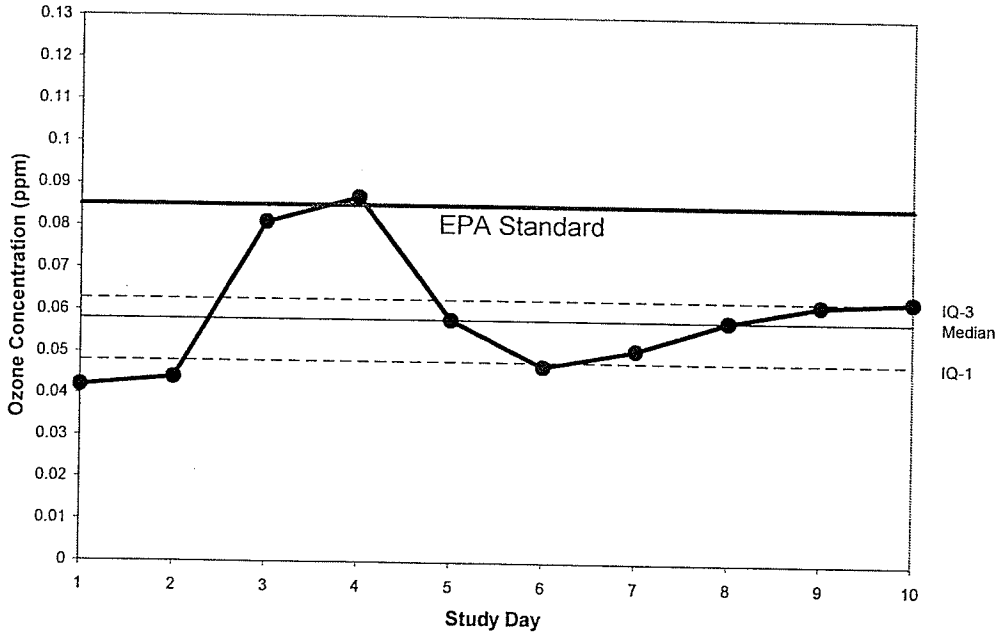


Figure 3. 1700 hour ozone concentration (1 hr avg.) by study day

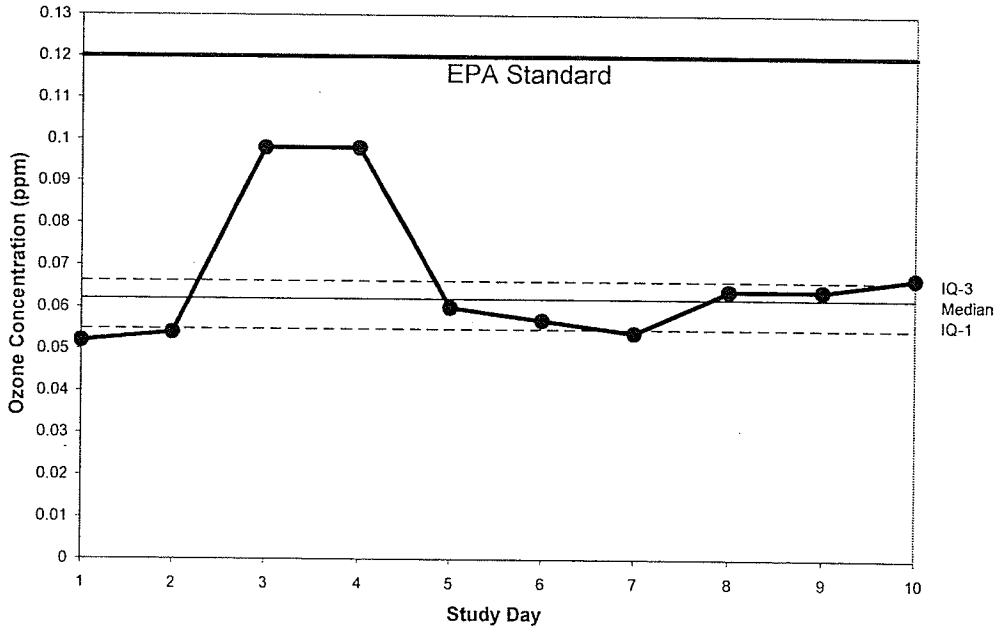


Figure 4. 1700 hour ozone concentration (8 hr avg.) by study day

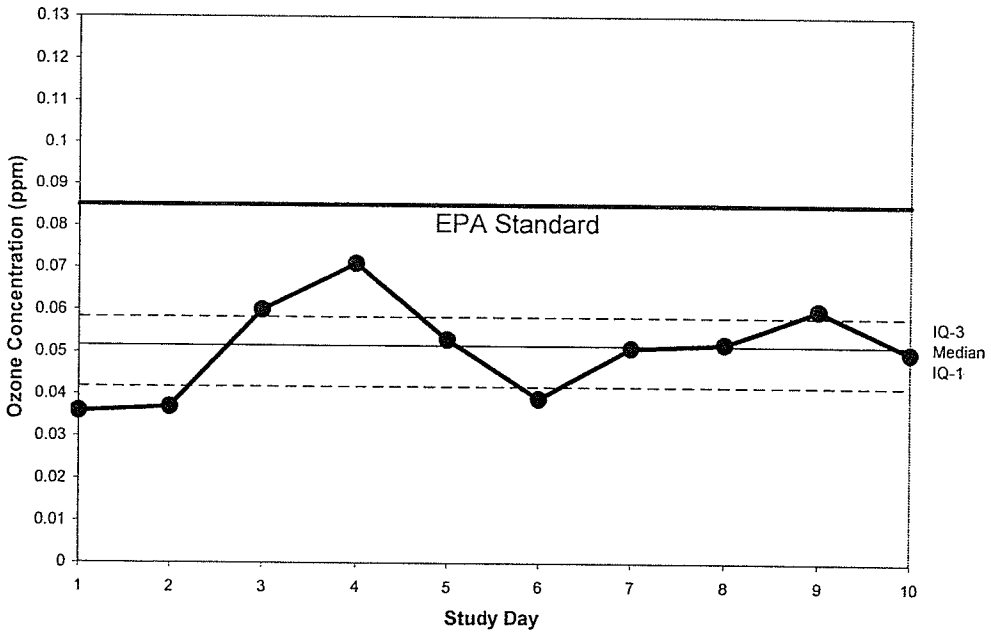


Figure 5. Maximum $PM_{2.5}$ (1 h avg.) concentration by study day

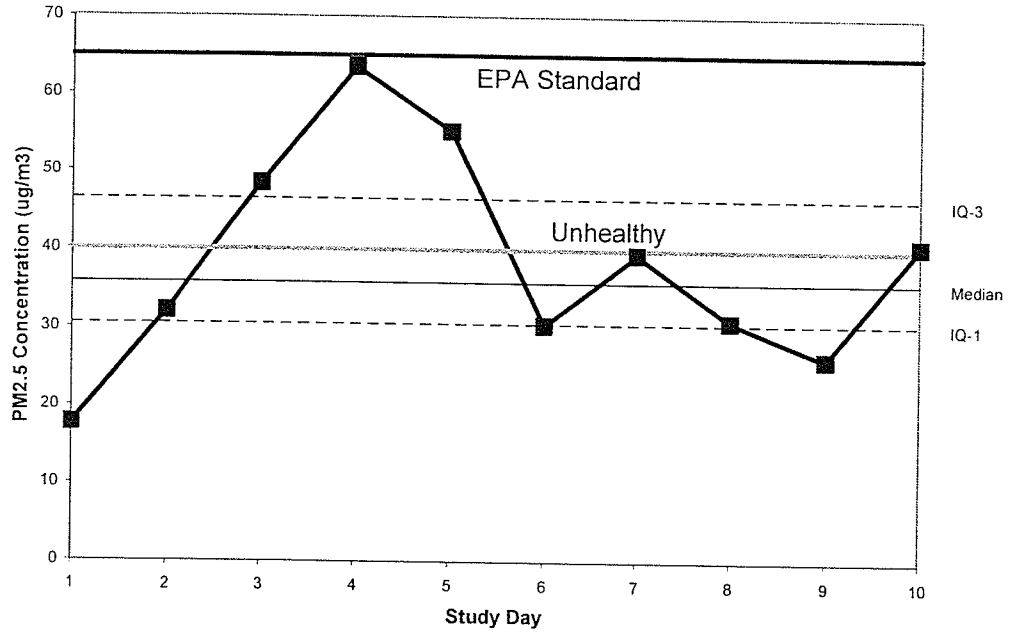


Figure 6. 1700 h $PM_{2.5}$ concentration (1 h avg.) by study day

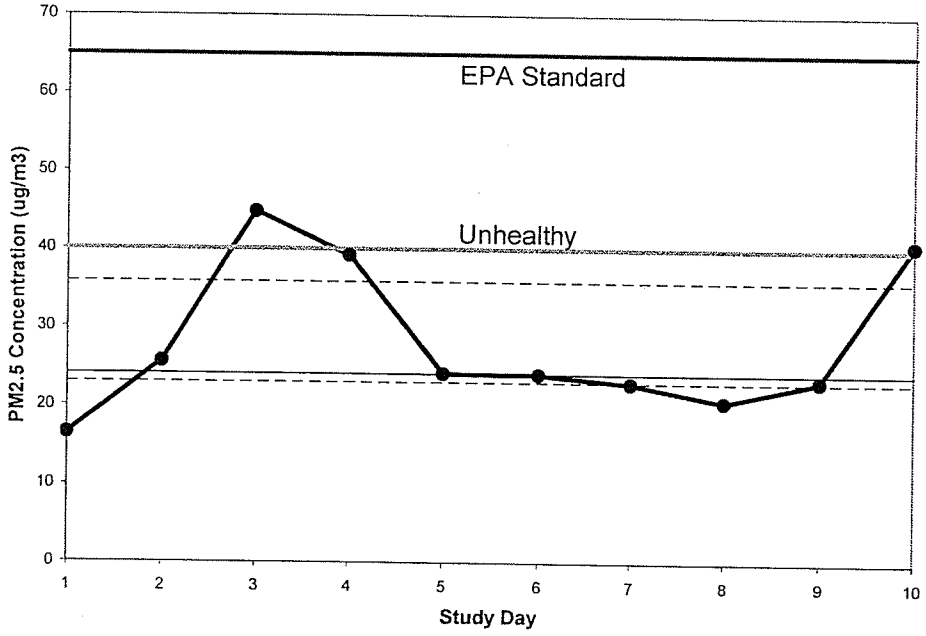


Figure 7. Maximum Air Quality Index by study day

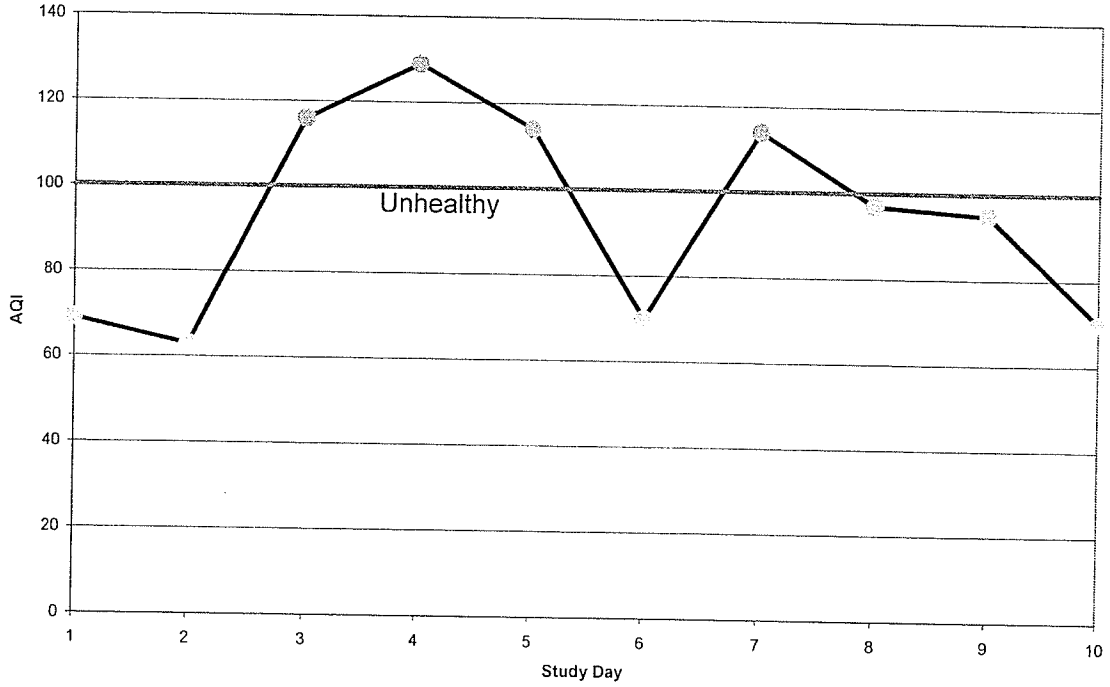


Figure 8. Change in FeNO and air quality measures by study day

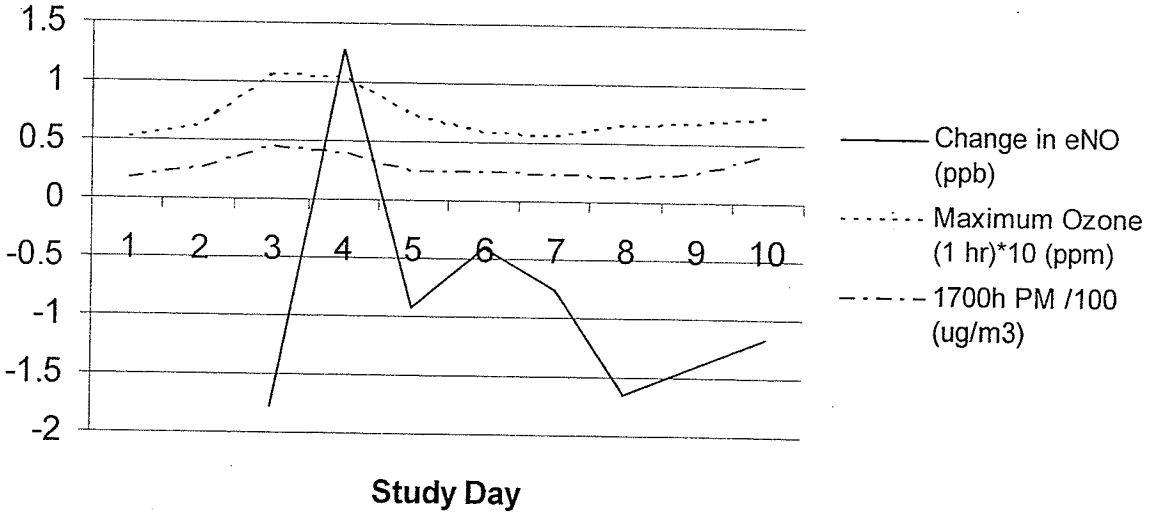


Table 2. Pearson correlation coefficients with post-practice FeNO

	Ozone (ppm)			$PM_{2.5}$ (ug/m^3)		
	Max (1 hr avg) No lag	**Max (1 hr avg) 1-day lag**	**Max (1 hr avg) 2-day lag**	**1700 h (1 hr avg) No lag**	**1700 h (1 hr avg) 1-day lag**	**1700 h (1 hr avg) 2-day lag**
n	9	9	9	9	9	9
r	0.49	0.12	0.09	0.58	0.28	0.21

Table 3. Pearson correlation coefficients with natural log of post-practice FeNO

	Ozone (ppm)			$PM_{2.5}$ (ug/m^3)		
	LN Max (1 hr avg) No lag	**LN Max (1 hr avg) 1-day lag**	**LN Max (1 hr avg) 2-day lag**	**LN 1700 h (1 hr avg) No lag**	**LN 1700 h (1 hr avg) 1-day lag**	**LN 1700 h (1 hr avg) 2-day lag**
n	9	9	9	9	9	9
r	0.51	0.08	0.09	0.59	0.24	0.32

Figure 9. Post-practice FeNO vs. maximum ozone (1 hr avg.)

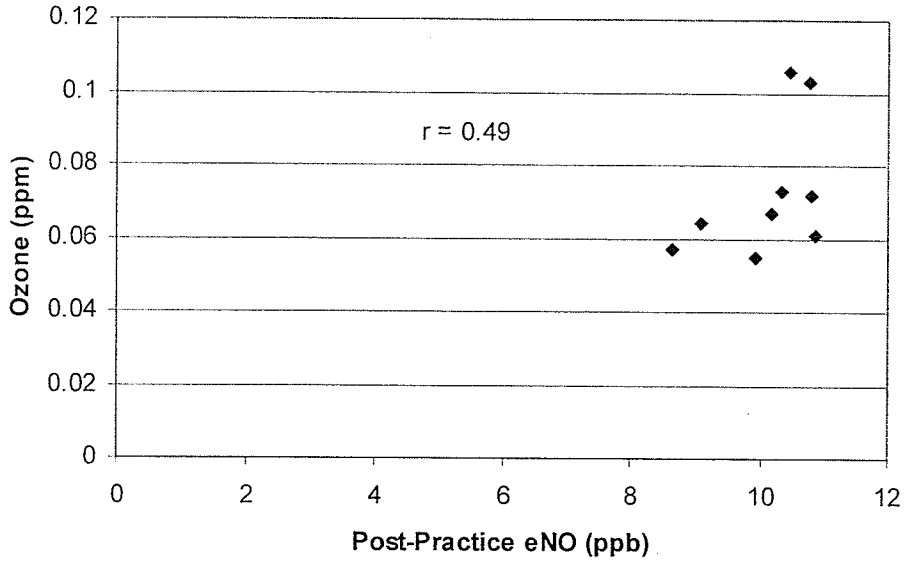


Figure 10. LN post-practice FeNO vs. LN max ozone (1 hr avg.)

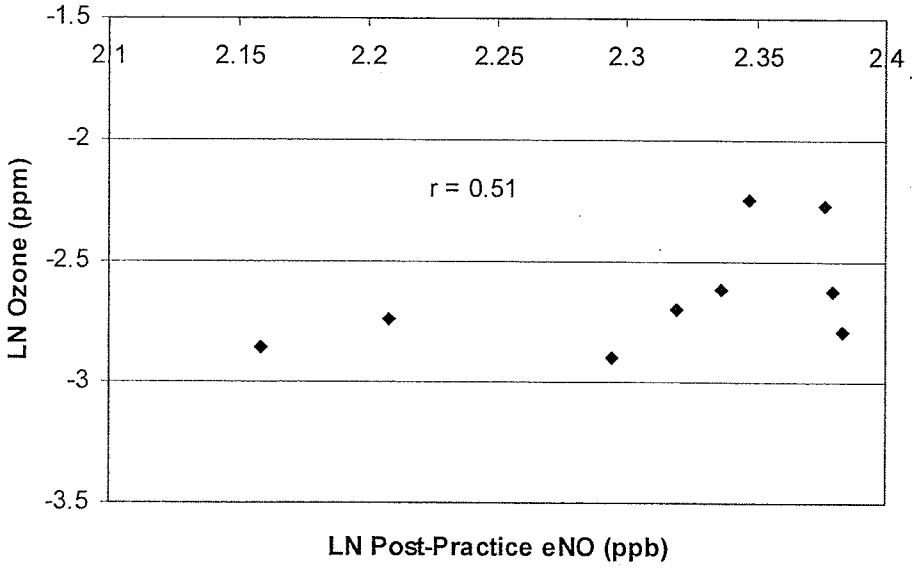


Figure 11. Post-practice FeNO vs. 1700 h PM

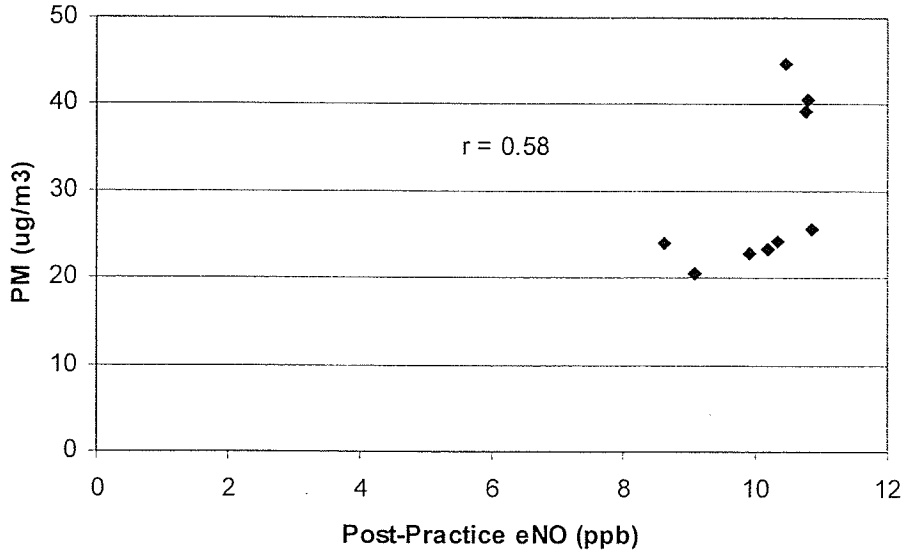


Figure 12. LN Post-practice FeNO vs. LN 1700 h PM