The Pennsylvania State University

The Graduate School

Intercollege Graduate Degree Program in Physiology

PULMONARY FUNCTION CHANGES IN CIGARETTE SMOKERS EXPOSED TO OZONE

A Dissertation in

Physiology

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

May 2008

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ABSTRACT

PURPOSE: The acute inhalation of ozone (O_3) by healthy nonsmokers compromises conducting airway function during exercise, as measured by forced expiratory volume in one second (FEV_1). Paradoxically, cigarette smokers have exhibited little to no decrement in FEV_1. We hypothesized that smoking-induced changes in the epithelial lining layer, such as increased thickness or lower antioxidant capacity, may allow O_3 to penetrate deeper into the lungs of smokers versus non-smokers, thereby reducing the conducting airway responsiveness. If O_3 penetrates deeper longitudinally, O_3 may alter markers of distal airway function in smokers, notably the normalized slope (S_N) of the CO_2 expirogram.

METHODS: We recruited 30 smokers (19M, 11F, 24 ± 4 years, 6 ± 4 total years smoking, 4 ± 2 packs/wk) and 30 non-smokers (17M, 13F, 25 ± 6 years) with clinically normal lung function, who had no history of respiratory or cardiovascular disease. Volunteers participated in two research sessions where they exercised for one hour on a cycle ergometer while breathing either filtered air or 0.30 ppm O_3 at a workload sufficient to elicit a minute volume equal to 15 liters per minute times body surface area (m^2). Exposure gases were delivered through a Hans Rudolph mask that allowed for oral breathing only. Breath-by-breath measures of tidal volume, breathing frequency, and the dose of O_3 retained by the lung were made. Before and after each exposure, subjects completed lung function tests, a symptom questionnaire, and a series of breaths during which the CO_2 expirograms were recorded. From the CO_2 expirograms, we calculated values of conducting airway volume (V_D) and S_N. Additionally, pre- and post- exposure

we sampled the nasal epithelial lining fluid (ELF) via nasal lavage and measured the ELF antioxidant capacity. Pre-O_3 exposure, we obtained blood plasma and quantified circulating uric and ascorbic acid concentrations. Uric and ascorbic acid concentrations were quantified by high performance liquid chromatography and the total antioxidant capacity was determined using the oxygen radical absorbance capacity (ORAC) assay.

In order to test our hypothesis, we developed a mathematical model which describes the longitudinal partitioning of the inhaled dose of O_3 to the conducting airways and alveolar airspaces. This model relates the fraction of the total O_3 dose reaching the alveolar region to the rate of transport of O_3 to the ELF in the conducting airways (Ka) and the ratio of the V_D to tidal volume (V_D/V_T) during exercise. Ka is determined largely by the availability of ELF antioxidants.

RESULTS: Both smokers and non-smokers experienced no significant changes in FEV_1, V_D, or S_N with air exposure. However, with O_3 exposure, we found smokers and non-smokers to be equally responsive in terms of FEV_1 (-9.5 ± 1.8% versus -8.7 ± 1.9%). While smokers were responsive in terms of V_D (-6.1 ± 1.2%) and S_N (9.1 ± 3.4%), non-smokers were not. We compared pre-and post-O_3 exposure values of V_D with values of V_T measured in the 10^{th} and 55^{th} minute of exposure and found that in the 10^{th} minute of exposure, smokers and non-smokers had similar values of V_D/V_T. However, in the 55^{th} minute of exposure, non-smokers increased V_D/V_T (16.4 ± 2.8%) while smokers did not (8.4 ± 4.2%). Post-O_3 exposure, smokers experienced fewer respiratory-related symptoms (shortness of breath, cough, and chest burning) compared to non-smokers.

In terms of antioxidant status, smokers and non-smokers were not different in terms of plasma or nasal ELF ascorbic or uric acids. The ELF of both smokers and non-smokers had similar ORAC values. This led us to conclude that, because ELF antioxidant capacities were similar between both groups, Ka is not different. In applying our findings to our model, we concluded that, because Ka and initial V_D/VT were not different in non-smokers and smokers, initial differences in longitudinal dose distribution are not responsible for the increased changes in S_N experienced by the smokers. However, because smokers fail to increase V_D/V_T over the course of the exposure, they receive a higher cumulative dose to the peripheral airspaces compared to non-smokers.

CONCLUSIONS: Young cigarette smokers retain their responsiveness to O_3 in terms of FEV_1. Uniquely, these smokers experience changes in V_D that lead to heterogeneity in airway morphometry. This conclusion is supported by the observed increase in S_N. We have determined that changes in the expirogram are not a result of initial differences in the dose penetrating to the peripheral airways. However, a decrease in V_D/V_T over the course of the exposure increases the dose to which the peripheral lung is exposed relative to the conducting airways. These findings demonstrate that young smokers are more sensitive to the health effects of O_3 than non-smokers. Because we have identified that this population of young smokers responds differently to O_3 in terms of FEV_1 than the populations of older smokers reported in the literature, future studies of the health effects of airborne pollutants in smokers should include a cohort of young smokers.

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ACKNOWLEDGEMENTS

There are a number of individuals to whom I am deeply indebted for the quality of education I have received while at Penn State. First and foremost, I would like to thank my thesis advisor, Dr. James Ultman for his guidance and wisdom. Dr. Ultman is not only a physiologist of the highest caliber, but has been a tremendous teacher, mentor, and role model. I am grateful that he has always told me what I needed to hear, but not always what I wanted to hear. I feel blessed to have had the opportunity to learn from him during these last years. I am grateful to him and his wife for the warm hospitality they have extended to my family.

I would like to thank my co-mentor, Dr. Aziz Ben-Jebria for his insights. I am grateful for his attention to detail and the scrutiny with which he has reviewed my work. I believe that the high expectations he has had for me have made me a better scientist. I am also indebted to my thesis committee members Dr. James Pawelczyk and Dr. Rebecca Bascom for their valuable feedback and support of my career.

My sincerest thanks go to the research participants who gave generously of their time to help me complete this work.

Penn State has a technical and medical staff whose expertise is unparalleled. Thank you to the staff of the General Clinical Research Center for their support in ensuring the safety of our research participants. Rick Ball, Senior Research Assistant at the GCRC Immunology Core Lab has been a tremendous source of information. I am grateful for his help in the implementation of my biochemical assays. I am grateful for the technical

assistance of Don Lucas, Engineering Aide in the Machine Shop in the Department of Chemical Engineering. Don is an invaluable resource and fantastically patient teacher. Amy Ciccarella, a nutritionist at the General Clinical Research Center, served as our study coordinator during a portion of the study. Her work recruiting volunteers and collecting biological samples is greatly appreciated.

Dr. Ali Fassih and Timothy Brenza were instrumental in the completion of this project. I am grateful for their dedication and senses of humor.

Finally, I could not have completed this project without the support of my family and the love and understanding of my husband and son. Thank you for teaching me to live every day like it's Shark Week and that nothing is impossible…

Except for dinosaurs.

Chapter 1 – Introduction

In pulmonary toxicological research, one of the most commonly used tools to assess the health effects of a substance is clinical spirometry. The inhalation of ozone (O_3), an oxidizing gas component of photochemical smog, causes alterations in forced expired parameters in healthy, non-smoking human subjects. Specifically, O_3 causes decrements in the forced expired volume in one second (FEV_1). Data from, Kerr *et. al.,* Foster *et. al.*, and Frampton *et. al.* suggest that smokers do not experience decrements in FEV_1 in response to O_3 exposure. These authors, and others, have speculated that cigarette smokers may be desensitized to the pulmonary effects of an oxidant insult.

In this clinical protocol we sought to further investigate whether smokers are, in fact, insensitive to O_3-induced lung injury. In healthy non-smokers, O_3 exposure not only has an effect on FEV_1 as a gross marker of conducting lung function, but also on indicators of conducting and distal airway function measured from gas wash-out techniques –-the Fowler dead space (V_D) and normalized slope of the alveolar plateau (S_N). This indicates that non-smokers are not only affected by O_3 at the level of the conducting airways, but in markers of gas distribution and peripheral airway function as well. These changes occur independently of changes in FEV_1 and demonstrate that investigation of FEV_1 alone may not be sufficient to rule out a lack of sensitivity to O_3. In this investigation, we used these parameters, taken from the CO_2 expirogram, in order to assess the effect of O_3 on gas distribution and conducting and distal airway function in

smokers. More specifically, we hypothesized that smokers would show larger changes in S_N than their non-smoking counterparts.

In the total lung, there is a dose-response relationship such that increases in exposure concentration are related to increasing decrements in FEV_1 and increases in airway resistance. There is, however, substantial inter-subject variability in response. Ultman, *et. al.* hypothesized that this could be explained by inter-subject variability in actual dose delivered to the lung, measured as the difference between the inhaled and exhaled dose. By exposing 60 non-smoking participants, the investigators demonstrated that some of the variability in the change in S_N could be explained by the variability in delivered dose. We hypothesized that, among smokers and non-smokers, differences in the regional delivered dose would be sufficient to explain the larger responses we expected to observe in cigarette smokers.

The regional uptake of O_3 within the lung is related to the chemical composition of the epithelial lining fluid (ELF). Within the ELF, O_3 reacts primarily with low- molecular weight, water-soluble antioxidants. Ozone is removed from inspired air within the conducting airways where the ELF is thickest and these antioxidants are most abundant. Cigarette smoking, however, can have important effects on the ELF composition. Smoking causes mucus hypersecretion and thickening of the ELF, potentially increasing the resistance to O_3 uptake. Additionally, smoking may depress the expression of low-molecular, weight antioxidants, further decreasing the uptake of O_3. Overall, we hypothesized that this would decrease O_3 uptake within the conducting airways, allowing O_3 to penetrate deeper into the respiratory airspaces. This difference in the

longitudinal distribution of O_3 would be responsible for an increased response in S_N in smokers.

In considering whether compromised ELF antioxidants could cause smokers to be differentially responsive to O_3 in their peripheral airways, we used the following combination of human exposure and biochemical techniques to investigate our hypotheses:

Hypothesis 1: Smokers will show decreased responsiveness in markers of conducting airway function, namely FEV_1 and V_D, but increased responsiveness in a marker of peripheral airway function (S_N) compared to non-smokers.

We exposed 30 non-smokers and 30 smokers to either filtered air or 0.30 ppm O_3 for 60 minutes on two separate days while they performed cycle exercise at a workload sufficient to elicit a minute volume equal to 15 L/min per square meter of body surface. Before and after the exposures, participants performed forced spirometric maneuvers from which we obtained FEV_1. Additionally, we obtained a series of CO_2 expirograms from which we calculated V_D and S_N. In order to verify the smoking status of our population, we collected smoking history questionnaires and measured plasma cotinine, a marker of nicotine exposure.

Hypothesis 2: Among smokers and non-smokers, differences in the regional delivered dose will be sufficient to explain larger responses in S_N in cigarette smokers.

Participants were exposed to air or O_3 via a mask apparatus that prevented respiration via the nose. During the air and O_3 exposures, we made real-time, breath-by-breath measurements of ventilation and inhaled and exhaled concentrations of O_3. From this, we calculated minute volume (V_E), tidal volume (V_T), and frequency (f). In addition, we calculated an overall delivered dose and efficiency of O_3 uptake. We compared these dosimetry variables to pre-to-post changes in V_D, S_N, and FEV_1 to determine the role that a dose-response relationship might play in differential responsiveness.

Hypothesis 3: Cigarette smokers experience a decreased antioxidant competency that prevents the normal removal of O_3 in the conducting airways. results in deeper penetration of the inhaled O_3 and larger responses in S_N.

We investigated the overall antioxidant competency of smokers by measuring the uric acid (UA) and ascorbic acid (AA) content of nasal lavage and plasma and the oxygen radical absorbance capacity (ORAC) of nasal lavage. We compared these to pre-to-post changes in FEV_1, S_N, and V_D and to indices of dose in order to determine the role that these antioxidants may play in determining differential responsiveness.

In addition to this chapter, this thesis contains five subsequent chapters. Chapter Two provides additional background information and a review of the literature relevant to this project. Chapter Three provides detailed information as to the methodology employed. Chapter Four contains the results of this investigation and an explanation of the statistical methods used. Chapter Five contains an in-depth discussion of our findings. Chapter Six outlines our conclusions and potential future directions.

Chapter 2 – Background

The purpose of this chapter is to provide the reader with a review of the relevant literature with respect to pulmonary function measures, the dose-response relationship, and the biochemical assays employed in this clinical protocol.

Tropospheric ozone (O_3), an oxidizing component of urban smog, is formed via a photochemical reaction between nitrogen oxide (NO_x) and volatile organic compounds (VOC) (See Figure **2-1**) (Sillman-2002)**.** Major sources of NO_x and VOC include automobile emissions, factory exhaust, and agricultural processes. Reflective of the role of sunlight in the production of O_3, ambient ground-level O_3 concentrations tend to be higher both diurnally and during the summer months (Kasibhatla-2000)**.**

The United States Environmental Protection Agency's National Ambient Air Quality Standards set a maximum one-hour average exposure limit of 0.12 ppm and a maximum eight-hour average exposure limit of 0.08 ppm. Regardless, 104 of 639 monitored U.S. counties violated the primary eight-hour standard between 2003 and 2005. An additional 398 of 639 US counties registered ambient O_3 concentrations greater than 0.075 ppm.

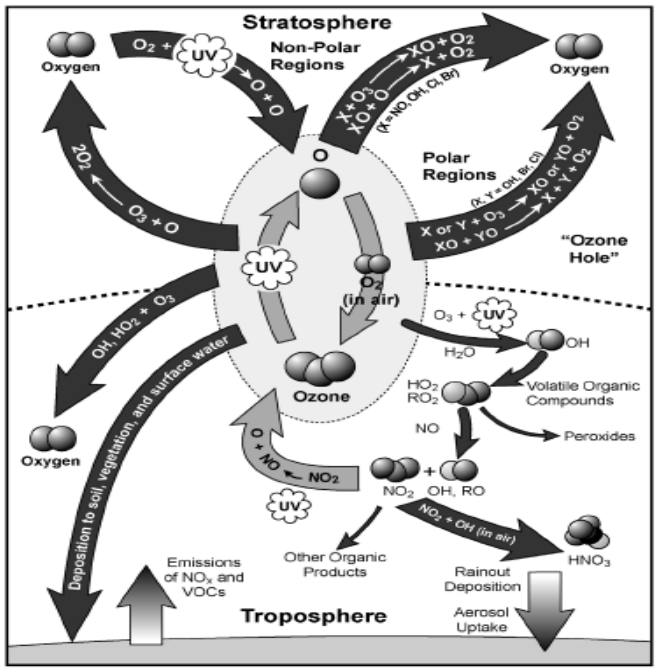


Figure 2-1: Stratospheric and tropospheric ozone formation (USEPA)

The inhalation of O_3 is associated with serious health effects, including decrements in pulmonary function, pulmonary inflammation, alterations in neonatal and childhood lung development, and potentially permanent lung damage (Kunzli-1997, Frischer-1999). In controlled human exposures, volunteers exposed to O_3 report moderate to severe cough, shortness of breath, and pain upon deep inhalation (McDonnell-1999). Epidemiological studies suggest that an elevation in ambient O_3 concentration is related to an increase in both the number of cardiovascular events and exacerbations of respiratory disorders like asthma or chronic obstructive pulmonary disorder (COPD) (Thurston-1997, Romieu-1997). From a socioeconomic standpoint, increases in the ambient O_3 concentration are related to an increase in emergency room visits and school absenteeism (Burnett-1997, Chen-2000, Delfino-1998).

The Environmental Protection Agency defines five populations that are at higher than average risk of suffering morbidity from O_3 exposure (USEPA-1999):

- Individuals with pre-existing lung (or cardiovascular) disease
- Senior citizens
- Individuals who exercise outdoors
- Children
- Individuals who exhibit larger than average responses to O_3 exposure

It is important to study the health effects of O_3 exposure, not only in healthy populations, but in populations that may be especially susceptible to health effects of exposure because of potential alterations to the normal pulmonary milieu (Bhalla-2002). For this reason, this work focused on studying the effects of O_3 on cigarette smokers compared to non-smokers.

Spirometry as an Indicator of Physiological Response to Ozone

The following section outlines the role of FEV_1 as a commonly used indicator of health effects induced by O_3 exposure. The mechanism by which O_3 alters FEV_1, the dose-response relationship between concentration and decrememnt in FEV_1, and the effect of O_3 on FEV_1 in smokers arediscussed here.

Measurement and Interpretation of the Forced Expired Volume in One Second

Perrhaps the most commonly measured indices of physiological response to O_3 come from forced expired spirometry. Specifically, pulmonary toxicologists often use the

forced expired volume measured in the first second (FEV_1) and forced vital capacity (FVC) as major indicators of lung health and airway function. FEV_1 is defined as the volume of air exhaled during the first second of a forced expired maneuver. FVC is defined as the total volume of air exhaled during a forced expired maneuver. These are shown graphically in Figure 2-2.

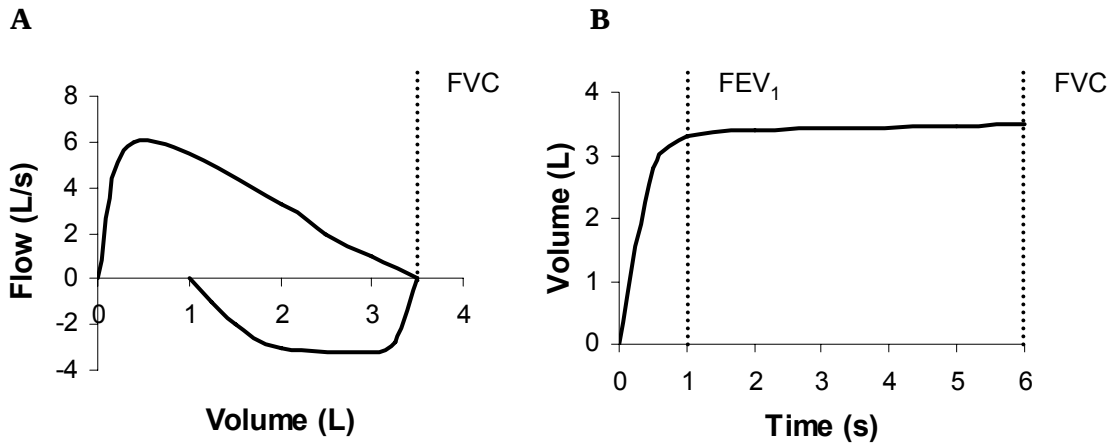


Figure 2-2: Spirometric results from a model subject having an FVC of 3.5 L and FEV_1 of 3.3 L. Panel A shows a flow versus volume loop. Panel B shows a volume versus time curve. FVC can be found by determining the point at which the expiratory phase of the flow-volume loop intersects the x-axis or by comparing the end of the volume-time curve to the y-axis. FEV_1 can be determined by integrating the volume-time curve to one second.

Clinically, the results from a spirometric test are typically assessed based on two criteria – the patient's value of FEV_1 compared to a predicted value obtained from a sample population, and the ratio of FEV_1 to FVC. Predicted values are calculated from regression equations generated by comparing spirometric parameters collected from a sample population with that population's anthropomorphic data (ie, sex, height, weight, age, and race) (West-2004).

The normal range of values for FEV_1/FVC are determined by considering 95% confidence interval of the reference population using Gaussian methods. Values which fall into the lower 5th percentile are then designated as "abnormal." By convention, a value of 0.70 is typically used because it is inclusive of the upper boundary of the 5th percentile for many of the commonly used reference equations (Pellegrino-2005). However, care should be used when applying this convention, because it may result in the overdiagnosis of ventilatory defects, especially in adults over age 40. Diagnostically, a FEV_1/FVC of 0.70 may only be considered significant if a patient presents with symptoms indicative of lung disease or the diagnosis can be confirmed with an additional clinical test (Crapo-2004). Values of FEV_1/FVC either in the 5th percentile or less than 0.70 are considered indicative of an obstructive defect. Values above the 5th percentile or greater than 0.70, with a concommitant deficiet in FEV_1 and FVC, are considered indicative of a restrictive defect.

Table 2.1: Severity ccores for percent predicted FEV_1 (Pellegrino-2005).

Degree of Severity	% Predicted FEV_1
Mild	> 70
Moderate	60-69
Moderately Severe	50-59
Severe	35-49
Very Severe	<35

Values of FEV_1 obtained from a patient's first spirometric asessment are typically evaluated by comparing the percent of the predicted value, calculated from some reference population, to previously described indices which consider two factors -- a person's ability to perform work and engage in daily activities, and the risk or morbidity and mortality at that value of FEV_1 (Becklake-1988, Traver-1979). Classifications based on The American Thoracic Society and European Respiratory Society's consensus statement on the interpretation of lung function tests are show in Table 2.1. Consecutive

measurements of FEV1 are typically compared to a patient's first, or baseline, measurement. Day-to-day and week-to-week variability can range from 5 – 11%. Therefore, in a patient without a prior diagnosis of lung disease, a change of >5% within a single day or a week-to-week change >12% is considered clinically important (Pennock-1981).

Changes in Forced Expired Volume with Ozone Exposure

Over past 30 years, a large amount of experimental evidence has been generated from the study of young healthy adults, indicating that O_3 exposure causes alterations in spirometric markers of lung function. In experiments conducted in resting human volunteers, exposure concentrations > 0.50 ppm are required in order to induce spirometric changes. Considering that environmental ozone concetrations rarely exceed 0.30 ppm, these exposure conditions yield important information, but are probably not physiologically relevant (Bates-1972, Goldsmith-1969). The addition of light to moderate constant or intermittant exercise during exposure, originally added in order to simulate light to moderate outdoor labor, causes decrements in FEV_1 at lower concentrations (ie, <0.50 ppm).

The mechanism by which changes in FEV_1 occur has three major components:

- Cholinergically-mediated bronchoconstriction
- An effort-dependant decrease in inspiratory capacity
- An additional non-effort-dependant decrease in inspiratory capacity

Bronchoconstrictive changes seem to be neurally mediated through bronchial muscarinic receptors and C-fiber activation. Beckett, et. al. exposed 25 healthy men, on four separate occasions, to 0.40 ppm O_3 or room air for 2.5 hours with intermittent treadmill exercise and measured the effect of the exposure on airways resistance (sR_{AW}) and FVC (Beckett-1985). During one pair of exposures (room air and 0.4 ppm O_3), participants received nebulized saline. During the second pair, participants received nebulized atropine. Atropine administration significantly prevented the increase in sR_{AW} associated with O_3 exposure but did not prevent the decrease in FVC. This indicates that acute changes in post-O_3 lung function are, in part, mediated by cholinergic mechanism and some additional mechanism(s) independent of the parasympathetic nervous system.

Hazucha, *et. al.* established that one of these additional mechanism was an effort-dependant and C-fiber-mediated decrease in inspiratory capacity (Hazucha-1995). Because the inhalation of O_3 causes chest discomfort, the authors hypothesized that participants performing post-exposure pulmonary function tests voluntarily minimized the inspiratory maneuver that precedes a forced expiratory maneuver in order to minimize their discomfort. Hazucha and colleagues exposed 14 healthy volunteers to 0.50 ppm O_3 for 2 hours with intermittent treadmill exercise. Post-exposure, participants received either nebulized saline or lidocaine at a dose sufficient to abolish response to inhaled citric acid. The inhalation of lidocaine restored some, but not all of the post-exposure decline in FVC and FEV_1. This demonstrates effort-dependant and potentially neurally dependent components of the decline in pulmonary function. However, because FVC was not fully restored by lidocaine administration, Hazucha speculates that there must be some final and concomitant mechanism by which O_3 causes a decline in inspiratory capacity and hypothesizes that it is neurally mediated but

not lidocaine sensitive. Regardless, these experiments demonstrate that the fall in FEV1 following O_3 exposure is both complex and multi-factorial in nature and changes in FEV_1 should not be interpreted as reflective of any mechanism of action.

The Dose-Response Relationship Between Ozone and Forced Expired Volume

Both meta-analyses and individual investigations have recognized the relationship between cumulative dose and response. McDonnell *et. al.* exposed 135 healthy male volunteers, performing intermittent heavy exercise (V_E = 65 L/min) to six concentrations of ozone for two hours and demonstrated a relationship between concentration and decrement in FEV_1 that could best be described by a sigmoidal fit.(Mc-Donnell-1983).

Changes in FEV_1 with exercise seem to be related to not only the exposure concentration, but to the cumulative inhaled dose, with higher cumulative doses being associated with larger decrements in lung function. For example, Horstman, *et. al.*, exposed subgroups of healthy volunteers to 0, 0.08, 0.10, and 0.12 ppm O_3 for 6.6 hours with intermittent exercise (Horstman-1990). While participants experienced no change in FEV_1 with air exposure, (+0.68%), for each of the exposure conditions, subjects experienced decrements in FEV_1 of 7.06, 6.83, and 12.3% respectively. Further analysis of this data by the authors indicates that the time course of the change in FEV_1 is related to components of cumulative dose -- both the exposure concentration and duration of exposure (Larsen-1991).

McDonnell, *et. al.* first described a model which unified the findings of many of the previous studies investigating the dose-response relationship (Mc-Donnell-1983). McDonnell considered data from 485 individuals exposed to six different O_3 concentrations over two hours performing one of three levels of intermittent exercise and fit a series of different models to the data. The best fit model is shown in Equation 2-1, where β_n represent the coefficients of a logistic fit, Age represents the age of the participant at the time of exposure, C represents the exposure concentration, V_E represents the minute volume, and T represents the time of exposure.

$$\Delta FEV_1 = \frac{\beta_1(1+\beta_2 Age)}{1+\beta_5 e^{-\beta_3(CV_E^{\beta_4})(1-e^{-\beta_6 T})}} \qquad \text{Equation } \mathbf{2-1}$$

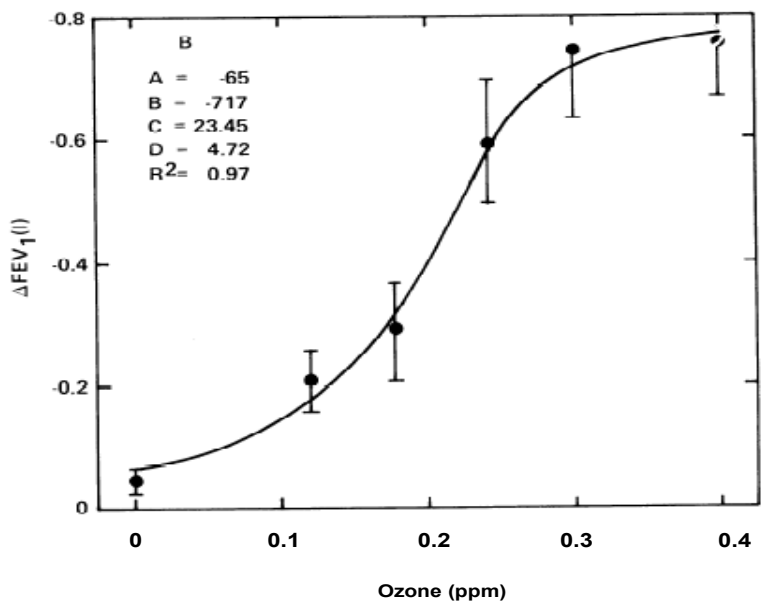


Figure 2-3: Dose-response curve comparing ozone concentration (ppm) to change in FEV1 (L). Data reflect results from 135 male volunteers exposed to one of six exposure concentrations. A, B, C, and D are coefficients from a logistic fit. (Mc-Donnell-1983)

McDonnell and others, however, have recognized that, even with multi-parameter dose-response models, there is substantial inter-subject variability in the response of FEV_1 to O_3 (McDonnell-1985). A dose-response curve, demonstrating the high degree of inter-subject variability is shown in Figure 2-3.

Changes in Forced Expired Volume with Ozone Exposure in Cigarette Smokers

In toxicological research, it is important to generate information which may be used to predict health effects in both healthy individuals and individuals who, because of some pathology or concomittant risk factor, may be more susceptible to health effects. Cigarette smokers, one group who because of smoking-induced pathology may be at an increased risk of health effects, have been the subject of three investigations measuring the effects of O_3 on pulmonary function. A summary of the data from these experiments is shown in Figure 2-4.

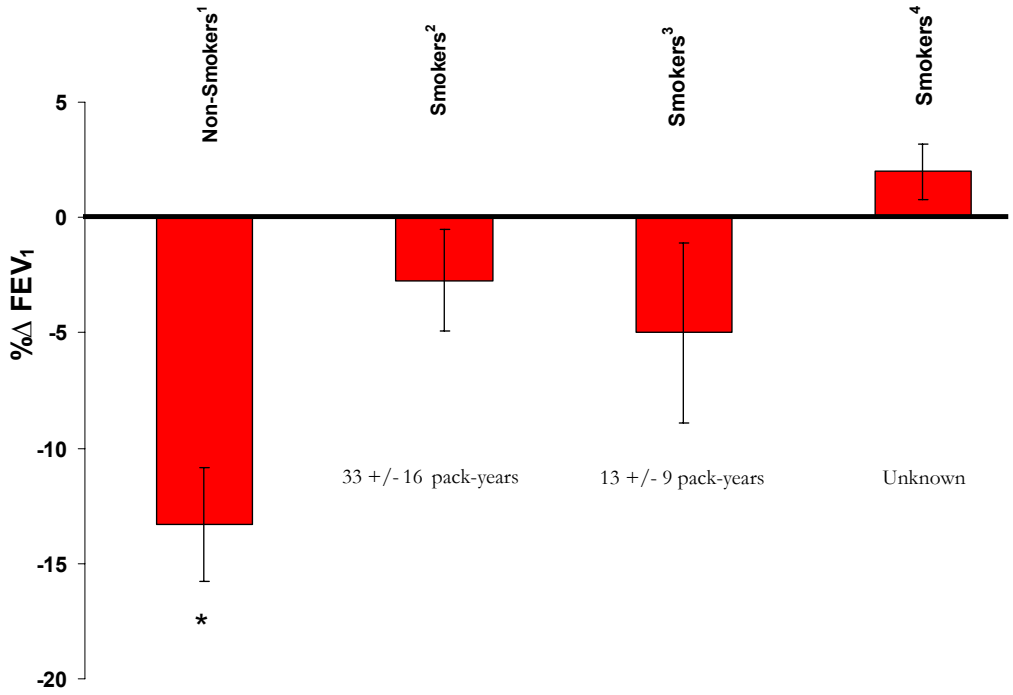


Figure 2-4: Changes in forced expiratory volume in smokers exposed to ozone. Perecnt change in FEV_1 data from smokers measured by [2]Foster, et. al., [3]Frampton et. al., and [4]Kerr, et. al.are compared to non-smokers measured by [1]Ultman et. al. Only the non-smokers demonstrate significant decrements in FEV1. When known, smoking history is shown below the data. Error bars are standard error about the mean.

In the earliest study, Kerr et. al. measured changes in pulmonary function in 20 volunteers (10 smokers and 10 non-smokers) with no history of pulmonary disease (Kerr-1975). While Kerr does not report percent predicted values, pre-exposure values of FEV_1 and FVC for the two populations were not significantly different. Participants were exposed to 0.50 ppm for six hours in an environmental chamber. The authors report that the population of 20 individuals had significant changes in markers of pulmonary function (ie, FEV_3, specific airway conductance, and airway resistance). However, although the study was not designed to investigate the difference between smokers and non-smokers, when smokers were considered as a separate population, they failed to demonstrate significant changes.

Subsequently, Foster *el. al.* and Frampton *et. al.* specifically investigated the impact of O3 exposure on the pulmonary function of cigarette smokers. Frampton, *et. al.* compared the FEV_1 responses of 56 never-smokers to 34 smokers with a an average pack-year history of 13 ± 9 pack-years (Frampton 1997). Participants were exposed to 0.22 ppm O_3 for 4 hours while performing intermittent exercise. As a population, smokers were less responsive in FEV_1 to than non-smokers. In number, fewer smokers (12%) than non-smokers (28%) experienced a change in FEV_1 greater than 15%. Smokers also experienced fewer respiratory symptoms then non-smokers. The authors hypothesize that smokers may represent a self-selected group who are generally less responsive to an oxidant insult. Alternatively, smoking may decrease the responsiveness to O_3.

Foster, *et. al.* exposed 34 smokers before and after completion of a smoking cessation program to 0.40 ppm O_3 for 2 hours (Foster-1991). Participants smoked an average of 33±16 pack-years. Before completion of the cessation program, smokers failed to experience a decrememnt in FEV_1 to O_3 exposure. Smokers who were exposed after completion of the six month cessation program did not signigicantly improve their baseline FEV_1 nor did they experience changes in FEV_1. This indicated that smoking cessation does not restore this O_3-induced response. Smokers did, however, improve baseline mid-maximal flow (MMF) with cessation and experienced decrements in MMF roughly equal to the degree of improvement. This indicates that smokers may regain small airway responsiveness with cessation.

Alteration of pulmonary function changes with cigarette smoking is probably not related to short-term exposure, but to pathological alterations in bronchial function caused by chronic exposure. For example, the FEV_1 response of individuals exposed concomittantly to cigarette smoke and O_3 is not different than the response of individuals exposed to O_3 alone (Shephard-1983). Consideration of these pathological adaptations to smoke exposure may be the key to understanding differences in response.

Smokers in these three studies clearly exhibited a lack of response in FEV_1 to O_3 exposure. However, caution should be used in saying that smokers are unresponsive to O_3. FEV_1 is an important and convenient clinical tool because of the availability of commercial spirometry equipment, but its interpretation has a few important limitations. Clinical spirometry is effort dependant and the assessment of test quality can be somewhat subjective. FEV_1 is dependant on total lung volume and inspiratory effort. Discernment of bronchoconstriction in light of a large decrement in FVC can be difficult. Finally, FEV_1 gives no information about respiratory airway function or ventilation distribution. In order to definitely determine whether O_3 causes health effect in smokers, it is important to investigate additional markers of pulmonary function.

The CO_2 Expirogram as an Indicator of Physiological Response to Ozone

Gas wash-out techniques offer important information about ventilation distribution and conducting and respiratory airway function. Fowler first described the three phases of the N_2 wash-out curve in 1948 (Fowler-1948). Since, nitrogen and other gas wash-out techniques have been used extensively to measure the volume of the conducting airways and provide information about respiratory airway function and the distribution of

ventilation. In our investigation, we considered the CO_2, rather than N_2, expirogram. Using the CO_2 expirogram has a number of distinct advantages. First, it requires no introduction of a test gas and an unlimited number of maneuvers can be collected without affecting alveolar gas tension. Because CO_2 is evolved from the lung, the CO_2 expirogram gives information about both gas distribution and exchange. Second, because it does not require the breathing of 100% O_2, it can be monitored continuously during exercise. Finally, because capnography is an important clinical tool, the quality and accuracy of measurement equipment available is quite high.

Dead Space and the Normalized Slope of the Alveolar Plateau

The following section gives information about the interpretation of V_D and S_N, both in terms of its significance in healthy populations and its presentation in pathology. Additionally, this section considers the effect of ozone exposure on the shape of the CO_2 expirogram.

Gas transport in the lungs occurs by two processes – convection and diffusion. Convective transport is a function of the linear velocity of the gas and is inversely proportional to the cross-sectional airway of the lung. Thus, as gas is transported into the lung, velocity decreases as a function of airway generation until net velocity becomes zero and gas is transported simply via diffusion. Upon inspiration, gas with a low P_{CO2} is transported into the lung. This low P_{CO2}, relative to the high P_{CO2} of the alveolar region, creates a concentration gradient (West-2004). The result is net diffusion towards the lung. These transport processes are shown schematically in Figure 2-5.

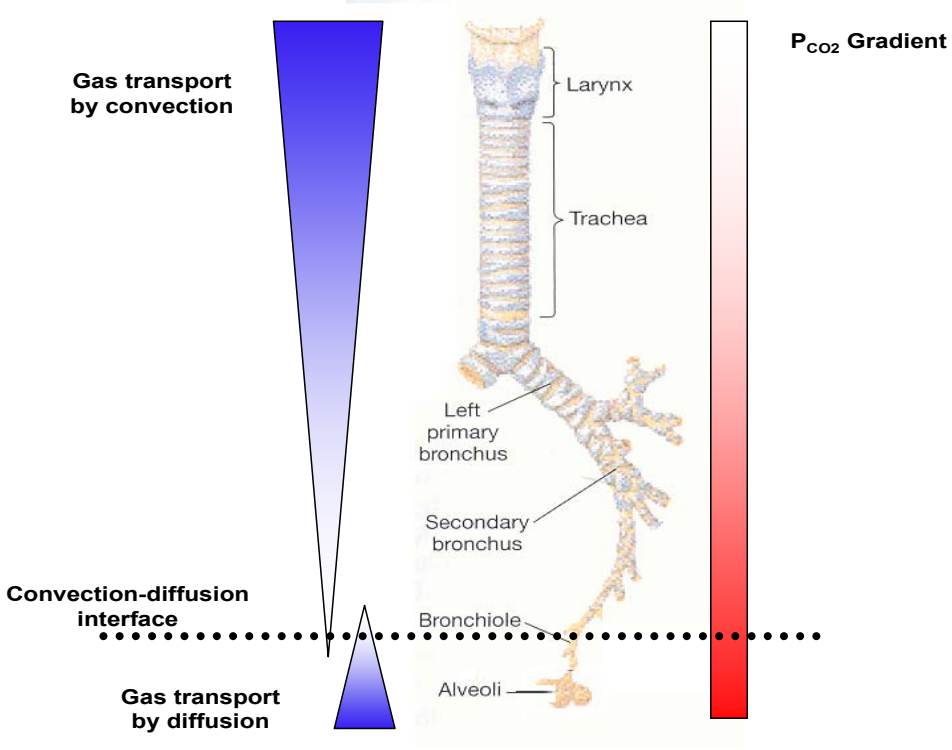


Figure 2-5: Gas transport in the lung. Upon inspiration, gas is transported toward the deeper lung primarily by convection, with linear velocity inversely related to cross-sectional area. The concentration gradient of CO2 results in net diffusion towards the mouth, with the transition from convection to diffusion occuring at approximately the level of the respiratory bronchioles.

A representative CO_2 expirogram is shown in Figure 2-6. The CO_2 expirogram is obtained by measuring the P_{CO_2} of expired gas and plotting it against cumulative expired volume. The result is a sigmoidal curve with three distinct phases, reflective of the gas transport processes in the lung. Phase I represents inspired gas with a low P_{CO_2}, which fills the conducting airways. Phase II represents the transition from conducting airway gas, having a low P_{CO_2} and transported mainly via convention, to alveolar gas, having a higher P_{CO_2} and transported mainly via diffusion. This interface is thought to occur at approximately the level of the respiratory bronchioles and is, thus, largely reflective of conducting airway volume or anatomic deadspace (V_D) (Folkow-1955). Phase III represents purely alveolar gas with a higher P_{CO_2} compared to ambient air and is often

referred to as the alveolar plateau (Fletcher-1981**)**. Phase III typically displays some degree of upward sloping. This has important physiological significance and is often analyzed by regressing phase III, normalizing by the average volume of CO_2 exhaled (V_{CO_2}) and describing a normalized slope of the alveolar plateau (S_N)

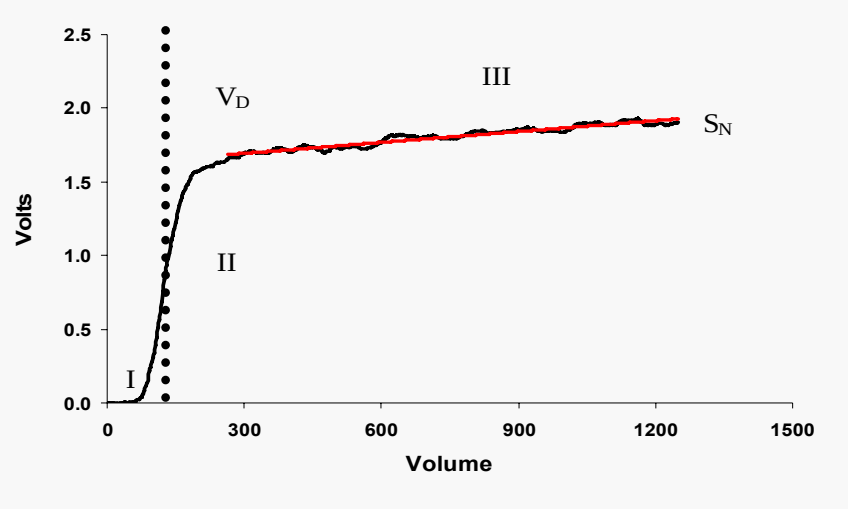


Figure 2-6: The CO_2 expirogram. Exhaled volume is shown on the x-axis and the voltage output of the capnometer is shown on the y-axis. Graph labels indicate the location of phases I, II, and III, the anatomic dead space (V_D), and the slope of the alveolar plateau (S_N).

The Significance of the Dead Space

Important to the interpretation of data presented in later sections is an understanding of the distinction between the different types of dead space. Enghoff described the volume of the dead space in 1938 as the "volumen inefficax" or "volume of wasted gas" (Enghoff-1938)**.** Serveringhaus and Stupfel later defined the dead space as "the portion of expired air which does not remove CO_2 from pulmonary blood (Severinghaus-1957). This dead

pace is comprised of two components – the anatomical dead space (also called the Fowler, airway, or series dead space) and alveolar dead space (also called the parallel dead space). The combination of these two dead spaces yield the physiological dead space (also called the Bohr dead space). A schematic of these relative dead spaces is shown in Figure 2-7.

Physiological dead space is calculated by solving the Bohr equation using arterial CO_2 in lieu of alveolar CO_2 and reflects both alveolar and anatomic dead space (Bohr-1909, Riley-1951). In healthy individuals, the alveolar dead space is negligible (Fishman-1954). Analysis of the two components of the physiological dead space can be done by independently considering phase II and phase III of the expirogram.

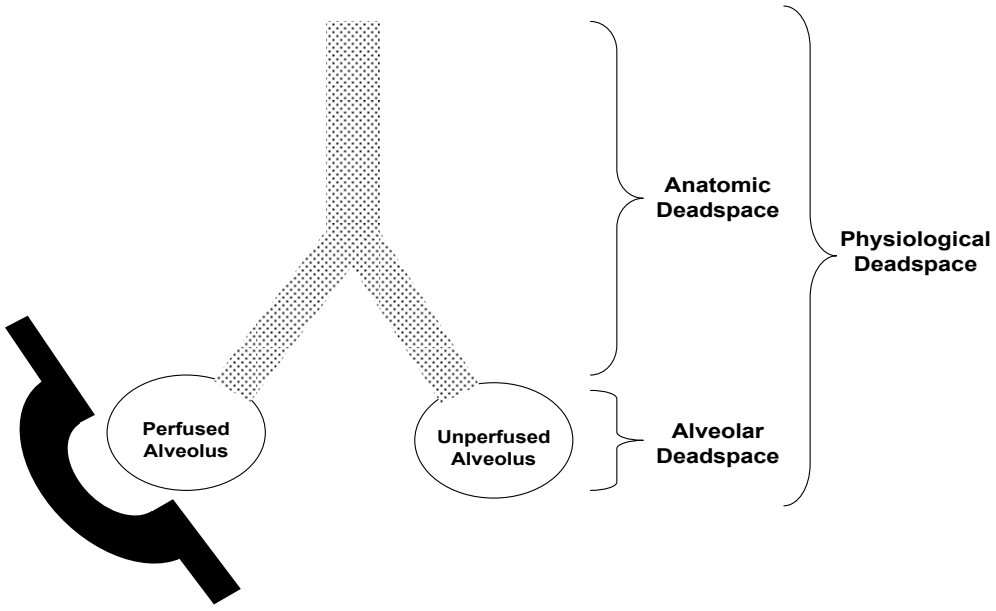


Figure 2-7: Schematic of the respiratory deadspaces. The physiological deadspace is the sum of the anatomical and alveolar deadspaces. The anatomical deadspace is comprised of the volume of the conducting airways while the alveolar deadspace is comprised of unperfused alveoli. Adapted from Severinghaus, *et. al.*

Anatomical dead space is identified by measuring the midpoint of phase II of the CO_2 expirogram, as described above, and is a function of the convective transport of gas distally in the lung and diffusive transport of gas apically during inspiration. The boundary condition between convection and diffusion typically occurs at the level of the respiratory bronchioles. For this reason, the value of V_D obtained is indicative of conductive airway volume. In humans and animals, the accuracy of V_D calculated from consideration of phase II of the capnogram has been validated both by comparison to values obtained from other wash-out tests (i.e., He, SF_6, and O_2). In animal models, V_D values have been compared to the volume of the conducting airways measured by filling the extrapulmonary airways with water. These measurements have been made both in healthy human subjects and in a feline model of acute lung injury (Folkow-1955, Bartels-

1954). In humans, the average value for V_D is 156 mL in men and 104 mL in women (Fowler-1948).

Anatomic dead space is dependant upon a number of parameters. First, and foremost, V_D is dependant upon lung size. Hart et. al. used a N_2 wash-out technique to measure V_D in 73 normal, healthy volunteers between 4 and 42 years of age (Hart-1963). Hart noted a strong relation ($r^2 = 0.92$) between V_D (mL) and height (cm). Height is related to lung size and is often used as a surrogate for markers of lung morphometry (West-2004). Factors which affect the convective transport of gas distally upon inspiration and the anatomy of the conducting airways also affect V_D. Factors which affect the airway geometry will affect the size of the dead space. For example, the administration of methalcholine, a bronchoconstrictive agent, or albuterol and isoproterenol, both bronchodilators, affect conducting airway diameter and, consequently, V_D (Folkow-1955, Lollgen-1978). Hypoxia constricts the bronchi and decreases V_D (Severinghaus-1957). The position of the head and neck, presence of any artificial dead space (e.g., the placement of endotracheal tube), and changes in transmural pressure affecting airway diameter can alter V_D (Hedenstierna-1975).

Finally, factors which influence the location of the boundary condition between convective and diffusive transport will alter V_D independent of changes in morphometry. Increasing or decreasing inspired flow will move the boundary distally or apically, respectively. Breath holding will allow for apically-directed diffusion of CO_2 and underestimating of V_D (Engel-1973, Shephard-1957, Bartels-1954). Therefore, in order to minimize inter- and intrasubject variability, it is important that V_D be measured using standardized flow rates and breathing patterns.

Significance of the Normalized Slope

Phase III of the CO_2 expirogram is typically used clinically in the determination and interpretation of end tidal PCO_2 with rtelation to both physiological dead space and acid-base homeostatis.. However, phase III, and specifically the slope of phase III (S_N), contains information about CO_2 elimination and distribution within the alveolar region.. This information can be useful in the assesment of health effects within the distal airspaces. In healthy humans with normal pulmonary function, S_N is positive and is a result of both the airway anatomy and the regional efficiency of gas exchange. Because regional gas exchange efficiency is determined both by the degree to which the lung is ventilated and perfused, alterations in either of these components can change S_N.

Krogh and Lindhard first measured the composition of alveolar air during the respiratory cycle and determined that the concentration of CO_2 and O_2 in an exhaled breath is proportional the time during which the sample is taken (Krogh-1914). When Aitken and Clark-Kennedy measured the partial pressure of exhaled CO_2 as a function of exhaled volume using a series of Douglas bags filled serially with exhaled gas, they noted the upward slope of phase III and attributed it to the continued evolution of CO_2 across the respiratory membrane (Aitken-1928). Specifically, the authors postulated that during exhalation, CO_2 continues to evolve across the respiratory membrane but, because alveolar volume is decreasing, the concentration of C_{O2} within the alveoli increases. Therefore, as exhalation continues, the P_{CO2} of the expirate rises. This would occur until alveolar and capillary CO_2 tensions become equal.

In mathematical models considering a lung with a single alveolus, the slope of the expirogram is well explained by the continued evolution of CO_2 (DuBois-1952, DuBois-1954). Additionally, work by Cochrane, *et. al.* indicates that with exercise, S_N increases (Cochrane-1982). The authors attribute this to an increase in the rate of CO_2 flux across the respiratory membrane. While, in healthy lungs free of obvious pathology, the continued evolution of CO_2 is mathematically sufficient to explain the slope of the plateau, experimental evidence suggests that the mechanism is more complex (Grønlund-1987) . Furthermore, continued evolution of CO_2 fails to describe the positive S_N observed in subjects with any degree of lung pathology (ie, asthma or COPD) (Guy-1976).

The lung does not behave as a single well-mixed alveolus. To assume this structure in the modeling of S_N fails to appreciate the complexity of the pulmonary morphometry. Both healthy and, to a greater extend diseased, lungs display non-uniformity in the degree to which different regions empty upon exhalation as a function of both alveolar density and airway diameter, and regional gas tensions. Considering the upright human lung, ventilation is distributed such that the basal lobes of the lung are ventilated more highly than the apical lobes. The result, in combination with non-uniformity in regional perfusion, results in an increase in CO_2 tension oriented apically to basally. These regions, because of differences in airway diameter and compliance (the product of which is the RC time constant, τ), empty at different rates (Engel-1983). The sequential emptying of parallel alveolar units, with differing P_{CO_2}s explains much of the intersubject variability not explained by the continued evolution of CO_2 (Grønlund-1987, Paiva-1981)

Changes in the Expirogram with Pathology

In subjects exhibiting pathology typically associated with changes in airway diameter and gas exchange efficiency, S_N increases. This has been validated in both human and animal models. Arnold, *et. al.* used a lamb-model of saline-induced lung injury to investigate the effects of acute lung injury on the shape of the expirogram (Arnold-2000). The investigators found that, in healthy animals, S_N was positively correlated with changes in lung volume, induced by altering V_T and measured by N_2 washout. This is supported published work in animal models (Stenz-1998) and in young children (Ream-1995). Additional mathematical models considering the lung as a single alveolus indicate that the cross-sectional area of the lung is related to S_N in humans (Farmery-1995). After saline-induced lung injury, however, the change in lung volume was no longer positively related to S_N. This indicates that the single alveolus model is not appropriate for use in models of pathology. Models which consider differences in acinar structure are a better fit (see above).

The slope of the alveolar plateau has been characterized in human populations with lung pathology. You, *et. al.* measured the slope of the alveolar plateau in 10 healthy and 30 asthmatic patients with varying degrees of bronchoconstriction (You-1994) and found that the slope of the alveolar plateau is inversely related to % predicted FEV_1. The investigators concluded that the slope of the plateau is related to the degree of heterogeneous airway obstruction.

Kars, *et. al.* measured S_N in 28 control patients, 12 patients with asthma, and 29 patients with emphysema. (Kars-1997). The investigators found that S_N in asymptomatic asthmatic patients is elevated compared to healthy controls. Compared to asymptomatic asthmatic patients, S_N is elevated in asthmatics during an exacerbation and comparable to S_N in patients with moderately obstructed emphysema. The normalized slope of the alveolar plateau is the highest in patients with severely obstructed emphysema. This indicates that, compared to asthmatic and control patients, the degree of pathology experienced by cigarette smokers is greater.

Verbanck, *et. al.* investigated the degree to which S_N is elevated in smoking populations by investigating S_N in multiple breath washouts (Verbanck-2004). They found that S_N begins to increase in patients with more than 10 pack-years. S_N in patients with >60 pack-years is four times higher than that of non-smoking controls. When cigarette smokers were studied using single breath He and SF_6 washout techniques, the slope of the plateau was found to be related to the degree of fibrosis and bronchiolar inflammation (Van Muylem-1992). Because He and SF_6 do not equilibrate with the blood compartment, the results from He and SF_6 gas washouts give information exclusively about airway function and support the idea that airway heterogeneity is responsible for the positive S_N in asthmatic and smokers with and without overt pathology.

Changes in the Expirogram with Ozone Exposure

Silverman *et. al.* first measured the effect of O_3 exposure on a gas washout. The investigators exposed 28 human subjects for two hours to a range of O_3 concentrations (0-0.75 ppm) while they performed light, intermittent exercise in an environmental chamber (Silverman-1976) and found that O_3 exposure causes an increase in S_N of a N_2 washout. Additionally, the investigators found that the slope of the plateau, expressed at the percent of the air exposure value, was related to the exposure dose. Like the change in FVC or FEV_1 seen with O_3 exposure, the relation between dose and slope could be described by a sigmoidal function. The investigators did not attempt to relate the change in slope to the change in FEV_1.

Taylor, *et. al.* measured the effect of continuous O_3 exposure on S_N, determined from the CO_2 expirogram (Taylor-2007). The investigators exposed 60 healthy human volunteers to 0.25 ppm O_3 while they performed cycle exercise at a sufficient workload to maintain V_E=30 L/min. As with measurement of the N_2 washout, O_3 caused an increase in S_N. The investigators found that individual dose was correlated to the increase in S_N with O_3 exposure (Ultman-2004). Additionally, S_N was marginally correlated to the change in FEV1 (p=0.051). The investigators concluded that the CO_2 expirogram offers a valuable tool for the assessment of O_3-induced health effects.

Antioxidant Status and Its Alteration with Smoking

The lung is an important boundary between the internal and external environments, across which gas exchange occurs. In order to support this process, the respiratory membrane must be sufficiently thin to allow for the rapid diffusion of CO_2 and O_2. The thinness of the membrane, however, makes it especially susceptible to damage from the external environment. The epithelial lining fluid (ELF) is important in the defense of the delicate, distal airspaces from damage from inhaled toxins and pollutants.

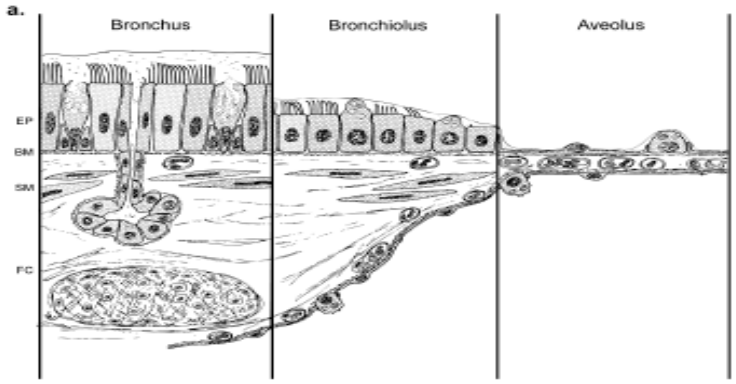
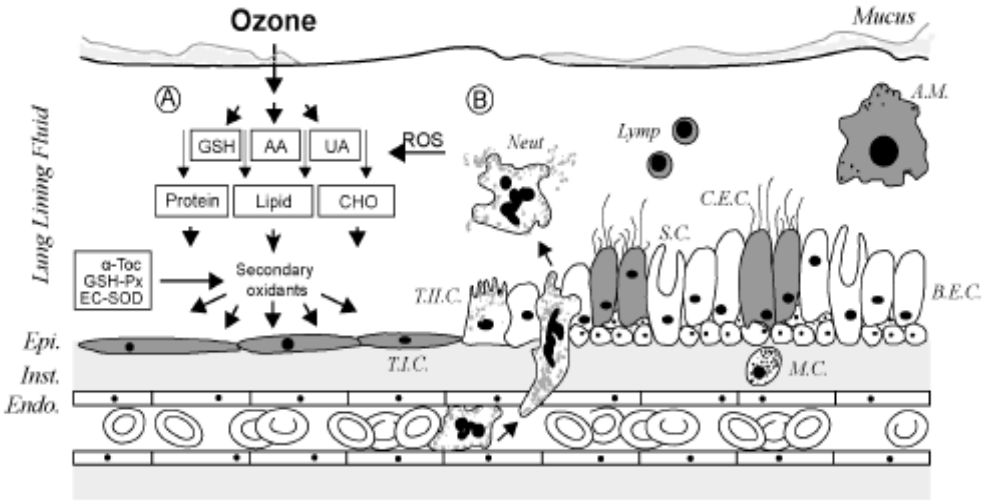


Figure 2-8: Regional airway anatomy. Compared to the distal airways, the bronchi contain cuboidal, cilliated epithelial cells, glands, and a thick fluid layer. In the alveoli, glands are absent and the epithlium and fluid layer are thinner **(**Weibel--1980**).**

The ELF consists of stacked gel-like and aqueous layers that coat the epithelium from the nose to the alveoli (Widdicombe-1997) (Figure 2-8). The ELF is heterogeneous such that its character and composition differ regionally. In terms of its antioxidant capacity, the ELF contains a number of water- and lipid-soluble, low molecular weight antioxidants, antioxidant enzymes, metal-binding proteins, reactive proteins, and unsautrated fatty

acids (Cantin-1987**,** Cross-1984); O_3, as an oxidizing gas, is scavenged by these antioxidants (Figure **2-9,** Panel A). Of particular interest in O_3-related research are the water-soluble, low molecualr weight antixodants uric acid (UA), ascrobic acid (AA), and glutathione (GSH) because of their high rate of reaction with O_3 (Giamalva-1985, Kermani-2006). Because they are preferentially oxidized by O_3, compared to many of the other antioxidants in the ELF, they are often referred to as "sacrificial" antioxidants (Kelly-1995) Reaction of O_3 with ELF antioxidants, especially polyunsaturated fatty acids, is the first step in a cascade of reactions (Figure 2-9, Panel B) in which subsequent free radical species may be produced. These subsequent free radicals are responsible for the toxicological effects of O_3 (Pryor-1991). Therefore, the antioxidant content of the ELF is important, not only in scavenging O_3, but in quenching the secondary radical species that are produced.

A



B

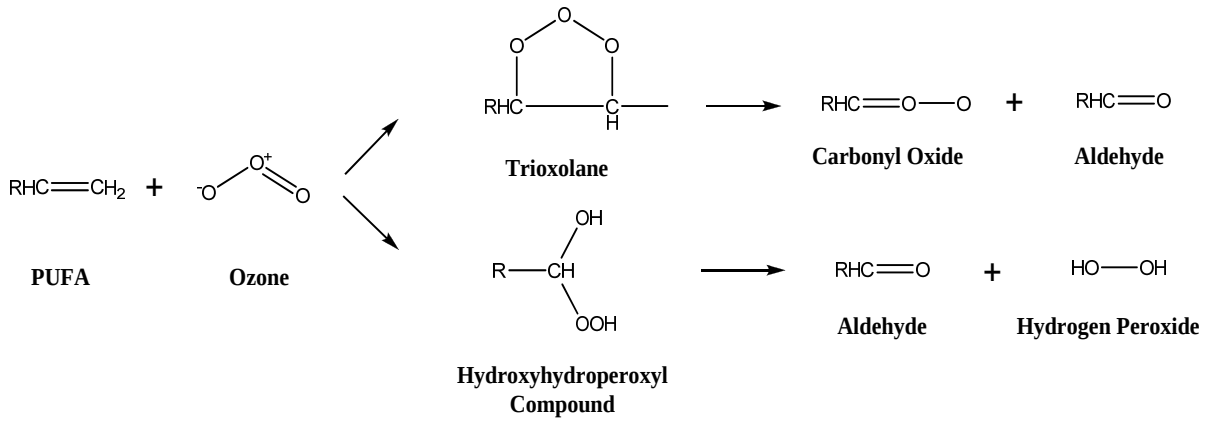


Figure 2-9: Interaction of ozone with the epithelial lining fluid. Ozone is scavenged, primarily, by low molecular weight, weight antioxidant and secondarily by proteins, lipids, and carbohydrates (Panel A) (Mudway-2000). The reaction with the ELF, especially polyunsaturated fatty acids (PUFA) generates secondary oxidant species, which are responsible for the toxicolofical effects of O_3 (Panel B) (Pryor-1991).

Uric acid is a byproduct of purine metabolism and is created from xanthine via a reaction catalyzed by xanthine oxidase (Figure 2-10). Purines are found in large quantities in meat products (Lee-2006) and, as a result, high plasma UA concentrations are often

associated with meat-rich diets (Eggebeen-2007). The intake of alcohol, in combination with a meat-rich diet, can further amplify blood UA concentrations. Because humans lack the enzyme uricase (although it is still expressed within the human genome), UA is not further metabolized and is excreted by the kidney (Halliwell-1996). While UA is typically soluble, a number of pathologies (including gout, arthritis and urolithiasis) are caused by its crystallization (Kelley-1973).

In aqueous solutions, O_3 degrades to form hydroxyl radicals (Hoigne-1976). Uric acid, upon reaction with hydroxyl radicals, is oxidized to form urea, allantoin, oxonic acid, and parabanic acid. Of these, allantoin and urea are the most common degradation products (Bernhard-1993, Meadows-1986) (Figure **2-10**). It is often referred to as a "suicidal" antioxidant because of the inability of UA to recycle itself (Kelly-1995).

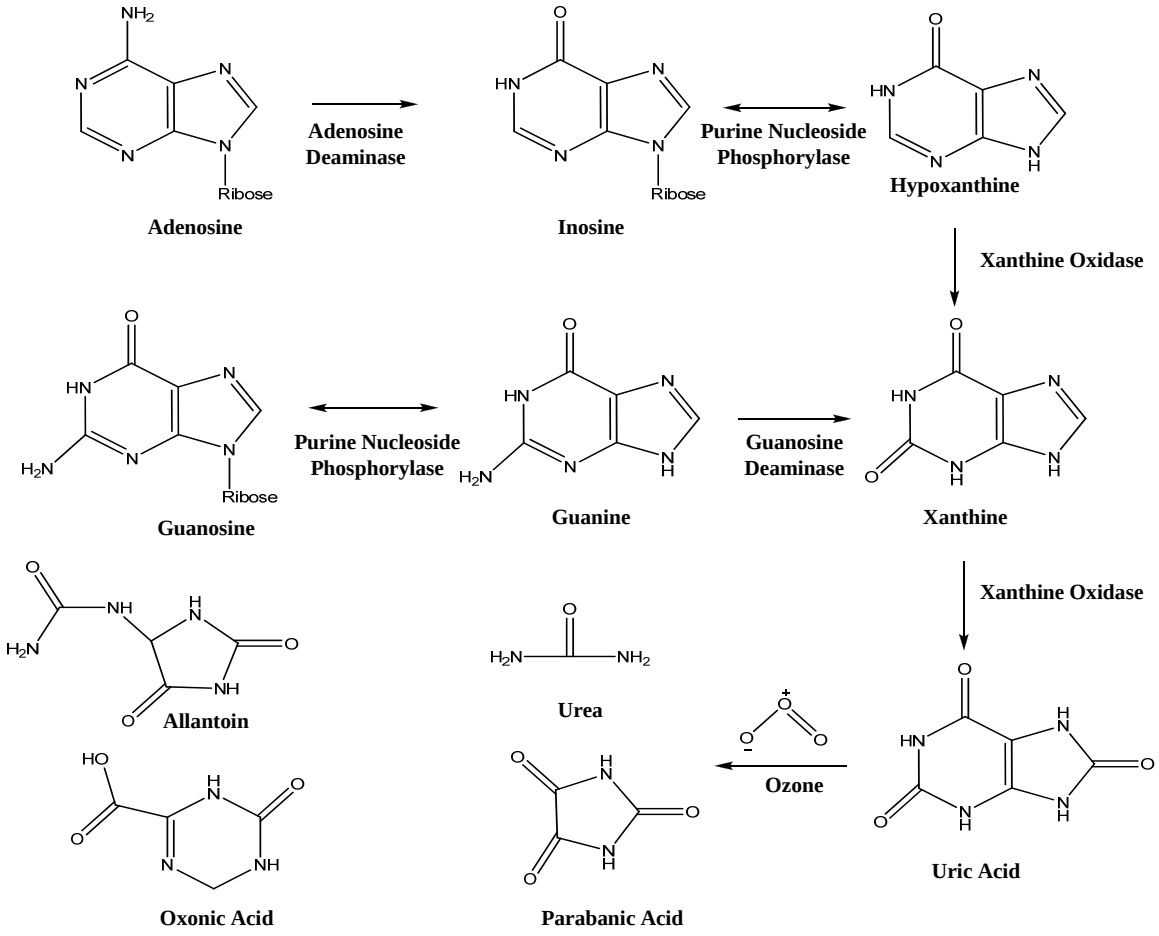


Figure 2-10: The synthesis and oxidation of uric acid. Uric acid is a byproduct of the metabolism of adenosine and guanosine. The oxidation of uric acid by ozone generates the by products allantoin, urea, oxonic acid, and parabanic acid.

Glutathione is a low molecular weight peptide containing glycine, cysteine, and glutamic acid and is an anion it physiological pH (Meister-1988). It is synthesized in two steps; first, glutamic acid is joined with cysteine in a reaction catalyzed by g-glutamyl-cysteine synthetase. In a subsequent reaction, glycine is added by glutathione synthetase (Figure 2-11). The first reaction is limited by the availability of cysteine and inhibited by the presence of GSH. Glutathione is an important intracellular antioxidant and plays a crucial role in the quenching of oxidants generated within the mitochondria. While

intracellular GSH is found in millimolar concentrations, the concentration of extracellular glutathione is < 2 μM (Kelly-1999). Depletion of intracellular GSH is thought to be associated with a myriad of diseases, including cirrhosis, diabetic nephropathy, and cardiovascular disease (Lash-2006).

Reduced glutathione provides protection in two unique ways. First, GSH may scavenge oxidizing radicals directly via reduction of its thiol group, resulting in the formation of glutathione disulfide (GSSG). Secondly, GSH may serve as a substrate for glutathione peroxidase. Glutathione peroxidase catalyses the reduction of H_2O_2, and other hydroperoxides. Oxidized glutathione is recycled to GSH by glutathione reductase (Figure 2-11) (Fridovich-1986).

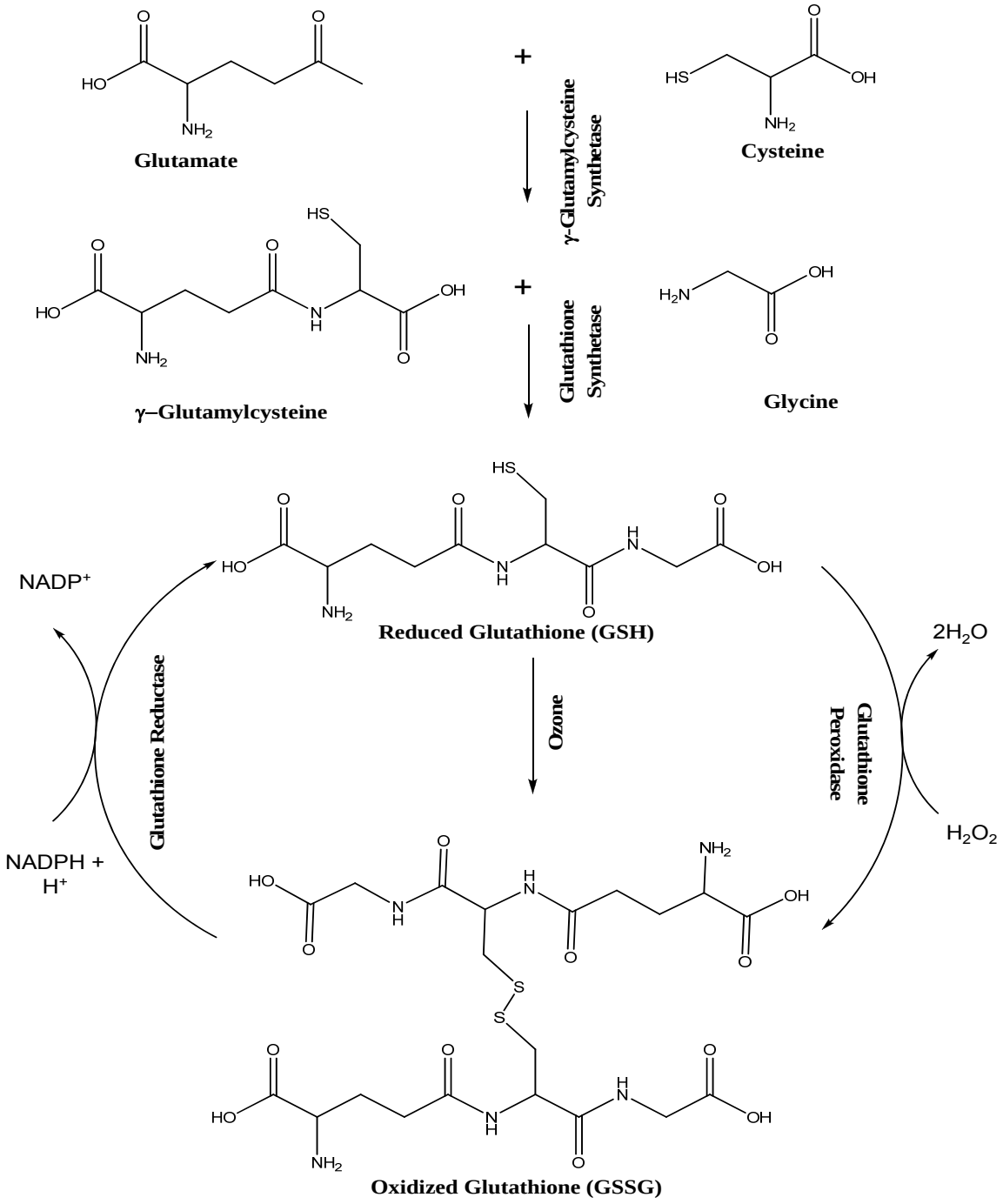


Figure 2-11: The synthesis, oxidation, and recycling of glutathione. Glutathione is synthesized enzymatically from cysteine, glycine, and glutamate. Glutathione may be oxidized directly or enzymatically using glutathione peroxidase. Oxidized glutathione is recycled by glutathione reductase.

Ascorbic acid, or vitamin C, is an important water-soluble vitamin and is not synthesized by humans. As a result, humans rely on dietary sources of AA. Most of the current understanding of the importance of AA comes from studies done in plasma; AA may protect the vascular bed from oxidative damage and is important in Vitamin E recycling (May-2003, Li-2003). Because of its high rate of reaction with radicals, high solubility in plasma, easy excretion by the kidneys, and low toxicity, AA is a commonly used by investigators studying the role of oxidative stress in various pathologies (Jablonski-2007). Deficiency of dietary AA results in scurvy (Wang-2007).

Ascorbic acid reduces free radials via oxidation of its carbon-carbon double bond. The first step in AA oxidation is the generation of the ascorbate free radical. (AFR) This product is stable at physiological pH. The AFR may reduce other AFRs, regenerating AA and dehydroascorbate (DHA), or continue to be oxidized by other free radical species to form DHA. Dehydroascorbate is unstable at physiological pH. Unless it is recycled, DHA rapidly degrades to 2,3-diketo-1-gulonic acid (DGA) (Buettner-1993). The oxidation of AA is completely reversible until the degradation of DHA to DGA, allowing for the recycling of DHA to AA (Figure **2-12**) (Mendiratta-1998).

The degree to which AA is recycled in the ELF is unknown. In plasma, AA concentrations are maintained through the recycling of the AFR and DHA to AA by red blood cells and the vascular endothelium. In the endothelium, the AFR is recycled to AA by thioredoxin reductase in an NADPH dependant reaction, or by mitochondrial AFR reductases in a NADH dependant reaction. In cases of oxidative stress, in which the normal endothelial mechanisms are not sufficient to recycle the AFR, AFRs may oxidize

each other, recreating AA and generating DHA. The DHA can then be reduced to AA by GSH (May-1998). In red blood cells, DHA can enter the cell via facilitated diffusion through the GLUT1 transporter and be reduced in either a GSH or NADPH dependant manner. Dehydroacorbate can be reduced by GSH alone or enzymatically using thioltransferase glutaredoxin and GSH as a cofactor. As in the endothelium, DHA can be reduced using thioredoxin reductase and NADPH as a cofactor (May-2000).

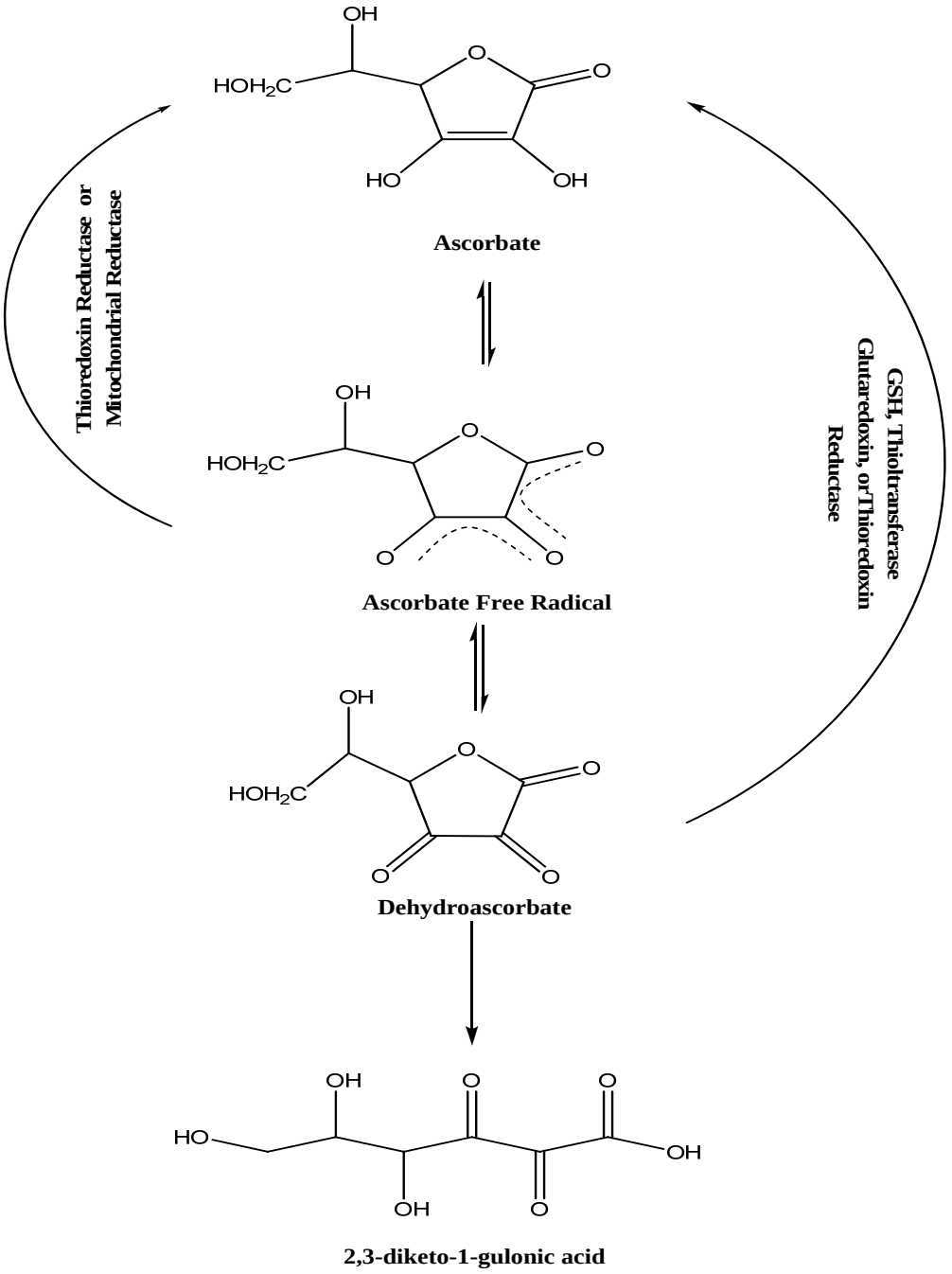


Figure 2-12: The oxidation and recycling of ascorbic acid. The oxidation of ascorbic acid forms the ascorbate free radical. The ascorbate free radical can auto oxidize to form dehydroascorbate and ascorbate or be enzymatically reduced to ascorbate. Dehydroascorbate either degrades to 2,3-diketo-1-gulonic acid or can be recycled enzymatically.

The ELF is typically sampled through lavage techniques in which some amount of isotonic saline is introduced into the level of the respiratory tract of interest and then collected. As a result, there is some discrepancy as to the absolute concentrations of these antioxidants in the lungs that is probably a result of both intersubject variability and differences in dilution between investigators. While the absolute concentrations of the antioxidants are debatable, their relative abundance is consistant. In the nose, uric acid is the principal antioxidant. In the lung, glutathione and ascrobic acid are more abundant (Cross-1984, Mudway-2000). Table 2-2 illustrates the difference in uric acid, glutathione, and ascorbic acid concentrations in the nose and lung and compares them to plasma.

Table 2-2 Mean concentration of uric acid, ascorbic acid, and glutathione in the nasal and lung lining fluids, and plasma. These values assume a 40X and 100X dilution factors in the nose and lung.

	Nasal Lining Fluid	Lung Lining Fluid	Plasma
Uric Acid (μM)	160	90	300
Ascorbic Acid (μM)	40	100	40
Glutathione (μM)	<10	100	1.5

Uric acid, ascorbic acid, and glutathione are each transported into the ELF by different mechanisms. This may, in part, explain their differential expression in the upper and lower respiratory tracts. Uric acid is secreted by glands that co-secrete mucin and lactoferrin and are sensitive to cholinergic stimulation (Peden-1993**,** Peden-1991). These density of these glands is variable throughout the respiratory tract, being more prevalent in the upper airways and rare in the distal airways (Figure 2-8)**.** As a result, the local

concentration of UA decreases as a function of longitudinal distance from the airway opening (Heffner-1989). Because the nose is vascularized by highly fenestrated capillaries and venous sinusoids, its is postulated that uric acid may easily diffuse between the glands and plasma. If this is the case, the the concentration of UA in the glands and plasma should be equal. (Mudway-1999, Eccles-1996).

Glutathione is much more abundant in the lung compared to both the nose and plasma. Within the lung, 95% of glutathion is found in the reduced form. While it seems unlikely that the high concentration of pulmonary GSH would be a result of simple diffuion between the plasma and pulmonary compartments, it is unclear how it is maintained both in terms of its high concentration and preferentially reduced state.. Pulmonary GSH may be secreted by type II alveolar cells, Clara cells, and/or macrophages. Its high concentration may be a result of the inability to remove GSH from the ELF either through decreased catabolism by g-glutamyl transpeptidase, or its inability to diffuse into the plasma compartment. Finally, the reduction of GSH may be efficeint such that very little GSSG is observed (Kelly-1999). Regardless, it seems clear that the mechanisms that cause GSH to be found in quantity in the lung are not present in the nose.

Like GSH, the mechanism by which AA enters the ELF and is replenished after an oxidant challeneged is unclear. Ascorbic acid is thought to diffuse freely between the plasma and ELF (Kelly-1995). As a result, some research has focused on oral Vitamin C supplementaion in order to alter the ELF (Kucera-2003). If this is the case, then the amount of AA in the nose and lung should be similar to that in plasma. Whether AA is recycled in the airways has not been clearly demonstated but it has been postulated that,

as in the plasma, it may occur through both enzymatic and non-enzymatic mechanisms (Rusakow-1995).

Within any region of airway, the relative importance of each antioxidant in scavenging free radicals is a function of both its relative abundance and the kinetics of the reaction. In terms of protection again O_3-induced injury, UA seems to be the most important antioxidant in the nose. This is a result of both its high expression and the fact that the rate at which it is oxidized is higher than that of other, non-low molecualr weight antioxidants (Mudway-1999). In the lung, where GSH, AA, and UA are found at more similar concentrations, the importance of each antioxidant may be a function of its kinetics.

In vitro work has investigated the rate of reaction between O_3 and model antioxidant solutions. Kermani, et. al. measured both the rates of O_3 uptake and antioxidant depletion using an interfacial reactor and a series of model solutions at physiologically-relevant concentrations and pH (Kermani-2006). The investigators found that the reactivity of the antioxidants with O_3 was such that UA was approximately equal in reactivity to AA (5.8×10^4 M^{-1} s^{-1} vs. 5.5×10^4 M^{-1} s^{-1}). Both were much more reactive than GSH ($57.5 \times 10^{-0.75}$ M^{-1} s^{-1}). These data are in agreement with work done by Mudway and Kelly (Mudway-1999). Mudway and Kelly exposed a series of model solutions consisting of individual antioxidants, antioxidants in combination, and antioxidants in the the presence of albumin, to 0-1500 ppb O_3 and measured their rates or reactivity.

While the absolute values of the rate constants determined by Mudway and Kelly are different from those determined by Kermani, *et. al.* (and may be a result of

methodological differences), the hierarchy of the rates is the same; UA and AA were determined to be approximately equal in their reactivities while both were much more reactive than GSH. In composite solutions of the three antioxidants, a heirarchy exists such that UA is more reactive than AA and AA is more reactive than GSH. Finally, Mudway and Kelly demonstrated that the reaction between antioxidants and O_3 is augmented in the presence of protein such that UA is much more reactive than AA, and AA and GSH are approximately equal in their reactivities in the presence of protein.

Mudway and Kelly conclude that UA, because of it high reactivity, is resposible for "scrubbing" O_3 from the air in the nasal and condcuting airways and that GSH is a poor substrate for O_3. The idea that GSH is poorly reactive with O_3 is well supported (Kanofsky-1991, Kanofsky-1995). These data indicate that events which alter the compostion of the ELF, either by changing the relative concentrations of AA, UA, and GSH, or by altering the abundance of protein, can have important effects on the ability of the ELF to scavenge O_3.

Smoking-Induced Changes in Antioxidant Status

The oxidant insult on the lungs of cigarette smokers has two sources. First, and most importantly, cigarette smoke contains 10^{17}-10^{20} oxidant molecules per puff, many with a sufficiently large half life to reach the distal airspaces (Pryor-1993). Second, cigarette smoking causes an increase in airway neutrophils and other inflammatory cells; inflammatory cells are a source of reactive oxygen and nitrogen species (Davis-1988). The following sections outline the compensatory and pathological effects of cigarette

smoking on the ELF, and the effect of smoking on the plasma antioxidant composition. Of note is the fact that investigations of the impact of cigarette smoking on the ELF have focused on the lung. Data exploring the effect on the nasal ELF are unavailable.

The total antioxidant capacity of the ELF of chronic smokers (24 ±4 pack-years) is elevated compared to that of non-smokers (Morrison-1999). The total antioxidant capacity of a solution is typically measured by comparing the ability of a solution to protect an indicator protein from oxidation by an oxidant producing compound to the ability of a model antioxidant solution to protect the indicator protein. Although measuring the total antioxidant capacity yields important information, the reaction between the oxidant and the solution is a complex combination of the reactions between the individual antioxidants and the oxidant. As such, it gives no information about the kinetics of the reaction. Additionally, it does not give information about the ability of the solution to react specifically with O_3. For this reason, it is important to consider the effect that smoking has on the individual antioxidants contained in the ELF.

Smoking causes an increase in the concentration of the protein component of the ELF both by increasing the epithelial permeability and by inducing new protein synthesis. As a consequence of epithelial damage and increased airway permeability, the concentration of albumin and other plasma proteins is higher in the ELF of smokers (Jones-1980, Morrison-1999). Additionally, smoking causes goblet cell hyperplasia and increases the production of mucin by activating the epidermal growth factor receptor (EGFR). The effect of cigarette smoke on the EGFR is especially detrimental in that its activation causes the transcription of the EGFR and the placement of more receptors on the cell surface. The increase in receptor number with each stimulation may be the cause of

mucous overproduction in smokers (Takeyama-2001). Mucin has antioxidant potential because of the large number of sulfhydryl groups it contains (Cross-1984). The degree to which it is important in protecting the lung from O_3 is unknown.

Smoking also has effects on the low-molecular weight, water-soluble antioxidants in the ELF. However, the extent to which this occurs is less clear. While one group of investigators has demonstrated that smoking increases the AA concentration of the ELF (Bui-1992), the majority of evidence comes from the study of GSH. Compared to non-smokers, the GSH concentration of the ELF of chronic smokers is two-fold higher (Cantin-1987, Rahmann-1999). Upon acute smoking, the ELF GSH concentration of the smokers becomes similar to that of non-smokers (Rahmann-1999).

The increase in ELF GSH is caused by an increase in the transcription of genes related to its synthesis and recycling. Genomic studies of the airways of cigarette smokers demonstrate an upregulation of glutathione peroxidase, glutathione synthetase, and γ-glutamylsynthetase with cigarette smoking (Hackett-2003). The transcription of these enzymes is regulated both by mediators of inflammation and by oxidants directly. Oxidants that reach the epithelium can activate the transcription factor AP-1. The genes coding proteins involved in glutathione synthesis and recycling contain AP-1 binding sites in their promoter regions. Both oxidants and inflammatory mediators can activate NF-kB, resulting the transcription of proinflammatory mediators like TNF-α and IL-1. TNF-α and IL-1 can cause intracellular oxidative stress at the mitochondrial level by causing the mitochondria to release oxidants that would normally be components of the electron transport chain. These oxidants can then activate NF-kB or AP-1 (MacNee-2000).

As mentioned above, the composition of the ELF may differ in smokers depending on the time since their last cigarette. Additionally, the composition of the ELF may differ depending on smoking history. While the majority of research investigating smoking-induced effects uses subjects with a substantial smoking history (i.e., 20 pack-years or more), an investigation of the role of smoking history on ELF glutathione demonstrated that, although total gluthatione was not different between older (37-77 years) and younger smokers (20-29 years), the ration of GSSG to GSH in the older smokers was much higher. This indicates that the age of the population studied may be an important factor in smoking-related research.

Like data investigating the effects of cigarette smoking on the antioxidant composition of the ELF, data on the cigarette-smoking induced changes to the plasma antioxidant composition are equally limited. In terms of the antioxidants present both in ELF and plasma, investigations have focused mainly on AA. There is no data available on the relationship between pulmonary and plasma antioxidants in this population.

A study of diet and plasma antioxidants in French men indicates that, while the smokers tended to eat fewer fruits and vegetables, cigarette smoking is the most important predictor of plasma AA concentration. In this study, plasma AA was inversely related to smoking intensity (Marangon-1998). A study investigating the plasma antioxidant status of Korean men yielded similar results. Specifically, the plasma AA concentration of smokers is lower than that of non-smokers. This study, however, also demonstrated that cigarette smoking affects the plasma antioxidant capacity of younger smokers as well. Teenage female cigarette smokers experienced decreased plasma AA, comparable to that

of adult male smokers, when compared to their non-smoking peers (Kim-2004). If AA in the ELF is in equilibrium with the plasma, then one would expect that, in cigarette smokers, AA should be lower in the ELF.

Verification of Smoking Status

Vital to the study of cigarette smoking-related effects is the verification of the smoking status of the subject population of interest. Self-reporting is not always reliable. For that reason, and additional measurement of smoking status is necessary to validate self-reporting (Apseloff-1994). Quantification of the nicotine metabolite cotinine is potentially valuable tool for distinguishing smokers from non-smokers. The following sections describe the metabolism of nicotine and the formation of cotinine, and factors that may influence the detection of this metabolite.

Nicotine Metabolism

Nicotine is an alkaloid found primarily in tobacco, but also in tomatoes, potatoes, eggplant, green pepper, and chocolate. Nicotine represents 0.6-3% of the dry weight of tobacco (Hoffmann-1991) Because the half-life of nictoine is two hours, plasma or urinary nicotine measurements are not useful for the determination of smoking status. For example, plasma sampled in the early morning, after eight hours of smoking abstinence, would contain little to no nictotine. This would result in the misclassification of a smoker as a non-smoker. In order to give reliable information about smoking status, a metabolite must be used with a sufficiently long half-life as to not be sensitive to the

diurnal changes in plasma concentration. The half-life should be suffiiciently short, however, that intermittant or sporadic second-hand exposure will only be detected in those who have been recently exposed, minimizing the number of false positives. Cotinine meets both of these requirements.

Nicotine is metabolized in two phases. An overview of the metabolism of nicotine is given in Figure 2-13. Phase I involves the metabolism of nicotine to cotinine or nornicotine via C-oxidation, or the metabolism of nicotine to other secondary products. Cotinine is formed when nicotine is oxidized by the hepatic enzyme cytochrome CYP2A6 (Nakajima-1996). Nicotine may also undergo N-oxidation by hepatic flavin-containing monoxygenase-3 to form nicotine-N-1'-oxide (Papadopulos-1964), or nicotine and cotinine can undergo N-methyl- or demethylation by an unknown enzyme to form demethylcotinine and cotinine-N-1'-oxide. (Gorrod-1999).

Approximately 70-80% of absorbed nicotine is converted to cotinine. The remainder is processed into secondary metabolites, or excreted directly in urine (Yildiz-2004). Cotinine has a half-life in plasma of 12-17 hours (Benowitz-1993). Approximately 10-15% is excreted directly into the urine and the remainder is further metabolized (Benowitz-1996). It binds minimally to plasma proteins (Benowitz-1983). It is soluble both in the body's aqueous compartments and fat such that total body cotinine values are typically 25-33% higher than the amount in the aqueous compartment alone (Bramer-2003).

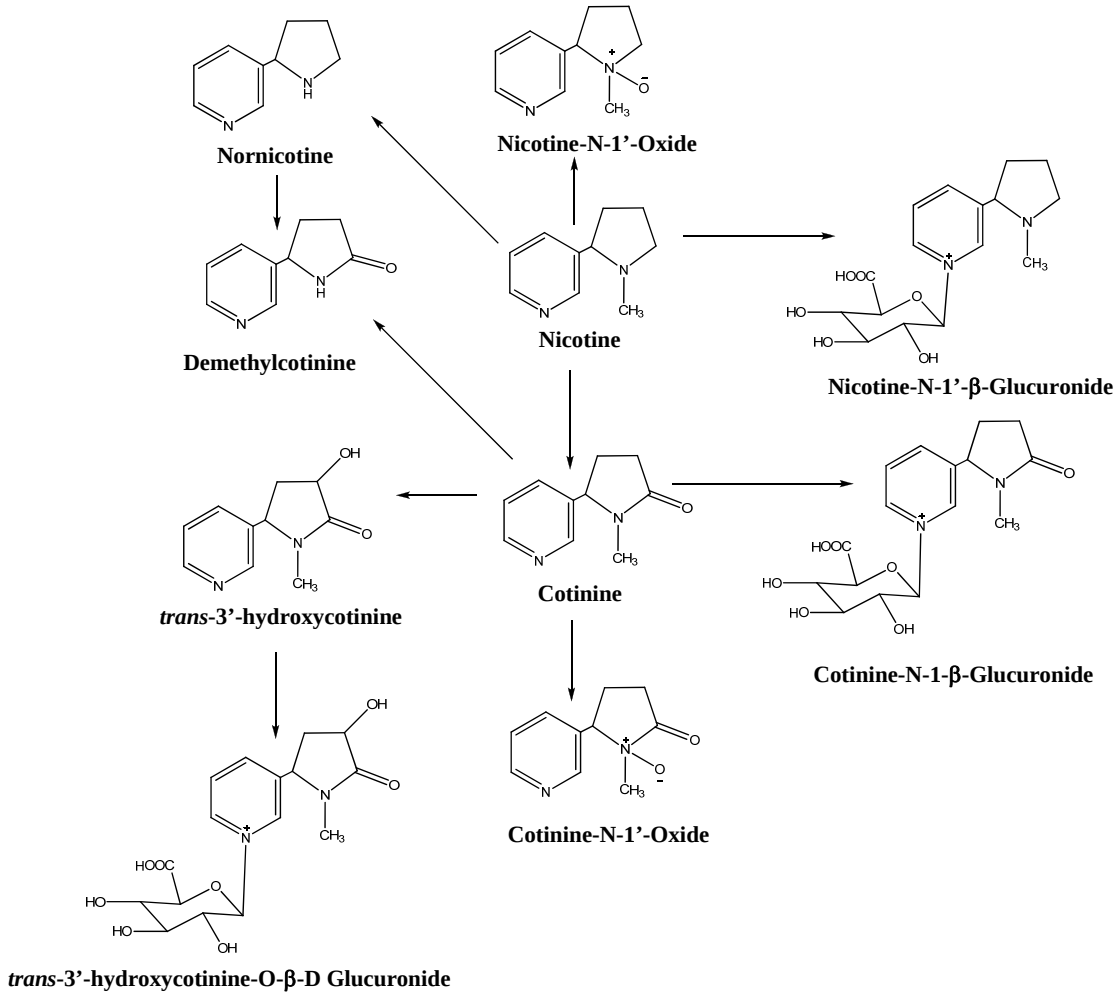


Figure 2-13: Nicotine and its major metabolites

Phase II of nicotine metabolism involves the glucuronidation of many of the Phase I metabolites. In this step, a glucuronide group is conjugated to the metabolite by UDP-glucuronosyltransferase. The addition of glucuronide, a carbohydrate rich in hydroxyl groups, increases the solubility of the metabolite and aids in its excretion by the kidney (Byrd-1992, Caldwell-1992).

Measurement of Cotinine and Sources of Variability

A number of methods have been used to validate smoking status, including the measurement of carbon monoxide, thiocyanate, and anatabine. These biomarkers are both expensive to measure, and not exclusively reflective of nicotine exposure (Etter-2001, Prignot-1987). Cotinine is a convenient biomarker because it is easily measured in a number of body fluids (saliva, blood, urine, etc.) using commonly available equipment (Bramer-2003). Cotinine, compared to many of the secondary metabolites, is present in larger quantities. The isolation of cotinine is relatively easy.

Cotinine, as opposed to other methods of validating smoking status, is specific to nicotine exposure. The average plasma concentration of a smoker exposed to 24 mg of nicotine per day is 300 ng/mL. The average cigarette contains approximately 1 mg of nicotine (Benowitz-1996). Conversely, the average plasma cotinine concentration of a non-smoker is <10 ng/mL. This accounts for dietary and environmental nicotine exposure (Eskenazi-1992). Therefore, studies that use cotinine as a biomarker typically designate non-smokers as those with plasma concentrations less than 10 ng/mL and smokers as those with plasma cotinine concentrations greater than 10 ng/mL.

Plasma cotinine is linearly related to nicotine exposure when nicotine is administered transdermally (r^2=0.77) or nasally (r^2=0.73) (Benowitz-1997). The degree to which plasma cotinine, however, corresponds to daily smoking habits is debatable. A study conducted by Rosa *et. al.* indicates that plasma cotinine is linearly related (r=0.92) to

daily estimated nicotine intake, based on number of cigarette smoked (Rosa-1992). Plasma cotinine, however, is poorly correlated to the estimated nicotine intake determined from machine-smoked cigarettes (Bramer-2003). These inconsistencies may be due to differences in smoking behavior between smokers. Clark, *et. al.* conducted a study in which they asked a cohort of black and white smokers to collect their cigarette butts for a week. Analyzing the butts, they found that, while black smokers tended to smoke longer cigarettes and more of each cigarette than their white counterparts, they smoked fewer millimeters of cigarette per day (Clark-1996).

However, even in cotinine measured after oral, nasal, or transdermal administration, there is notable intersubject variability. This may be due to differences in nicotine metabolism. The CYP2A6 gene is highly polymorphic and, in humans, there is a high degree of variability both in terms of the degree to which CYP2A6 is expressed and the activity of the protein. Variants of CYP2A6 that have reduced metabolic activity tend to be more highly expressed in populations of Asian descent, compared to population of African and Caucasian descent (Kwon-2001, Schoedel-2004). Individuals with different CYP2A6 variants may self-select in terms of their smoking intensity. A genetic study of Japanese adults indicates that smokers with a CYP2A6 variant associated with reduced metabolic activity tend to smoke less than those with a variant associated with higher metabolic activity (Fujieda-2004). Finally, there is intersubject and interracial variability in the rate at which nicotine metabolites are glucuronidated and cleared by the kidney. Individuals of African descent clear nicotine more slowly, and clear less glucuronidated metabolites, compared to smokers of Caucasian descent (Benowitz-1999).

Chapter 3 — Materials and Methods

This chapter will outline the general methodology employed in this experiment. Sixty smoking and non-smoking human subjects participated in a two-part health screening process followed by two research sessions. During the first research session they were exposed to filtered air. During the second research session they were exposed to 0.30 ppm O_3. The following sections detail the design of the individual sessions, the equipment and derivation of the response endpoints, and biochemical assays.

Experimental Design

Cigarette smokers and non-smokers were recruited from the student and staff population of The Pennsylvania State University and surrounding community via flyer postings and advertisements placed in local and campus newspapers. Participants were initially screened in-person to ensure that they met the inclusion and exclusion criteria listed in Table 3-1. Flyers, the pre-screening questionnaire, and additional questionnaires, may be found in Appendix B. All procedures, flyers, questionnaires, and the informed consent were approved by the Office of Research Protections of The Pennsylvania State University.

After meeting with an investigator and reviewing and signing an informed consent form, participants underwent two health screenings, designated the pre-screening and health-screening sessions.

Table 3-1: Inclusion and Exclusion Criteria for Human Participants

Inclusion	Exclusion
• FEV_1 > 80% of predicted Knudsen value and FEV_1/FVC > 0.70 **Non-Smokers:** • 18-35 years old • Pack-year history < 0.5 and no tobacco use during previous three years. **Smokers:** • 21-35 years old • Current smoker and daily tobacco user for at least one year	• Current or history of cardiovascular, respiratory disease, or any other chronic disease • Current upper or lower respiratory infection • Regular medication use, including over the counter pain relievers or anti-histamines but excluding hormonal birth control. • Pregnancy • Latex allergy

All screening and experimental procedures were performed at Penn State's General Clinical Research Center (GCRC) under the supervision of a clinician (either a medical doctor or nurse practitioner) and nursing staff. The purpose of the screening sessions was to both ensure that participants had no apparent respiratory disease and that they were at minimal risk for a cardiovascular event while completing the protocol. During the pre-screening session, participants performed a clinical pulmonary function test (VMAX229 Legacy, SensorMedics, Yorba Linda CA) to verify that their lung function met or exceeded the inclusion criterion. Participants completed medical and smoking history questionnaires. Venous blood samples were drawn from an antecubital vein and sent to a commercial lab (QUEST Diagnostics) for the assessment of clinical chemistry and

blood count. Because blood was already being drawn for screening purposes, an additional 7-mL of blood was drawn and analyzed for makers of nicotine metabolism and antioxidant status. This portion of the venous blood sample was preserved and later analyzed as described below.

The medical history information and blood cholesterol and triglyceride values obtained from the clinical chemistry analysis were used to perform a standard assessment of cardiac risk. This risk assessment was used by the GCRC clinician to determine both if it was safe for the participant to proceed with the health screening session and the level of clinical supervision required for the session.

During the health screening session, participants were given a standard, 12-lead electrocardiogram and physical exam by the GCRC clinician. Female participants were given a urine pregnancy test. Providing the ECG and physical exam revealed no cardiovascular abnormalities, or other chronic disease, participants performed an exercise tolerance test from which a continuous 12-lead ECG and continuous and maximal rates of oxygen uptake (VO_{2max}) were determined. The test was conducted using a cycle ergometer and standard clinical equipment (VMAX229 Legacy, SensorMedics, Yorba Linda CA) and ramp protocol. Blood pressure, pulse, and ECG were monitored by the clinical staff and the test was terminated when the participant reported reaching maximal perceived exertion on the Borg scale.

Research Sessions

After completing the pre-screening and health screening sessions, volunteers participated in two experimental sessions. The air and O_3 exposure sessions were separated by at least one week and the air exposure always proceeded the O_3 exposure session. By requiring that participants complete the air exposure session first, individuals with exercise-induced asthma could be identified and excluded before participating in the O_3 exposure session. As such, participants that experienced >12% decrement in FEV_1 during the air exposure session were prevented from participating in the O_3 exposure session. We excluded one non-smoking participant who experienced a decrement in FEV_1 of 16% with air exposure. A timeline of events is shown in Figure 3-1.

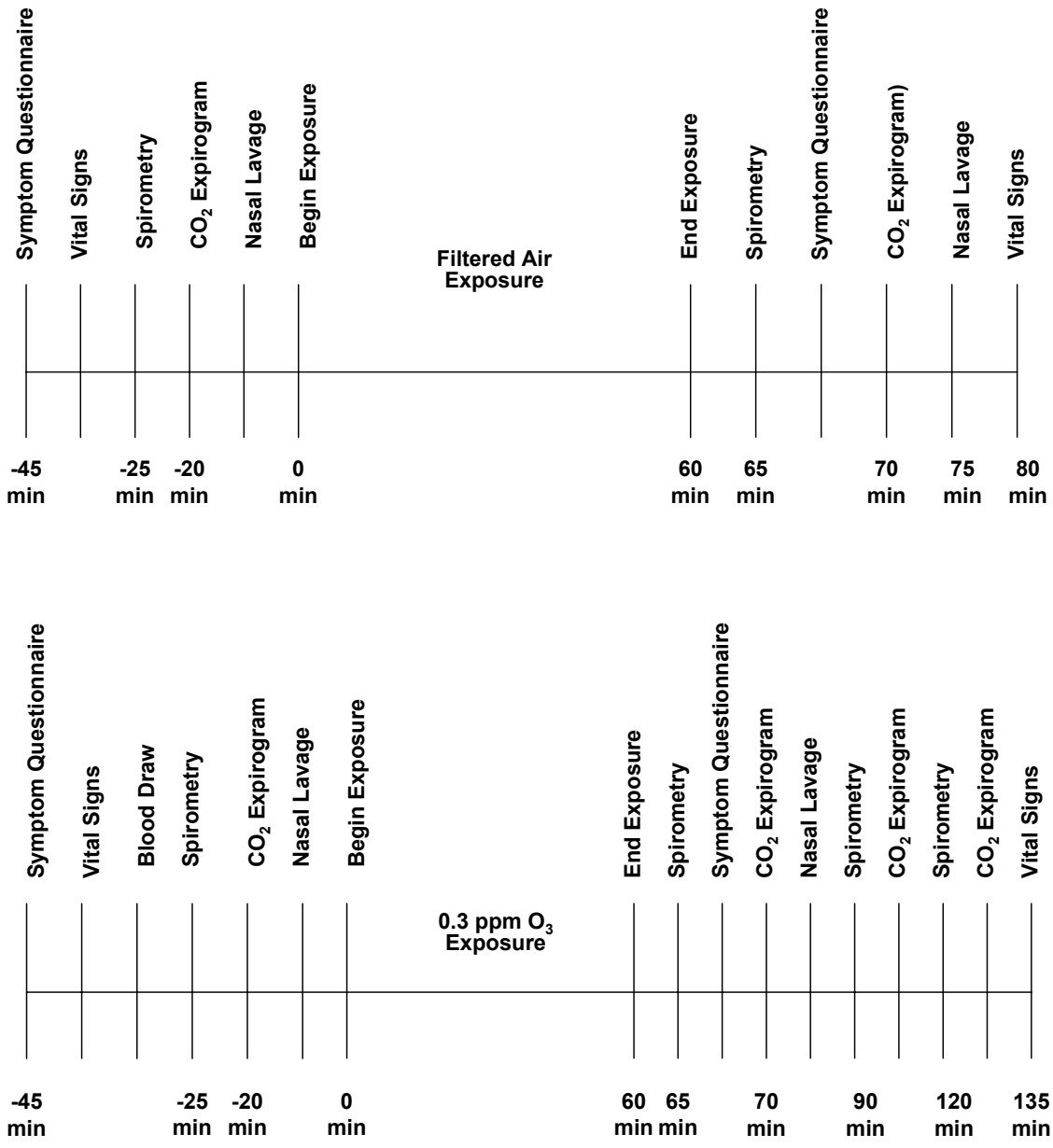


Figure 3-1: Timeline of events for the air and ozone exposure sessions. Events for the air exposure session are shown in the top panel and events for the ozone exposure session are shown in the lower panel

Upon arrival at our lab for either the air or O_3 exposure session, participants completed a symptom questionnaire. If a participant presented with apparent upper or lower respiratory tract infection, or any other condition which would prevent them from participating safely, the session was cancelled and rescheduled. After obtaining the symptom questionnaire and before each exposure, a research nurse assessed the participant's pulse, arterial oxygen saturation, and blood pressure. Prior to O_3 exposure, participants then had blood drawn from an antecubital vein. Blood was preserved and analyzed in the manner described in a subsequent section. A series of forced expiratory maneuvers and CO_2 expirograms were collected in the manner described below. Nasal lavage fluid was collected and the participant was fitted with the exposure apparatus.

During both sessions, participants exercised for one hour on a cycle ergometer at a constant cadence of 60±5 revolutions/min. The workload was set to elicit a minute volume equal to 15 L/min/m² of body surface (BSA) ± 2 L/min. Body surface area was calculated using the DuBois equation (Equation 3-1). During the air exposure session, volunteers breathed clean air. During the O_3 exposure session, volunteers breathed a nominal concentration of 0.30 ppm O_3. A nurse assessed the participant's vital signs every 15 minutes during the exposure.

$$BSA[m^2] = 0.007184 * H[cm]^{0.725} * W[kg]^{0.425} \qquad \text{Equation } \mathbf{3-1}$$

After the end of the exercise period, participants were given a four minute cool-down period. The post-exposure lung function and CO_2 expirogram, symptom questionnaire,

and nasal lavage were then collected in the order described in Figure 3-1. Post-O_3 exposure, additional expirograms and pulmonary function tests were collected 30 and 60 minutes post-exposure.

Measurement of the CO2 Expirogram and Forced Spirometric Parameters

Forced spirometric data was collected using a commercial clinical-grade spirometer, containing a brass Fleisch-type pneumotach, and PC-based software package (KoKo Model and Pulmonary Data System, Ferraris Corp). The device was calibrated before each session using a three liter air-tight syringe, accounting for the temperature, humidity, and atmospheric pressure in the room. The participant's height, weight, date of birth, and race were entered into the software package, which then calculated a set of predicted values based on Knudsen's data (Knudsen-1976).

Participants inhaled and exhaled through a mouthpiece just proximal to the pneumotach. To perform the forced expiratory maneuver, participants first inhaled and exhaled tidally for four cycles. Participants then performed a maximal inhalation from functional residual capacity (FRC) to total lung capacity, followed by a maximal exhalation, and a second maximal inhalation. From the maximal exhalation, values of FEV_1, FVC, and FEV_1/FVC were calculated. The participant continued to perform the maneuver until two matching tests were obtained. The quality of the tests was evaluated according to the 1986 American Thoracic Society Guidelines for the measurement of lung volumes.

The CO_2 expirogram was measured according to the method described by Taylor, et al (Taylor-2006). To complete the maneuver, subjects inhaled and exhaled through a mouth piece connected in series to a screen pneunmotach (Model R4500C, Hans Rudolph), capnometer cuvette and infrared sensor (Model M14365A, Hewlett Packard) for two respiratory cycles at a fixed flow rate of 250 mL/s. Beginning at FRC, participants performed one inhalation and exhalation, with each phase lasting three seconds. Participants then performed an additional three second inhalation followed by a prolonged exhalation during which a minimum volume of 1250 mL was exhaled. In order to assist participants in performing the maneuver correctly, a real-time signal, indicating the individual's flow rate, was displayed on a monitor in front of the subject. The maneuver was repeated until a minimum of four maneuvers had been collected. The accuracy of the pneumotach was verified before each session using a stream of filtered air at a known flow rate measured by an electronic mass flow meter (GFM-1133 Mass Flowmeter, Dwyer Instruments, Inc.). The capnometer was calibrated before each use using an on-board CO_2-filled cell supplied by the manufacturer. The voltage signals from both components was recorded via a data acquisition system (Keithley Instruments, Cleveland, Ohio) and the second collected breath was truncated at 1250 mL and analyzed with a macro of our own design.

Specifically, the volume of the Fowler dead space was calculated according to the method first described by Aitken and Clarke-Kennedy (Aitken-1928). The numerical solution to this is shown in Equation 3-2**,** where S represents the slope of the alveolar plateau and B is its intercept. A represents the area under the curve between V_D and V_{TE}, determined by integrating the flow signal as a function of time. Its numerical equivalent is shown in Equation 3-3. V_{TE} represents the end tidal volume.

$$V_D = -\frac{B}{S} + [\frac{B}{S}^2 - (2\frac{A}{S} - V_{TE}^2 - 2B\frac{V_{TE}}{S}]^2$$ Equation **3–2**

where:

$$A = 0.5[S(V_{TE} + V_D) + 2B][V_{TE} - V_D]$$ Equation **3–3**

Both the determination of V_D and S depend on the transition between phase II and phase III of the expirogram, or the point at which dead space gas is completely washed out and only alveolar gas is subsequently exhaled. ($V_{II/III}$). Based on the assumption that the dead space gas is completely washed out in a volume equal to $2V_D$, V_D and $2V_D$ were calculated via an iterative process. A value of $V_{II/III}$ was randomly chosen as a starting point and a value of V_D then calculated using Equation3-2. The iteration continued until the resultant value of V_D was equal to half $V_{II/III}$. Values of V_D and S_N were determined for each participant at the pre-exposure and post-exposure timepoint and were averaged to determine the mean value.

The Continuous Exposure Apparatus

In order to measure the uptake of O_3, minute volume (V_E), tidal volume (V_T), and respiratory rate (f), we used a previously described, novel piece of equipment to which we made a few improvements (MacDougal-1998,Rigas-2000) Specifically, improvements were made to the pneumotach as described below. An image of the exposure equipment can be found in Figure 3-2.

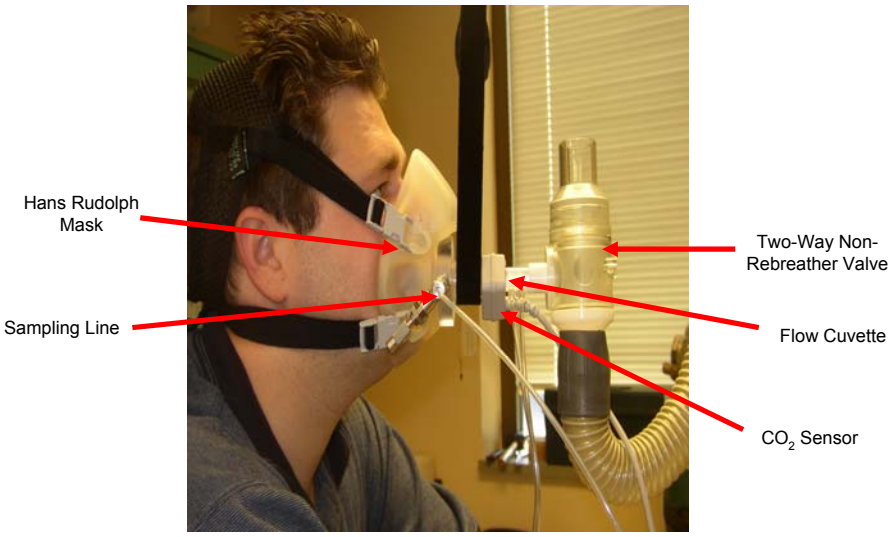


Figure 3-2: Exposure apparatus indicating the location of the mask, sampling line, two-way non-rebreather valve, CO_2 sensor, and flow cuvette.

Prior to the exposure, participants were fitted with a Hans Rudolph mask (series 7900, Hans Rudolph) which separated the mouth from the nose and allowed for only oral breathing. The following components were then attached in-line with the mask opening:

- A sampling line which ran between the mask assembly and our chemiluminescent O_3 analyzer. Samples obtained here were used in the determination of inhaled and exhaled concentration and dose.

- A flow cuvette (NICO, Adult) from which the voltage signal was digitally assessed on a breath-by-breath basis (NICO, Cardiopulmonary Management System Model 7300, LabView, National Instruments) to determine values for V_E, V_T, and f.

- A two-way non-rebreather valve with an inspiratory and expiratory port. Through the inspiratory port, we delivered the exposure gas of interest. Ozone was generated using a commercially available generator (03V1-0, OREC, Phoenix, AZ) that operates by passing room air over a UV light. Ozonated air was delivered to one port of a piping tee at a rate of 250 L/min. During the air exposure session the generator was turned off such that the piping tee received only room air. The second port of the piping tee delivered the exposure gas via a hose to the inspiratory port of the non-rebreather valve. The third port of the piping tee vented ozonated air generated in excess of the participant's demand to the outside environment. The expiratory port of the non-rebreather valve vented to the room.

Cumulative uptake was calculated by considering the difference in the amount of O_3 inhaled and exhaled by the participant. This was determined by numerical integration of the concentration and flow signals as a function of time.

The exposure equipment was calibrated before each session. The O_3 analyzer was calibrated using a generator provided by the United States Environmental Protection Agency (49PS, Thermo Environmental Instruments, Franklin MA). The voltage signal from the flow cuvette was verified using known airflows measured via a digital mass flow meter (GFM-1133 Mass Flowmeter, Dwyer Instruments, Inc.). The accuracy of the cycle

ergometer was verified regularly using a series of standard weights and a standard calibration procedure.

Preservation and Analysis of Biological Samples

Blood was collected from an antecubital vein, using a vacutainer system, by a research nurse or certified technician at the GCRC. During the pre-screening session, 15 mL of blood was drawn into a tube containing K_2EDTA, a tube containing sodium heparin, and a serum seperation tube. During the O_3 exposure, 15 mL of blood was drawn into tubes containing sodium heparin. Blood drawn into the tube containing K_2EDTA and the serum seperation tube were processed according to standard clinical practice and sent to a commercial lab for blood count and clinical chemistry analysis. Blood drawn into tubes containing sodium heparin was used for the analyses specifically detailed in this thesis.

Blood drawn into sodium heparin-containing tubes was centrifuged at 3000xg for 15 minutes in order to serparate the plasma from the cellular fraction. The plasma was then divided into two aliquots. The first aliquot was treated to preserve ascorbic acid by mixing 100 μL plasma with 300 μL of a solution containing the reducing agent dithiotreitol (DTT)(1g/L) and metaphosphoric acid (MPA)(30g/L). The second aliquot was divided into 1 mL and 600 μL fractions. All samples were stored in snap-top cryovials in a -70C freezer.

The nasal epithelial lining layer was sampled via nasal lavage. During the sampling, the participant sat semi-reclined in a chair while tilting the head back and pressing the

tongue against the roof of the mouth. This closes the velum in order to prevent swallowing of the saline. Five mL sterile inhalation saline (0.9% NaCl) was then introduced into one side of the nose with a sterile syringe. The saline was held in the nose for 10 seconds, after which the particpant leaned forward and deposited the saline into a sterile specimen cup. The procedure was repeated on the opposite side. The lavage was then passed through a 0.45 μM hydrophilic polypropylene fileter (Acrodisc Brand, Pall Corporation) to remove large debris.

The lavage fluid was divided into two aliquots. The first aliquot was treated to preserve ascorbic acid by mixing 200 μL lavage with 600 μL of the DTT/MPA solution described above. The second aliquot was divided into 1 mL fractions. All samples were stored in snap-top cryovials in a -70C freezer.

Measurement of Plasma Cotinine

Plasma cotinine was measured by adapting a method described by Ghosheh *et. al.* for use in our lab (Ghosheh-2000). The method involves a liquid phase extraction in an organic solvent and analysis via high performance liquid chromatography (HPLC) (Model HP1100, Agilent Technologies) coupled with a UV detector (Model G1315A). 2.4 mL of plasma, collected at either the pre-screening or ozone exposure session, was defrosted in 20C water and pippetted into a 15-mL centrifuge tube. As an internal standard to correct for differences in sample recovery during the liquid phase extraction, 40uL N,N diethylnicotinamide (NNDEN) was added. NNDEN, while once used in the treatment of barbituate overdose, is generally considered a toxin and should not be

found in normal plasma (Martindale-1989). 0.5 mL NaOH was added to the tube, followed by 10 mL dichloromethane. The tube was vortexed in order to ensure sufficient mixing of the aqeous and organic phases and centrifuged for 15 minutes at 3000xg.

The organic phase was pippetted into a new 15 mL centrifuge tube and dried in a vacuum liphilizer at 37C. The sample was reconsituted with 600 μL nanopure water and 100 μL of the sample was injected into the HPLC for analysis. Analytes were seperated over tandem C-18 guard (Model AJ0-4286, Phenomenex Inc), C8 (Eclipse XDB-C8 4.6 x 150 mm, 5μm pore size, Agilent Technologies), C18 (Supelcosil LC-C18 4.6 x 150 mm, 5μm pore size, Supelco), and sulfonic acid (Zorbax 300-SCX 4.6 x 250 mm, 5μm pore size, Agilent Technologies) bonded phase columns. A schematic of the column arragement is shown in Figure **3-3**. Samples were run in a mobile phase consisting of 70% nanopure water, 15% methanol, and 15% 0.3M $(NH_4)_2HPO_4$ adjusted to pH=4.8 with triethylamine

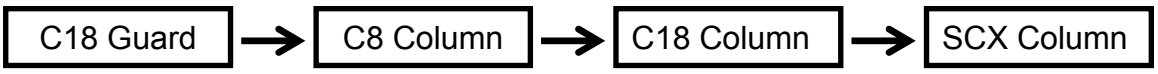


Figure 3-3: Orientation of columns used to separate cotinine from interfering compounds in plasma. Arrows indicate the direction of mobile phase flow.

at a flow rate of 1.1 mL/min.

In HPLC, analytes within a sample are separated based on two factors -- the time they are retained on the column, which is a function of the rate at which they are adsorbed and desorbed and is related to the bonded phase of the column and the character of the analyte, and whether they are quantified by the detection method chosen. In this case, Ghoshesh and colleagues described a method by which cotinine was sufficiently

separated from caffeine and 3'-transhydroxycotinine, two major interfering analytes, by a column with a sulfonic acid bonded phase (SCX). Cotinine could then be detected without difficulty at 254 nm. However, we were unable to detect cotinine with a suffient degree of sensitivity at 254 nm. Because of the conjugated ring that it contans, cotinine absorbs UV light maximally between 210 and 230 nm. For that reason, we chose to detect cotinine at 210 nm in order to increase the lower limit of detection. At 210 nm, the peak for cotinine overlapped with unknown interfering peaks when the sample was separated using the SCX column alone. In order to resolve cotnine from these substances, we added C8 and C18 columns in tandem with the SCX column.

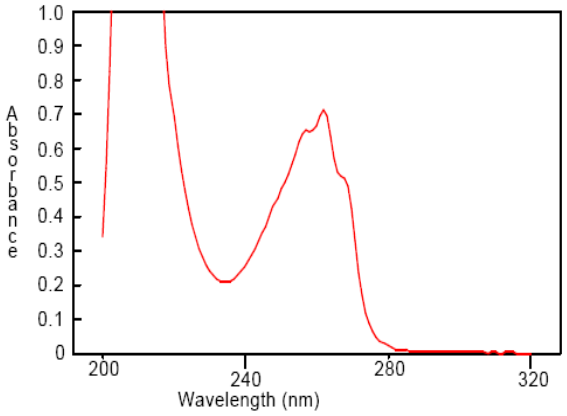


Figure 3-4: UV absorbance spectrum of cotinine. Provided by Sigma Chemicals Inc.

Samples were run in triplicate and the absorbance signal numerically inetegrated as a funciton of time to yield a value for the area under the cotinine peak. The elution times for cotinine and the internal standard were 20 and 40 minutes, respectively. The average area under the curve (AUC) was compared to a standard curve generated from a minimum of six concentrations. Unknown sample peaks were identified by comparing their elution times to those obtained from the standard solutions.

To generate the standard curve, aliquots of drug standard grade cotinine in methanol (1 mg/mL) (Catalogue #C0430, Sigma Chemicals) were dissolved in human plasma collected from non-smoking human donors who reported no medication or caffeine use prior to their donation (Valley Biomedical Supplies), processed, and analyzed via HPLC as described above. As mentioned above, NNDEN was used as an internal standard to correct for differences in sample recovery in the liquid phase extraction step between samples. This was done by assuming an equilibrium partitioning coefficient for NNDEN (k_1) and cotinine (k_2) between the different processing steps and determining a total partitioning coefficient for both analytes (k_T). This is is outlined in Figure **3-5** and Equations 3-4 to 3-8. As such, the concentration of cotinine in a sample (C_C) is equal to the product of k_T, the ration of the AUC of the NNDEN peak (O_S)to the cotinine peak (O_C), and the concentration of NNDEN in the sample (C_S).

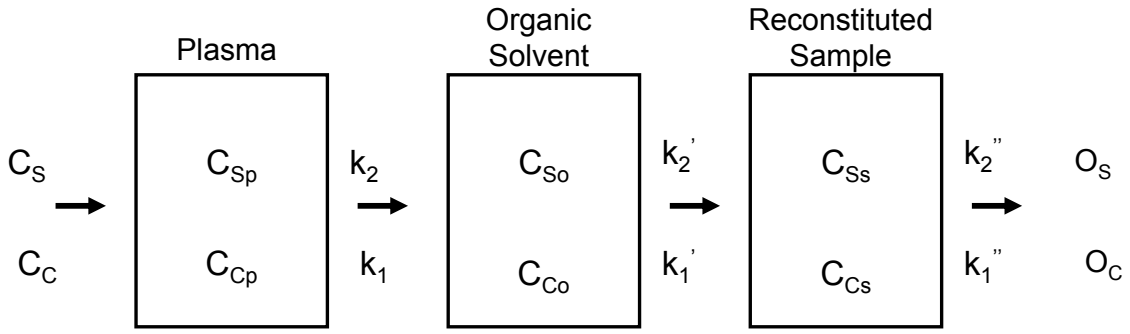


Figure **3-5**: Transfer of cotinine and the internal standard during the liquid phase extraction. k1 describes the partitioning coefficient of cotinine and k2 describes the partioning coefficient coefficient of the internal standard at each stage in the extraction. C_{Sp}, C_{So}, and C_{Ss} represent the concentration of the internal standard in the plasma phase, organic phase, and reconsituted sample. C_{Cp}, C_{Co}, and C_{Cs} represent the concentration of cotinine in the plasma phase, organic phase, and reconsituted sample. O_S and O_C represent the relative UV absorbances of cotinine (O_c) and the internal standard (O_s)

$$O_C = k_1 k_1' k_1'' C_C \qquad \text{Equation } \mathbf{3-4}$$

$$O_C = k_1 k_1' k_1'' C_C$$ Equation **3–5**

$$\frac{O_S}{O_C} = \left(\frac{k_2 k_2' k_2''}{k_1 k_1' k_1''} \right) \frac{C_s}{C_C}$$ Equation **3–6**

$$\frac{k_2 k_2' k_2''}{k_1 k_1' k_1''} = k_T$$ Equation **3–7**

$$C_C = k_T \left(\frac{O_C}{O_S} \right) C_S$$ Equation **3–8**

Standard curves were acceptable if the r^2 value for the correlation between the x and y-axes was greater than 0.99. A sample standard curve can be found in Appendix C .

The absorbance peak associated with cotinine was visible at concentrations higher than or equal to 0.032 μg/mL. This was assumed to be the lower end of detection. The resolution and sensitivity of the assay were verified from the standard curve by calculating the standard error of the absorbance output for each concentration. The assay was considered to be sufficiently sensitive to detect a particular concentration if the mean output minus two times the standard error was larger than zero. Our assay was sufficiently sensitive to detect cotinine at 0.032 μg/mL.

Measurement of Nasal and Plasma Uric and Ascorbic Acids

Uric acid was measured according to the chromatographic method described by Iriyama, *et al*, coupled with electrochemical detection (Iriyama-1984). For each analysis, 100 μL of lavage, or plasma diluted 8-fold in nanopure water, was injected into the HPLC and separated using a C18 column (Supelcosil LC-C18 4.6 x 150 mm, 5μm pore size, Supelco) and a mobile phase consisting of 0.2M $Na_2H_2PO_4$ (pH=2.1), flowing at 2 mL/min. The elutiuon time for uric acic was 2 minutes. The electrocehmical detector utlized a $AgCl/Ag^+$ reference electrode and was set at a voltage of +0.8V. The area under the uric acid peak was determined by integrating the current signal as a function of time. Samples were run in triplicate and the average area under the curve was compared to a standard curve run using a minimum of six concentrations. Unknown sample peaks were identified by comparing their elution times to those obtained from the standard solutions. Standard curves were acceptable if the r^2 value for the correlation between the x and y-axes was greater than 0.99. A sample standard curve can be found in Appendix C.

Measurement of the Oxygen Radical Absorbance Capacity in Nasal Lavage

The oxygen radical absorbance capacity assay (ORAC) was first described by Cao and associates (Cao-1993). The antioxidant capacity of a sample is determined by measuring its ability to protect the fluorescent protein β-phycoerythrin (β-PE) from being oxidized by the hydroxyl-generating compound azobis(2-methylpropion-amidine)dihydrochloride (AAPH). This is compared to the ability of Trolox, a water-solube vitamin E analogue, to

protect β-PE such that one ORAC unit is equal to the protection offered by 80 μM Trolox. The fluorescence of β-PE was measured using a fluorometer fitted with 535 nm excitation and 565 nm emission filters. Previously, this assay was performed in our lab using 5-mL cuvettes. In order to process the large number of samples collected during this protocol we adapted the assay to 96-well microplates. A description of the microplate method is given below.

All samples and reagents were prepared using a 100 mM NaH_2PO4 buffer solution (pH=7.4). 13.8 μL of β-PE stock solution (4 mg/mL in 60% sat. ammonium sulfate, 50 mM potassium sulfate) (P-800, Invitrogen Corp) was dissolved in 50 mL buffer solution and warmed in a water bath until the solution reached 45C. Lavage samples, a blank consisting of the buffer solution, and 80μM Trolox were placed on a 96-well plate in 25 μL aliquots. 150 μL of the β-PE was then added to each well. A schematic of the plate set-up can be found in Figure D-3 . In order to maintain a consistent temperature in the wells containing sample, the outer wells were filled with water and not used for analysis. Samples were plated in duplicate oriented vertically to each other on the plate. To each sample-containing well, 25 μL of 75μM AAPH was added and the plate was immediately read using a plate reader capable of detecting fluorescence at the appropriate wavelength. Plates were subsequently read every 8 minutes for the first 90 minutes. After 90 minutes, the plates were read every 16 minutes until the fluorescence of the well was equal to the mean fluorescence of the water-containing outer wells. In between readings, plates were incubated in an oven at 50C.

The antioxidant capacity of the sample was determined by calculating the quotient of the area under the sample decay curve and the area under the Trolox decay curve. Both curves are corrected using the decay curve of the blank. This is shown numerically in Equation 3-9.

$$ORAC = \frac{Sample - Blank}{Trolox - Blank} \qquad \text{Equation } \mathbf{3-9}$$

The microplate assay was verified by visually inspecting the shape of the decay curves and by comparing the ORACs of a series of standard solutions of uric acid (See Figure D-4). The concentration of uric acid in each sample is linearly related with the associated ORAC units.

Quantification of Total Protein and Urea

Total urea was determined in plasma and lavage from the continuous ozone exposure session using a commercially available kit (DIUR-500, Bioassay Systems). This kit employs the Jung method of urea detection (Jung-1975) In this method, the colorimetric reaction between urea, o-phthalaldehyde, and N-(1-naphthyl)ethylenediamine is measured at 520nm in 96-well plates using a standard plate reader. Samples were run in duplicate and compared to a standard curve constructed from at least six known samples and fit with with a logistic model. Standard curves were acceptable if the r^2 value for the correlation between the x and y-axes was greater than

0.99. The dilution of the lavage was corrected by assuming that the concentration of urea in lavage and plasma is equal.

Total protein was determined in lavage obtained during the continuous air and ozone exposure sessions using a commercially available kit (BioRad 500-001). This kit employs the Bradford method of protein detection (Bradford-1976). In this method, the absorbance of an indicator dye, Coomasie Blue, is altered by the binding of protein. Coomasie blue, which is red in color and absorbs at 465nm in the absence of protein, binds to basic and aromatic residues on the surface of proteins. The indicator dye, which becomes blue in color in the presence of protein, is then read at 600nm. Lavage samples were diluted 23-fold , plated in duplicate, and compared to a standard curve constructed from at least six known samples and fit with with a logistic model. Known samples were generated from stock solutions of bovine gamma globulin provided by the kit manufacturer. Standard curves were acceptable if the r^2 value for the correlation between the x and y-axes was greater than 0.99.

Statistical Analysis

Statistical analysis was performed using the Minitab statistical analysis package (Version 13, Minitab Inc, State College, PA). In general, data from this investigation was analyzed using a few basic statistical tests. Pulmonary function changes with O_3 and air exposure and differences in values obtained from the biochemical assays were evaluated using t-tests. Paired t-tests were used when comparing participants' responses to their own pre-exposure values and unpaired t-tests were used with comparing the responses of

different groups. Linear regression methods were employed to describe the significance of the the relationship between one or more factors and the response paramter of interest. The square of the Pearson's correlation coefficient (r^2) were used to describe the degree to which two or more variables were linearly related. Finally, analyses of variance and covariance (ANOVA and ANCOVA) were employed to describe the significance of subject, gender, and smoking status effects. Statistical significance was assessed at $p < 0.05$.

Chapter 4 – Results

This chapter describes the results of our investigation. It is structured so that the major sections contain results specifically pertaining to the three hypotheses outlined in Chapter One.

Population Characteristics and Smoking Status Verification

Population Characteristics

We recruited 30 non-smokers and 30 smokers based on the criteria outlined in Table 3-1. Summary anthropomorphic information is given in Table 4-1.

T-tests of the data indicate that smokers and non-smokers were not different in terms of height, weight, FEV_1, FEV_1/FVC or age. Considered as subpopulations of the smoking and non-smoking groups, men and women were different in terms of height ($p<0.000$), weight ($p<0.000$), BSA ($p<0.000$), and FEV_1 ($p<0.000$). Our populations contained an equal number of minority volunteers (1 Hispanic, 1 Black, and 5 Asian non-smokers and 1 Hispanic, 3 Black, and 3 Asian smokers).

Table 4-1: Anthropomorphic information (±SD) for the population separated by smoking status and sex. Packs/wk and Years Smoking represent values self-reported during the screening session. FEV1 and FEV1/FVC represent the pre-exposure value, averaged from the two research sessions. (†) indicates that the male subpopulations are significantly different than the female populations ($p < 0.05$).

	Non-Smokers (n=30)			**Smokers (n=30)**		
	Men (n=17)	Women (n=13)	Total	Men (n=19)	Women (n=11)	Total
Age (yrs)	26 (7)	23 (5)	25 (6)	24 (4)	24 (4)	24(4)
Packs/Wk	0 (0)	0 (0)	0 (0)	4 (2)	4 (2)	4 (2)
Years Smoking	0 (0)	0 (0)	0 (0)	6 (4)	7 (4)	6 (4)*
Height (cm)	179.3 (6.3)	163.9 (5.5) †	172.6 (9.7)	179.4 (6.2)	165.1 (5.0) †	174.2 (9.1)
Weight (kg)	81.7 (12.9)	61.6 (11.3) †	73.0 (15.7)	77.3 (11.3)	68.3 (16.1) †	74.0 (13.7)
BSA (m^2)	2.0 (0.2)	1.7 (0.2) †	1.9 (0.2)	2.0 (0.2)	1.7 (0.2) †	1.9 (0.2)
FEV_1 (L)	4.43 (0.60)	3.06 (0.33)†	3.8 (0.9)	4.52 (0.64)	3.35 (0.56)†	4.1 (0.8)
FEV_1/FVC (%)	84.6 (10.6)	82.3 (10.6)	83.6 (1.1)	80.2 (7.2)	81.7 (7.2)	80.1 (0.7)

In order to determine if the smoking and non-smoking populations had similar pre-exposure airway characteristics, we compared pre-exposure V_D and S_N by averaging values measured pre-air and O_3 exposure. Figure 4-1 and Figure 4-2 illustrate average pre-exposure values of V_D and S_N in the smoking and non-smoking populations. Figure 4-1 compares average V_D, obtained pre-air and O_3 exposure, with height and compares the relation between these parameters in our population with a historical comparison made by Hart, et al (Hart-1963). The correlation between height and V_D described by Hart is shown as a solid line. Hart and colleagues indicate that, in their population, all values of V_D are within 16.9% of the regression line.

Within our populations, seven non-smokers and five smokers had values of V_D greater than 16.9% of the value estimated by Hart's equation. Two non-smokers and three smokers had values of V_D less than 16.9% of the value estimated by Hart's equation. The fact that the majority of the values of V_D obtained from smokers and non-smokers fall within the boundaries of Hart's correlation indicates that they have similar conducting airway morphometry. Additionally, a two sample t-test indicates that average pre-exposure V_D is not significantly different between non-smokers (159.2±4.9 mL) and smokers (162.2±5.4 mL) ($p=0.68$).

In calculating pre- and post-exposure values of V_D, we collected a series of four to six expirograms and determined a value of V_D for each one (see Chapter 3). We then calculated a mean value of V_D using these individual values. In order to understand the variability of the measurement, we used the individual pre-exposure expirograms to calculate the degree to which each individual value of V_D within a series deviated from the mean. We did this by calculating the absolute value of the percent difference from the mean. We found that, on average for each participant, individual values of V_D varied ±4.8% from the participant's mean value.

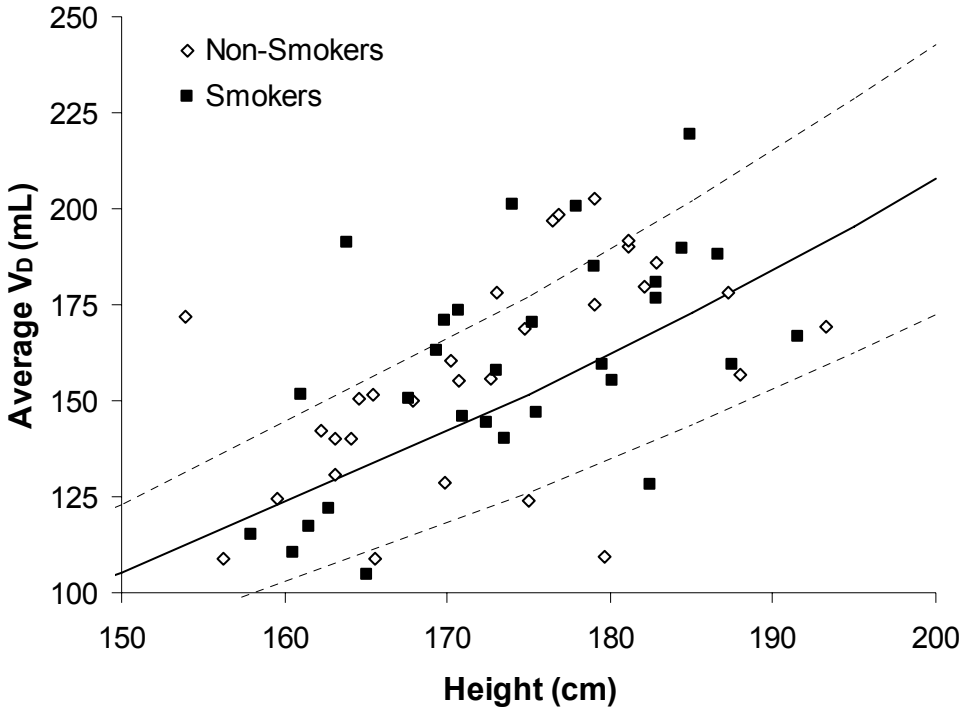


Figure 4-1: Comparison of average pre-air and ozone dead space (V_D) with height. The correlation described by Hart, et. al. [V_D (mL) = (7.585 x Ht (cm) $^{2.363}$) x 10^{-4}] is indicated by the solid line. Plus or minus 16.9% from the regression line is indicated by the dotted lines.

Figure 4-2 compares average pre-exposure S_N with height. An analysis of covariance (ANCOVA) using smoking status as a factor and height as a covariate indicates that height is a significant predictor of S_N (p=0.02) but smoking status is not (p=0.64). This result demonstrates that smokers and non-smokers are also similar in distal airway function. As with V_D, we calculated values of S_N from each of the individual expirograms we obtained pre- or post exposure (see Chapter 3) and then used these individual values to calculate a mean value. Again, in order to understand the variability of the measurement, we used the individual pre-exposure expirograms to calculate the degree to which each individual value of S_N within a series deviated from the mean. Again, we did this by calculating the absolute value of the percent difference from the mean. We

found that, on average for each participant, individual values of S_N varied ±10.4% from the participant's mean value.

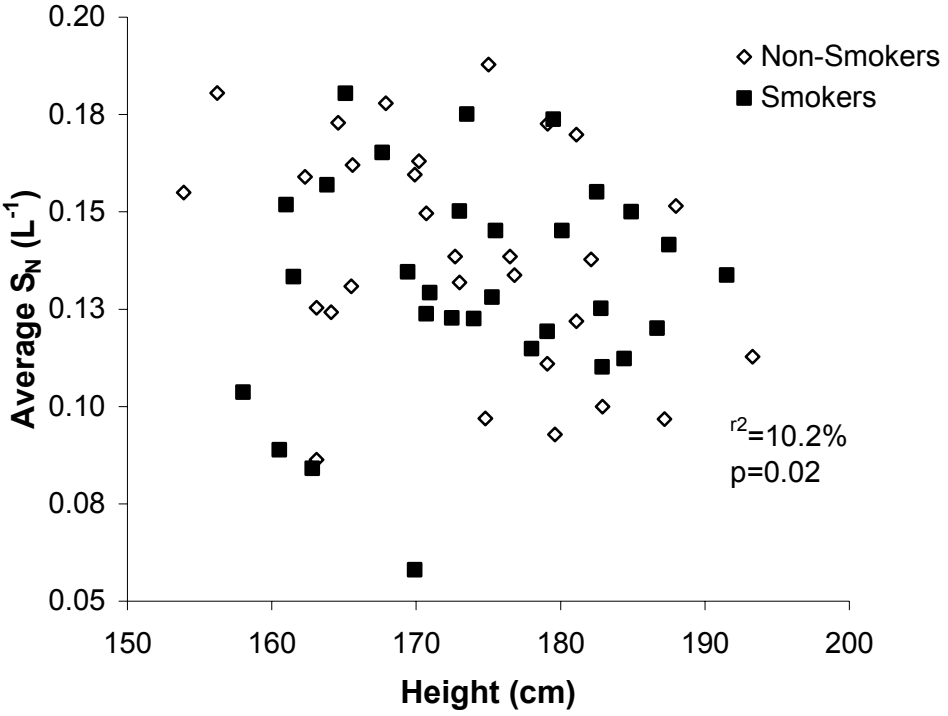


Figure 4-2: Comparison of average pre-air and ozone normalized slope (S_N) with height. S_N is correlated with height in the total population, but the addition of smoking status does not improve this relation.

When considered as a single population via ANCOVA using smoking status as a fixed factor and V_D as a covariate, S_N is significantly related to V_D (p=0.003) but not to smoking status (p=0.50).

Smoking Status Verification – Plasma Cotinine and the Smoking History Questionnaire

Reliability of the Plasma Cotinine Measurement

We used measurements of plasma cotinine in order to verify our participants' self-reported smoking statuses. Table 4-2 illustrates the number of participants testing positive for cotinine (>32 ng/mL) at the pre-screening and ozone exposure sessions. As was discussed in Chapter 3, in previous work investigators have employed a cut-off point of 10 ng/mL. However, because 32 ng/mL was the lower end of detection for our assay, we used values higher than or equal to this as indicative of cigarette smoking. At the pre-screening session, 26 non-smokers and 5 smokers tested negative. Four non-smokers and 25 smokers tested positive. At the O_3 exposure session, 23 non-smokers and 2 smokers tested negative. Six non-smokers and 28 smokers tested positive. This demonstrates that plasma cotinine values are largely consistent with self-reported smoking histories. However, both smokers and non-smokers were more likely to test positive on the day of the continuous O_3 exposure compared to the day of the pre-screening session.

Table 4-2: Number of participants testing positive or negative for plasma cotinine (>32 ng/mL) during the pre-screening and ozone exposure sessions. Of positive non-smokers, 2 tested positive at both sessions. Of negative non-smokers, 1 tested negative at both sessions.

	Pre-Screening		Ozone	
	Non-Smokers	**Smokers**	**Non-Smokers**	**Smokers**
Negative	26	5	23	2
Positive	4	25	6	28

In addition to verifying self-reported smoking status at the pre-screening and continuous O_3 exposures, we sought to determine the reproducibility of the cotinine measurement between the two time points. Figure 4-3 illustrates the relation between plasma cotinine measured in the smoking subjects during the pre-screening and O_3 exposure sessions. Contained within the population of smokers is an individual who, at the pre-screening session, had a plasma cotinine concentration 12 times higher than the population mean (female participant #114, pre-screening cotinine = 6.8 μg/mL). Regression analysis indicates that plasma cotinine values measured at the pre-screening and ozone exposure sessions are unrelated when #114 is included in the data set (r^2=0.01, p=0.60) and when #114 is removed from the data set (r^2=0.11, p=0.07).

Finally, we compared the population averages on the pre-screening and continuous O_3 exposure days. An important difference between the two sessions is that, on the pre-screening day, subjects were instructed to fast for 12 hours before completing the session. On the day of the continuous O_3 exposure, however, participants were instructed to eat a light meal approximately two hours beforehand. This was done to ensure that participants were not fasted before completing the exercise requirements of the session.

Table 4-3 illustrates plasma cotinine values measured in the pre-screening and O_3 exposure sessions and considers the population divided by smoking status. Two sample t-tests of the data reveal that during the prescreening session, non-smoker and smokers were not significantly different (p=0.06), nor were male and female smokers (p=0.24). During the O_3 exposure session, smokers were significantly different than non- smokers (p<0.000).

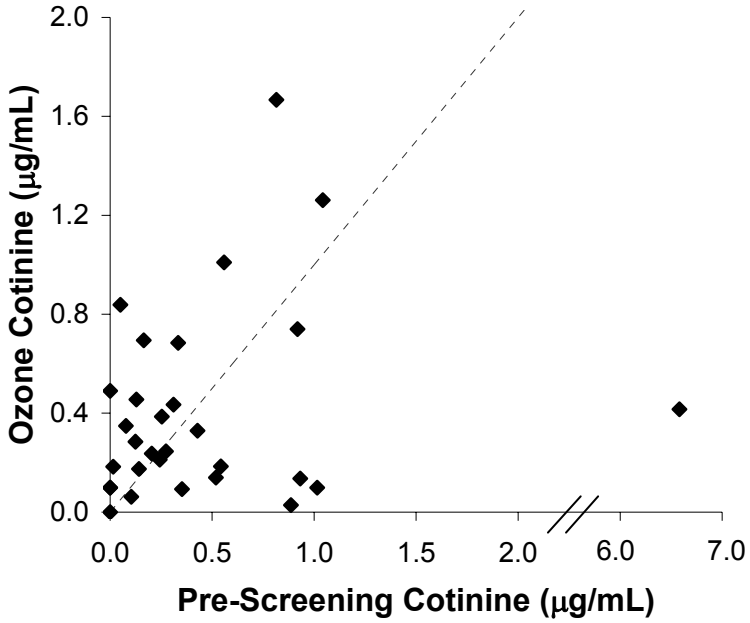


Figure 4-3: Relation between plasma cotinine measured at the pre-screening and ozone exposure sessions in smokers. The dotted line indicates the line of identity. Pre-screening cotinine values are unrelated to values measured in the ozone exposure session (r^2 = 0.01, p=0.6).

A paired t-test of the data reveals that values obtained during the pre-screening and ozone exposure sessions are not significantly different for the smokers (p=0.45). This

does not change when participant #114, whose pre-screening cotinine value was unusually high, is removed from consideration (p=0.61). Because the data obtained on the O_3 exposure day is consistent with what we would have expected, and we suspected that requiring our participants to fast at the pre-screening session may have affected the plasma cotinine results obtained in this section, future analyses used only the plasma cotinine values obtained at the continuous O_3 exposure session.

Table 4-3: Plasma cotinine values (μg/mL ± SD) in the pre-screening and ozone exposure sessions divided by smoking status and sex. (*) indicates that smokers are significantly different than non-smokers (p<0.000).

	Smokers	**Non-Smokers**
Pre-Screening (Fasting)	0.57 (1.20)	0.11 (0.36)
Ozone * (Non-Fasting)	0.40 (0.39)	0.07 (0.18)

Reliability of the Smoking History Questionnaire

In order to assess the reliability of self-reported answers to our smoking history questionnaire, we administered the questionnaire at two time points – at the pre-screening session and on the day of the continuous O_3 exposure. Results of the smoking questionnaire obtained during the pre-screening and O_3 exposure sessions are shown in Figure 4-4. Panel A of Figure4-4 illustrates answers to the question, "Within the last 2 months, on average, how many packs of cigarette per week did you smoke?" 61.3% of the

variability in the response to this question during the ozone session is explained by the variability in the response during the pre-screening session (r^2=0.61, p<0.000). Panel B of Figure 4-4 illustrates the answers to the question, "How many years have you been smoking cigarettes?" 96.7% of the variability in the response to this question during the ozone session is explained by the variability in the response during the pre-screening session (r^2=0.97, p<0.000). This indicates that responses to our questionnaire are largely reproducible between the two sessions.

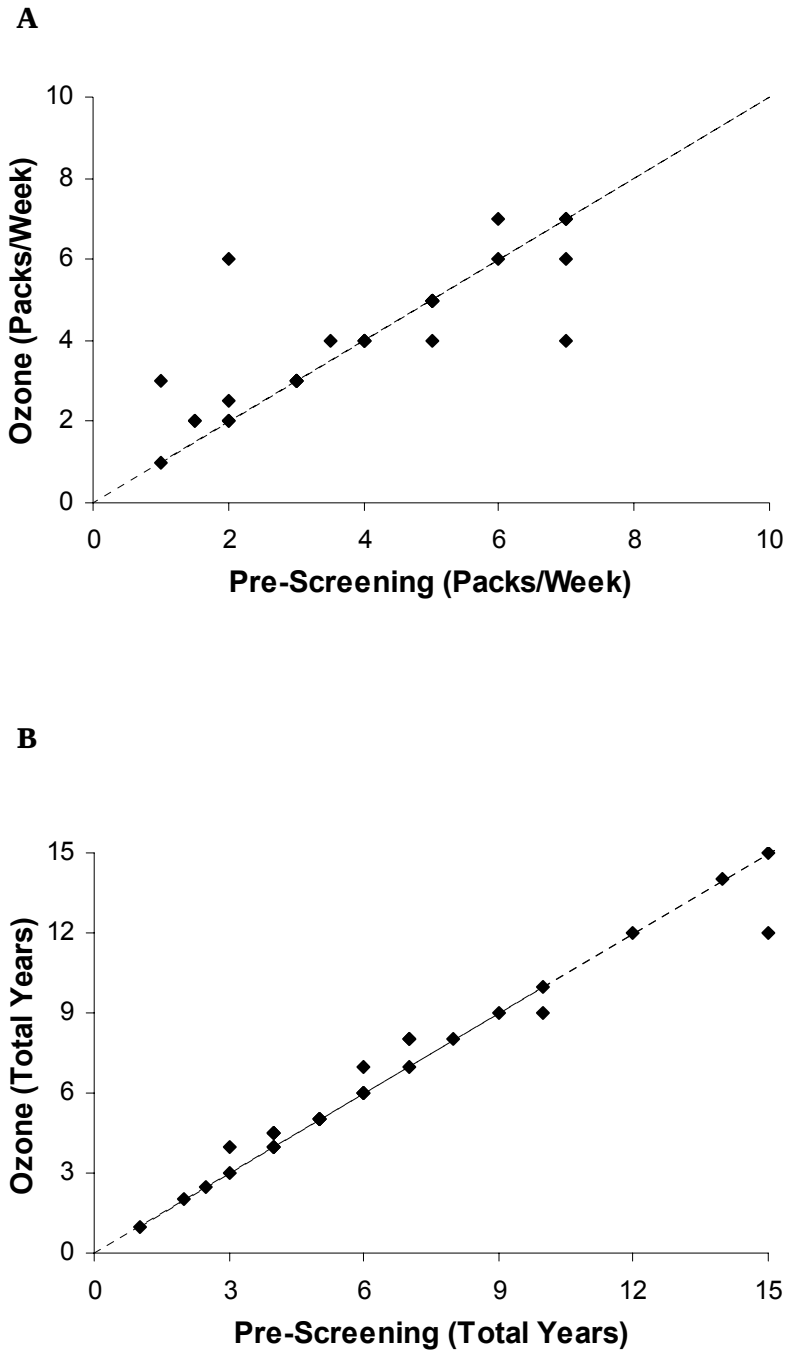


Figure 4-4: Comparison of smoking questionnaire responses obtained during the pre-screening and ozone exposure sessions. Panel A compares self-reported packs/week (r^2=0.61, p<0.000) and Panel B compared self-reported total years smoking (r^2=0.97, p<0.000). The dotted lines represent lines of identity.

As was discussed in Chapter 2, the degree to which plasma cotinine values are reflective of cigarette smoke intake is debatable. We sought to determine if, among our smoking population, reported smoking history in terms of packs per week smoked or total years smoking were related to plasma cotinine concentration. Comparisons of self-reported responses to the smoking history questionnaire and the plasma cotinine values are shown in Figure 4-5. Plasma cotinine values were regressed with self-reported smoking histories expressed in packs/week, total years smoking, and the product of the two (packs/week*years).

Plasma cotinine measured during the O_3 exposure session is not correlated with packs/week (r^2=<0.00, p=0.67), total years smoking (r^2=<0.00, p-0.96), or the product of the two (r^2=<0.00, p=0.73). These results indicate that plasma cotinine should not be used as a surrogate for self-reported smoking histories.

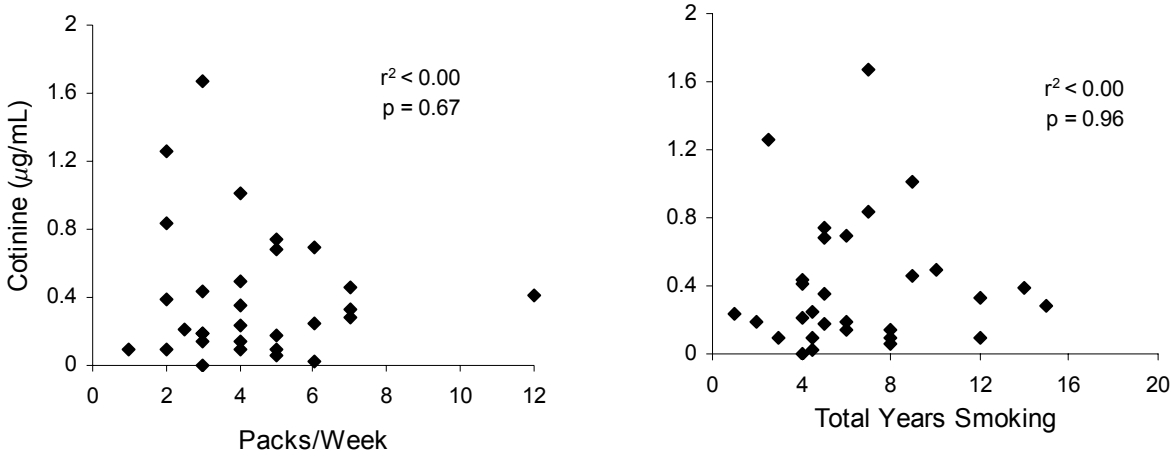


Figure 4-5: Comparison between plasma cotinine values and self-reported smoking history at ozone exposure session. The right hand panel compares cotinine to packs/week. The left-hand panel compares cotinine to total years smoking. Individual r^2 and p-values are given on each plot.

Effect of O_3 on the Pulmonary Function of Smokers - (Hypothesis One)

We hypothesized that with O_3 exposure, smokers would fail to experience changes in markers of conducting airway function but would experience changes in markers of peripheral airway function. The subsequent sections describe the composition of our population and the analyses we employed to verify that we had appropriately assigned participants to the smoking or non-smoking cohorts. Additionally, O_3-induced changes in FEV_1, V_D, and S_N are described.

In determining if smokers and non-smokers are differentially responsive to O_3, we first considered the mean response within each population with continuous air and O_3 exposure. Figure 4-6 illustrates the pre-to-post exposure changes in V_D, S_N, and FEV_1. One sample t-tests of the data indicate that, post-air exposure, neither non-smokers nor smokers experienced changes in V_D ($\Delta = 1.9 \pm 1.1\%$, $p = 0.09$ and $\Delta = -1.8 \pm 1.1\%$, $p = 0.10$), S_N ($\Delta = -5.8 \pm 3.2\%$, $p = 0.08$ and $\Delta = -1.9 \pm 2.6\%$, $p = 0.48$), or FEV_1 ($\Delta = -0.3 \pm 0.8\%$, $p = 0.74$ and $\Delta = 0.5 \pm 0.6\%$, $p = 0.48$), compared to baseline. Post-O_3 exposure, non-smokers experienced a significant change compared to baseline in FEV_1 ($\Delta = -8.7 \pm 1.9\%$, $p < 0.000$) but not in V_D ($\Delta = -0.1 \pm 1.4\%$, $p = 0.92$) or S_N ($\Delta = 0.7 \pm 2.6\%$, $p = 0.78$). Smokers experienced significant changes compared to baseline in V_D ($\Delta = -6.1 \pm 1.2\%$, $p<0.000$), S_N ($\Delta = 9.1 \pm 3.4\%$, $p = 0.01$), and FEV_1 ($\Delta = -9.5 \pm 1.8\%$, $p<0.000$). A two sample t-test of the data indicates that the percent change in FEV_1 experienced by the smokers post O_3 exposure was not different from that of the non-smokers ($p = 0.77$).

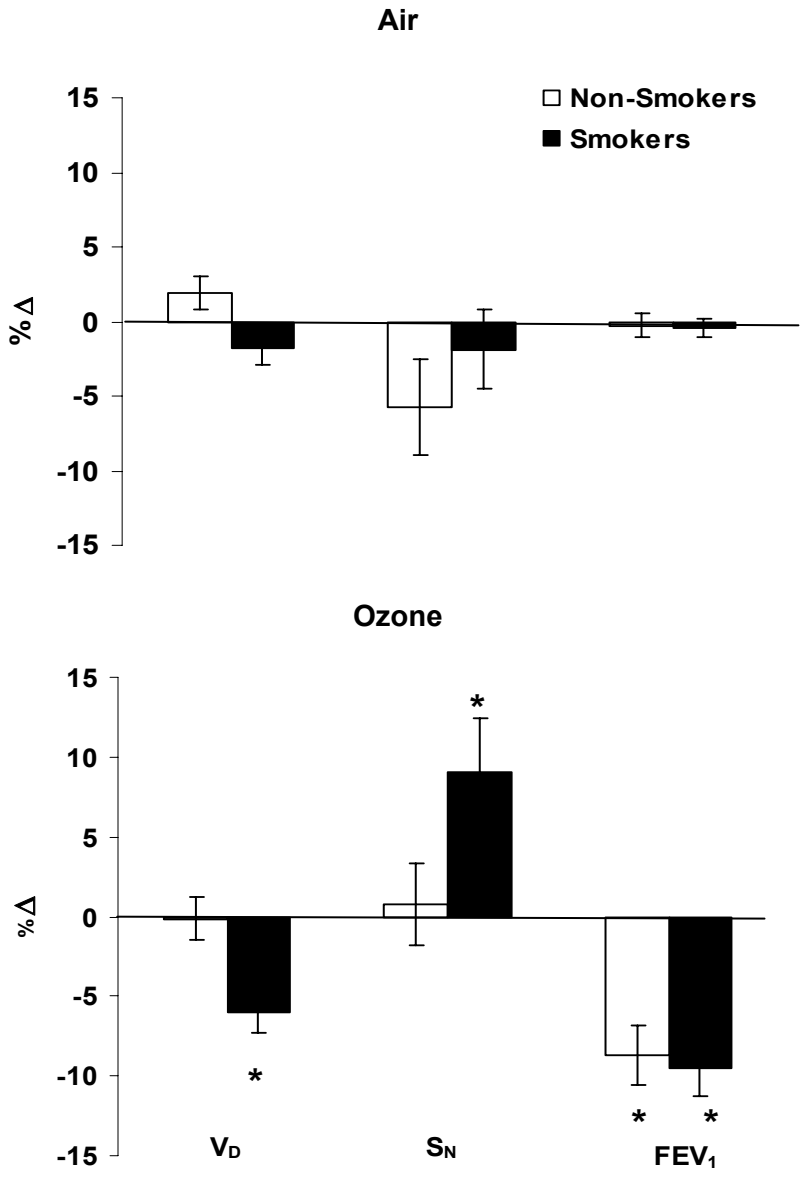


Figure 4-6: Pre-to-post changes in dead space (V_D), normalized slope (S_N), and forced expired volume (FEV_1) in smokers and non-smokers. The upper panel depicts changes with air exposure while the lower panel depicts changes with ozone exposure. (*) indicates a significant change from baseline ($P<0.05$). Error bars represent the standard error about the mean.

Because each cohort contained a few individuals whose self-reported smoking history was inconsistent with the results of the plasma cotinine analysis, we sought to investigate whether these individuals' responses were different than those of other individuals

within the respective cohort. This was done to ensure that they were not unduly biasing the group response data.

First, we plotted the individual data based both on reported smoking status and plasma cotinine results. This is shown in Figure 4-7. Data points representing individuals whose self-reported history was inconsistent with their plasma cotinine results do not appear to be visual outliers compared to the rest of the population. However, in order to be sure that these individuals were not biasing the group response results, responses were considered by analysis of variance (ANOVA) using plasma cotinine status (either positive or negative) as a fixed factor. Because of the limited number of smokers testing negative for cotinine, only the population of non-smokers testing positive for smokers were considered and compared to non-smokers testing negative for cotinine. In terms of percent change in V_D, S_N, and FEV_1, the non-smoking population testing positive for cotinine was not significantly different from the population of non-smokers testing negative for cotinine. Because of this, in subsequent analyses we considered smokers and non-smokers based on their self-reported status.

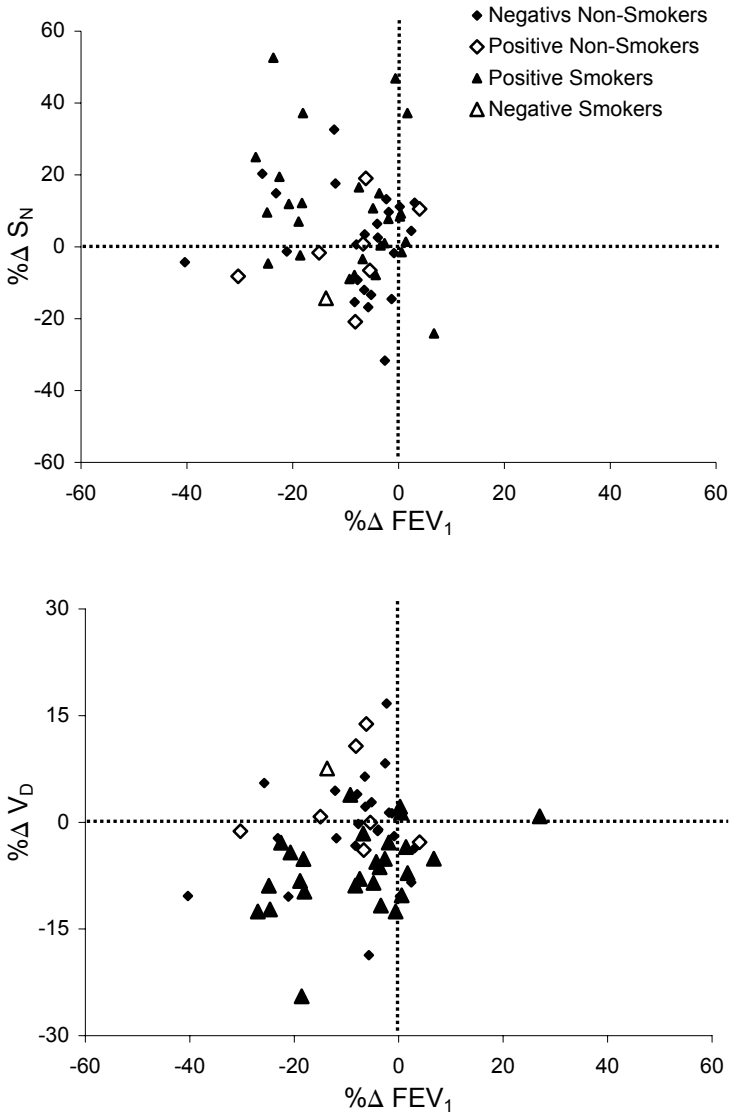


Figure 4-7: Changes in forced expired volume compared to changes in dead space and normalized slope. Individuals whose reported smoking history agreed with their plasma cotinine results are indicated with closed symbols. Individuals whose reported history or plasma cotinine results were inconsistent are shown with open symbols.

Recovery from Ozone-Induced Changes in Pulmonary Function

Following the O_3 exposure, participants performed forced spirometry and the CO_2 expirogram maneuver immediately post exposure and again at 30 and 60 minutes post exposure. We performed these measurements in order to characterize the recovery process from O_3-induced pulmonary function changes. Figure 4-8 illustrates the recovery of FEV_1, S_N, and V_D post-O_3 exposure. Both non-smokers and smokers experienced decrements in FEV_1 immediately post exposure (%Δ=-8.5±1.9%, p<0.000 and %Δ=-9.5±1.8%, p<000). Although these changes trended towards baseline, %ΔFEV_1 remained significant at 30 minutes (%Δ=-4.6±1.2%, p<0.000 and %Δ=-3.9±1.2%, p=0.002) and 60 minutes (%Δ=-2.4±0.8%, p=0.009 and %Δ=-2.5±1.1%, p=0.03) post exposure.

Smokers experienced a significant increase in S_N immediately post exposure (%Δ=9.1±2.6%, p=0.01) that persisted at both 30 minutes (%Δ=5.8±2.3%, p=0.02) and 60 minutes (%Δ=5.5±2.1%, p=0.01) post exposure. Non-smokers experienced no changes in S_N immediately (%Δ=0.7±3.4%, p=0.78), 30 minutes (%Δ=-1.7±2.3%, p=0.48), and 60 minutes (%Δ=-4.4±2.7%, p=0.12) post exposure.

Smokers experienced significant decreases in V_D immediately (%Δ=-6.1±1.2%, p<0.000) and 30 minutes (%Δ=-4.1±1.4%, p=0.006) post exposure, but had recovered to baseline by 60 minutes (%Δ=-2.2±1.4%, p=0.12) post exposure. Non-smokers experienced no change in V_D immediately (%Δ=-0.1±1.4%, p=0.94) or 30 minutes (%Δ=3.3±1.7%, p=0.06) post exposure, but a significant increase in V_D at 60 minutes (%Δ=3.9±1.7%,

p=0.03) post exposure. These data indicate that, under these exposure conditions, both smokers and non-smokers experience equivalent changes in FEV_1 that recover at equivalent rates. However, only smokers experience changes in V_D and S_N. Changes in V_D recover by 60 minutes post-exposure. Changes in S_N, however, do not.

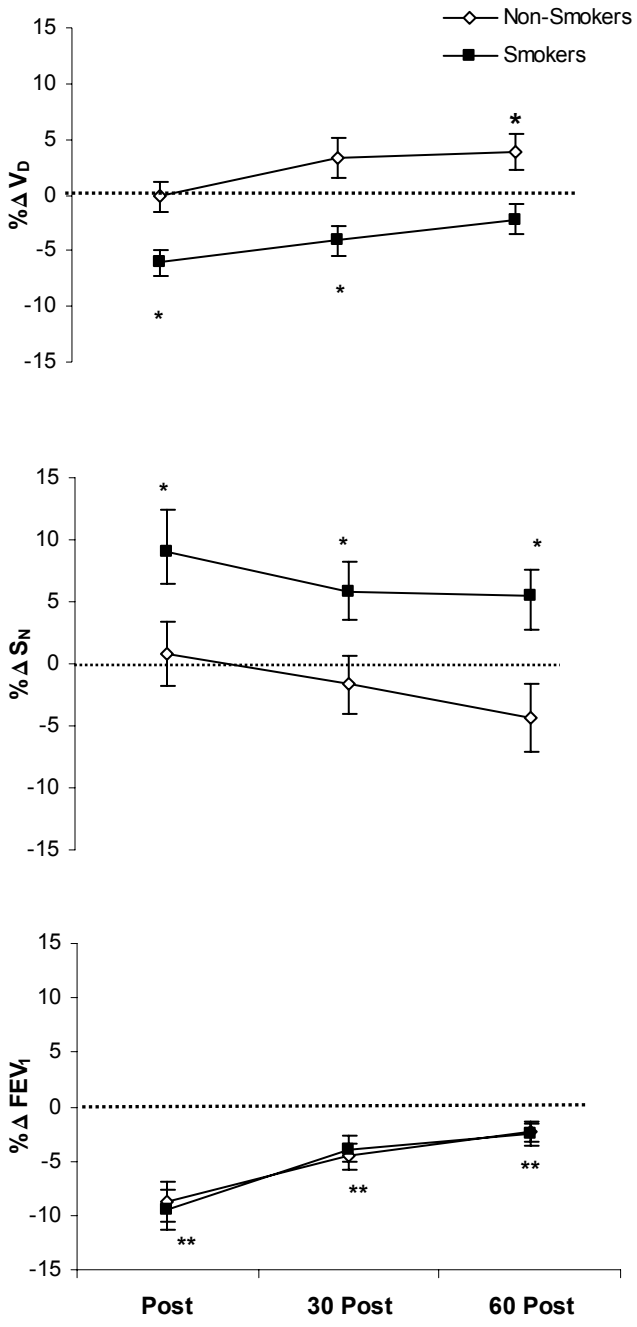


Figure 4-8: Change from baseline in forced expired volume (FEV_1), the normalized slope (S_N) and dead space (V_D) immediately, 30 minutes, and 60 minutes post exposure. * indicates a significant change from baseline for either smokers or non-smokers ($p<0.05$). ** indicates a significant change from baseline for both smokers and non-smokers ($p<0.05$).

Correction of the Ozone Exposure Data for Pulmonary Function Changes Induced by Exercise

It is common in air pollution research to perform controlled exposures with human participants over two separate sessions – during one of these sessions the participant is exposed to filtered air in order to assess changes in the measured endpoint that are related to the experimental conditions and not necessarily related to the pollutant. During the second, the participant is exposed to the pollutant of interest. These sessions may or may not be randomized. The effect of the pollutant is then corrected for changes induced by the experimental conditions independent of the pollutant.

Pre-to-post changes observed post-O_3 exposure were compared using linear regression methods to pre-to-post changes observed post-air exposure to determine if the exercise performed during both sessions had any biasing effect on the data. In smokers and non-smokers, changes in FEV_1 (r^2 <0.00, p=0.98 and r^2<0.00, p=0.74) and V_D (r^2=0.03 p=0.34 and r^2 <0.00, p=0.80) with air exposure are unrelated to changes with O_3 exposure. In smokers, changes in S_N with air exposure are unrelated to changes with O_3 exposure (r^2<0.00, p=0.95). However, in non-smokers, changes in S_N with air exposure were related to changes with O_3 exposure (r^2=0.14, p=0.047).

Because of the relation between the change in S_N with air and O_3 exposure, the effect of correcting the O_3 exposure data by the air exposure data was investigated. To do this, we considered the delta of the deltas ($\Delta\Delta$) by subtracting the percent change in S_N with air exposure from the percent change with O_3 exposure. For comparison sake, we performed this for both the smokers and non-smokers. In the smokers, correction of S_N increased the mean change in S_N (ΔS_N=9.1% vs $\Delta\Delta S_N$=11.3%). Although $\Delta\Delta S_N$ is statistically significant (p=0.01), the correction of the O_3 data by the air data inflated the standard error (ΔS_N=3.4% vs $\Delta\Delta S_N$=4.6%). In the non-smokers, correction of S_N increased the mean percent change in S_N (ΔS_N=0.7% vs $\Delta\Delta S_N$=6.6%), however $\Delta\Delta S_N$ was not statistically significant (p=0.06). Additionally, the correction of the O_3 data by the air data inflated the standard error (ΔS_N=2.6% vs $\Delta\Delta S_N$ = 3.3%).

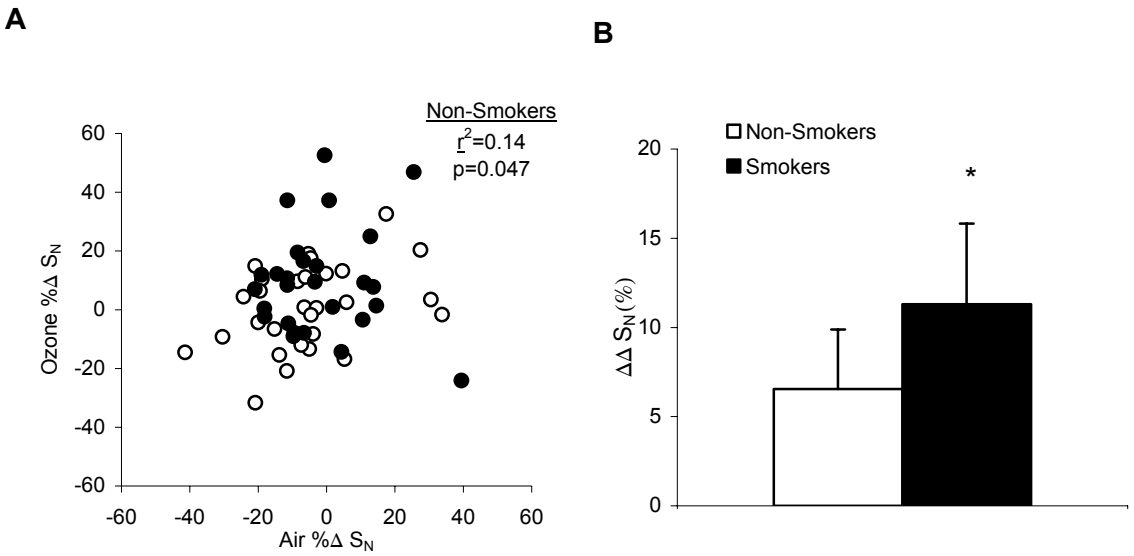


Figure 4-9: Comparison between changes normalized slope (S_N) with air and O_3 exposure. Changes in S_N with air and O3 exposure are significantly related in non-smokers (r^2=0.14, p=0.047). Panel B shows the change in S_N post-O_3 exposure, after correcting for the air exposure. Non-smokers fail to demonstrate a significant change in S_N in spite of the correction (p=0.059). Changes in the smoking group remain significant (p=0.01) although the standard error is inflated.

The Mechanism of Ozone-Induced Changes in Pulmonary Function

Because, among non-smokers, changes in FEV_1 may be related to both bronchoconstriction and inspiratory limitation, we investigated the potential mechanism by which smokers and non-smokers experienced decrements in FEV_1. We did this by relating post-O_3 exposure changes in FEV_1 to post-O_3 exposure changes in FVC.

Figure 4-10 compares the absolute change in FEV_1 with the change in FVC with O_3 exposure in smokers and non-smokers. The change in FVC is significantly related to the change in FEV_1 in non-smokers (r^2=0.73, p<0.000). The slope of the regression equation is 0.91 and the intercept is not significantly different from zero (p=0.35) (ΔFEV_1=-0.0461 + 0.912 ΔFVC). The change in FVC is significantly related to the change in FEV_1 in smokers (r^2=0.78, p<0.000). The slope of the regression equation is 1.00 (ΔFEV_1=-0.0306 + 1.00 ΔFVC) and the intercept is not significantly different from zero (p=0.54). As will be discussed in more detail in the following chapter, the fact that changes in FEV_1 are almost nearly equivalent to changes in FVC in smokers non-smokers indicates that the fall in FEV_1 is caused by a limitation of inspiratory capacity.

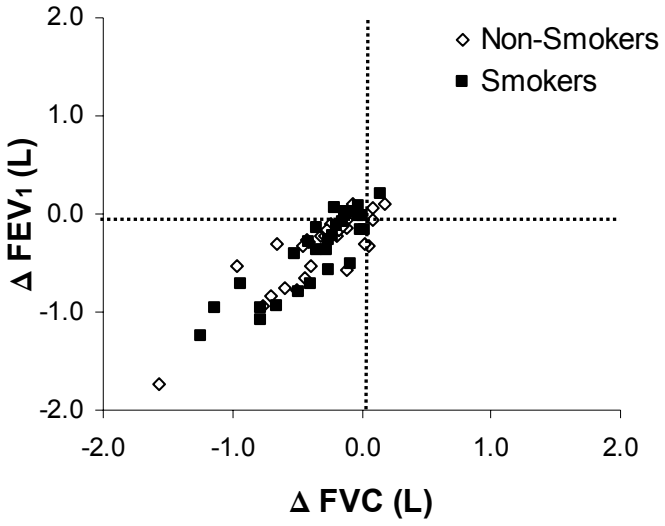


Figure 4-10: Correlation between the change in forced vital capacity (ΔFVC) and forced expired volume (ΔFEV1) in smokers and non-smokers with ozone exposure. ΔFVC is significantly related to ΔFEV1 in non-smokers (r^2=0.73, p<0.000) and smokers (r^2=0.78, p<0.000). In both cases, the intercept is not different from zero (p=0.35 and p=0.54).

In addition to investigating the manner by which O_3 induces changes in FEV_1, we investigated a potential mechanism by which S_N was altered in the smoking population. We did this by investigating the degree to which changes in S_N are related to changes in V_D. In the smoking population, the change in V_D was correlated with the change in S_N (r^2=0.24, p=0.006). These variables were uncorrelated in the non-smokers (r^2<0.00, p=0.70). This is shown in Figure 4-11**.** As will be discussed in the subsequent chapter in greater detail, this indicates that the increase in S_N among smokers may be caused by alterations in conducting airway diameter that cause regional ventilation heterogeneity.

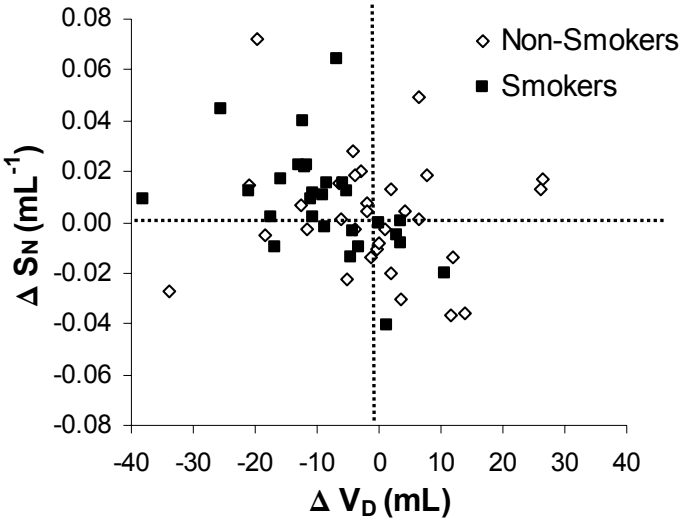


Figure 4-11: Changes in dead space (V_D) compared to changes in normalized slope (S_N). Changes in SN are unrelated to changes in VD in non-smokers (r^2=0.06, p=0.70) but are significantly in smokers (r^2=0.24, p=0.006).

The Presentation of Symptoms Associated with Ozone Exposure

During the course of this investigation we became interested in whether smokers and non-smokers, who are differentially responsive to O_3, experience different degrees of O_3-induced discomfort The following section outlines answers given to the symptom questionnaire (see Appendix B) administered pre-and post-air and O_3 exposure. We calculated the number reporting symptoms by subtracting the value of symptoms reported pre-exposure from those reported post-exposure and describing a change in symptoms experienced.

Figure 4-12 illustrates the proportion of the population experiencing symptoms post-air and O_3 exposure. Post-air exposure, one non-smoker reported headache, three reported runny nose, four reported shortness of breath, one reported cough, one reported chest burning, and six reported nausea. No smokers reported headache, one reported runny nose, two reported shortness of breath, one reported cough, one reported chest burning, and four reported nausea.

Post-O_3 exposure, four non-smokers reported headache, none reported runny nose, 18 reported shortness of breath, 12 reported cough, 11 reported chest burning, and seven reported nausea. Three smokers reported headache, one reported runny nose, eight reported shortness of breath, eight reported cough, five reported chest burning, and one reported nausea.

Table 4-4 and Table 4-5 demonstrate the number of participants reporting symptoms at each level of severity. Table 4-4 illustrates responses post-air exposure while Table 4-5 illustrates responses post-O_3 exposure.

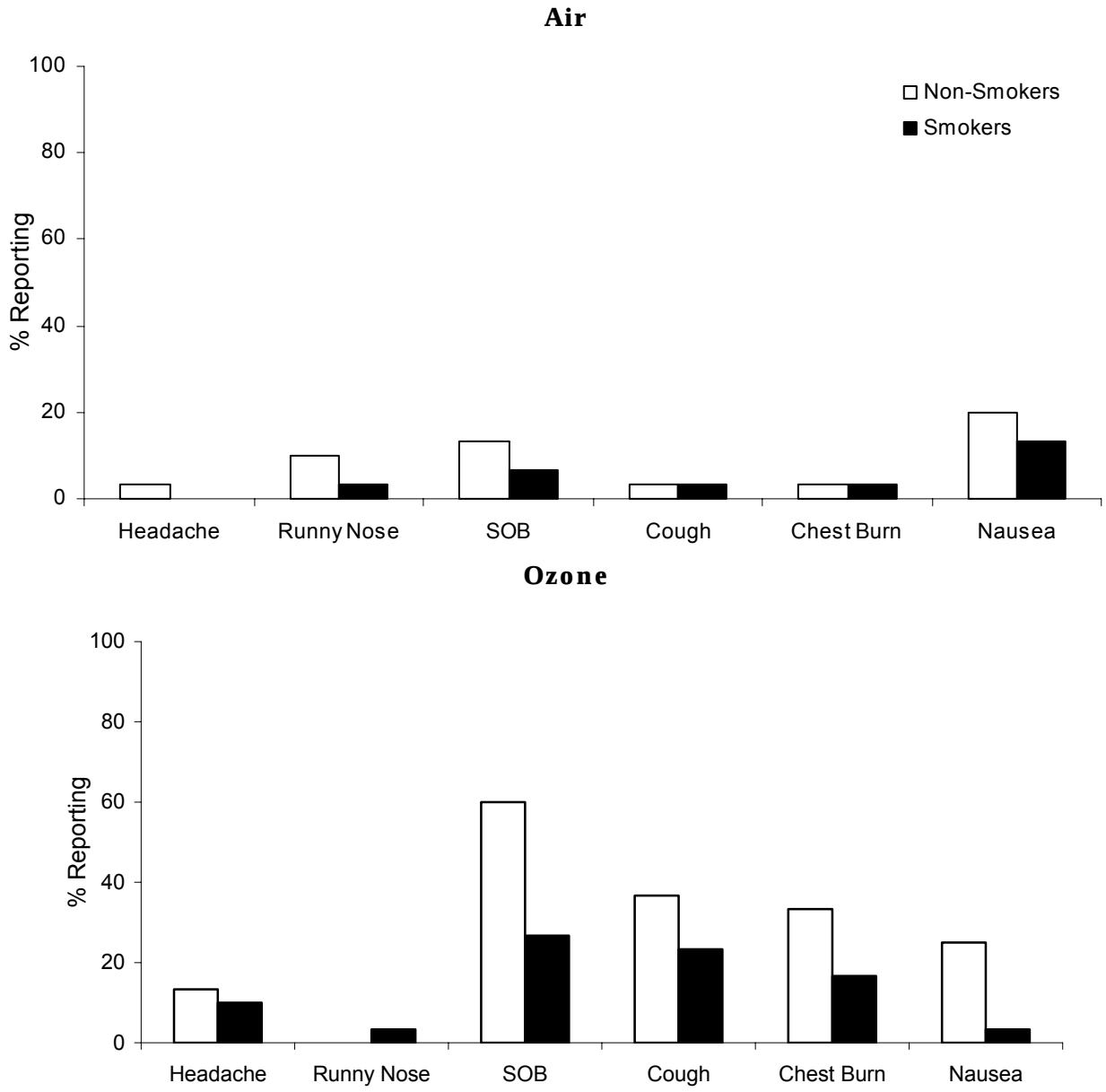


Figure 4-12: Response to symptom questionnaire post-air and ozone exposure. Post air exposure, an equal number of smokers and non-smokers reported chest burning. More smokers reported cough, but more non-smokers reported shortness of breath (SOB) Post-O_3 exposure, more non-smokers reported SOB , cough, and chest burning.

Table 4-4: Number of participants reporting symptoms per severity level post-air exposure

		Headache	Runny Nose	SOB	Cough	Chest Burn	Nausea
Non-Smokers	None	29	27	26	29	29	24
	Just Perceptible	1	3	4	1	1	6
	Distinctly Perceptible	0	0	0	0	0	0
	Nuisance	0	0	0	0	0	0
	Offensive	0	0	0	0	0	0
	Unbearable	0	0	0	0	0	0
Smokers	None	30	29	29	21	29	26
	Just Perceptible	0	1	1	1	1	4
	Distinctly Perceptible	0	0	0	0	0	0
	Nuisance	0	0	0	0	0	0
	Offensive	0	0	0	0	0	0
	Unbearable	0	0	0	0	0	0

Table 4-5: Number of participants reporting symptoms per severity level post-ozone exposure

		Headache	Runny Nose	SOB	Cough	Chest Burn	Nausea
Non-Smokers	None	26	30	12	18	19	24
	Just Perceptible	4	0	7	9	8	4
	Distinctly Perceptible	0	0	7	2	1	1
	Nuisance	0	0	3	1	2	0
	Offensive	0	0	1	0	0	0
	Unbearable	0	0	0	0	0	1
Smokers	None	27	29	22	21	25	29
	Just Perceptible	3	1	2	2	3	1
	Distinctly Perceptible	0	0	1	3	1	0
	Nuisance	0	0	2	3	1	0
	Offensive	0	0	3	1	0	0
	Unbearable	0	0	0	0	0	0

Comparison of Response to Ozone and Smoking History

Among our smoking population, we were interested in whether smoking history had an effect on responsiveness to O_3. Specifically, because our population consisted mainly of younger smokers, we were interested in whether, with increases in smoking history, individuals within the population because less sensitive in terms of FEV_1 and more sensitive in terms of parameters derived from the capnogram. We considered response as a function of the variables relating to smoking history. Because the half-life of cotinine is approximately 12-17 hours, we considered plasma cotinine to be indicative of smoking intensity over the previous 24-36 hours. We considered self-reported packs-week history to be indicative of smoking intensity over the previous two months, and self-reported total years smoking to be indicative of long-term smoking history.

Percent changes in V_D, S_N, and FEV_1 are not linearly related to plasma cotinine. Because of the variability in the data, we also tried binning the data as a means of decreasing the variability. Data from individuals testing positive for cotinine for were partitioned into the following five approximately equal sized bins:

1) 0.032 to 0.10 mg/mL (n=5)
2) 0.10 to 0.20 mg/mL (n-5)
3) 0.20 to 0.33 mg/mL (n=5)
4) 0.33 to 0.49 ng/mL (n=5)
5) > 0.49 mg/mL (n=7)

One-way ANOVAs comparing response, using bin number as a factor, indicate no differences in response between the bin.

Data were then analyzed by ANCOVA using total years smoking and packs/week as covariates and including a term describing an interaction between the two covariates. History in terms of packs/week is not related to the percent change in V_D ($p=0.97$), S_N ($p=0.16$), or FEV_1 ($p=0.94$). Additionally, history in terms of total years smoking, and the interaction term are not related to the percent change in V_D ($p=0.344$ and $p=0.72$), S_N ($p=0.34$ and $p=0.35$), or FEV_1 ($p=0.97$ and $p=0.83$). Because ANCOVA requires an assumption of colinearity among the data, we plotted each smoking history predictor against each response variable in order to verify that a nonlinear model did not better explain the data. No apparent nonlinear model provided a better explanation of the data. Provided as representative plots, Figure 4-13 compares changes in the response parameters with history reported in packs/week.

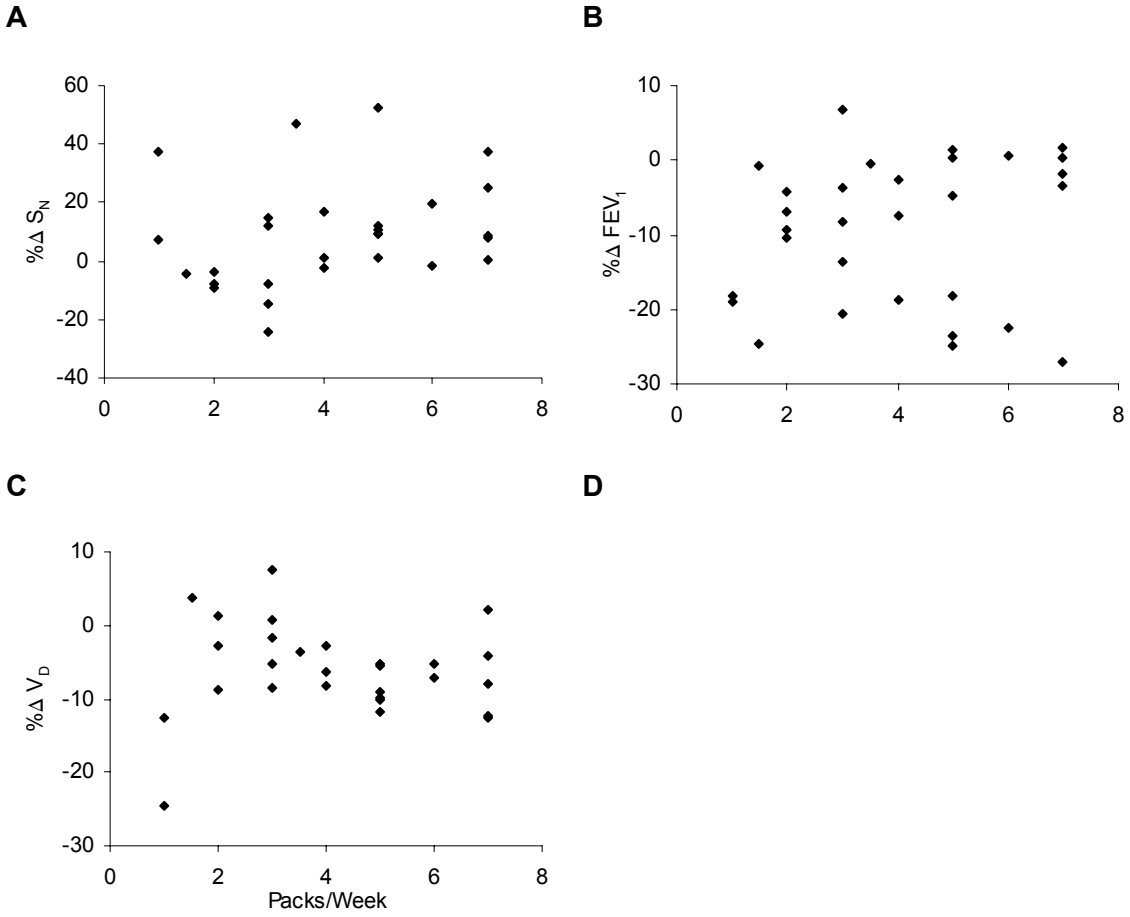


Figure 4-13: Relation between packs per week and percent change in response in smokers. Changes in response parameters are not related to history in terms of packs/week smoked, obtained the day of the continuous ozone exposure

Dependence of Pulmonary Function Changes on Inhaled Dose (Hypothesis Two)

We hypothesized that differential changes in peripheral airway function among smokers and non-smokers would be explained by differences in dose between the populations. Retained dose is determined by ventilatory pattern, inhaled concentration, and the fraction of O_3 retained. This next section describes real-time changes in ventilation that

occur with air and O_3 exposure. Additionally, it describes the consequences of altering regional airway mechanics on regional ventilation. Finally, it describes the dose of O_3 received by each population and its relation to response.

Changes in Ventilation with Ozone Exposure

Figure 4-14 illustrates changes in ventilatory parameters with air and O_3 exposure. Air exposure results are shown in the right-hand panels while O_3 exposure results are shown in the left-hand panels. Breathing frequency (f) is shown in the top panels, tidal volume (V_T) is shown in the center panels, and minute volume (V_E) is shown in the lower panels. Data are averaged over a five minute period, beginning five minutes into the exposure. Thus, averaged data shown at the ten-minute time point reflects data collected between minutes five and ten. In all cases, V_T and V_I were obtained from the inspiratory phase.

First, each session was considered separately and changes in f, V_T, and V_E were analyzed by ANCOVA considering time as a covariate and smoking status as a fixed factor. During the continuous O_3 exposure, f was related to time ($p<0.000$) but not to smoking status. V_T was also related to time ($p<0.000$) but not to smoking status ($p=0.16$). V_E, however, was not related to time ($p=0.24$) or smoking status ($p=0.21$). During the continuous air exposure, f was related to time ($p=0.004$) but not smoking status ($p=0.413$). V_T ($p=0.005$) was also related to time but not to smoking status ($p=0.191$). As with O_3 exposure, V_I was not related to either time ($p=0.24$) or smoking status ($p=0.24$).

When considered by ANCOVA using time as a covariate and exposure type (either air or O_3) and smoking status as fixed factors, f was related to exposure type ($p<0.000$) and time ($p<0.000$) but not to smoking status ($p=0.30$). V_T was also related to exposure type ($p=0.01$) and time ($p<0.000$) but not to smoking status ($p=0.09$). V_I was not related to exposure type ($p=0.62$), time ($p=0.09$), or smoking status ($p=0.08$). During the O_3 exposure, f increased 15% and V_T decreased by 14% over the 50 minute period. During the air exposure, f increased 6% and V_T decreased 8% over the 50 minute period.

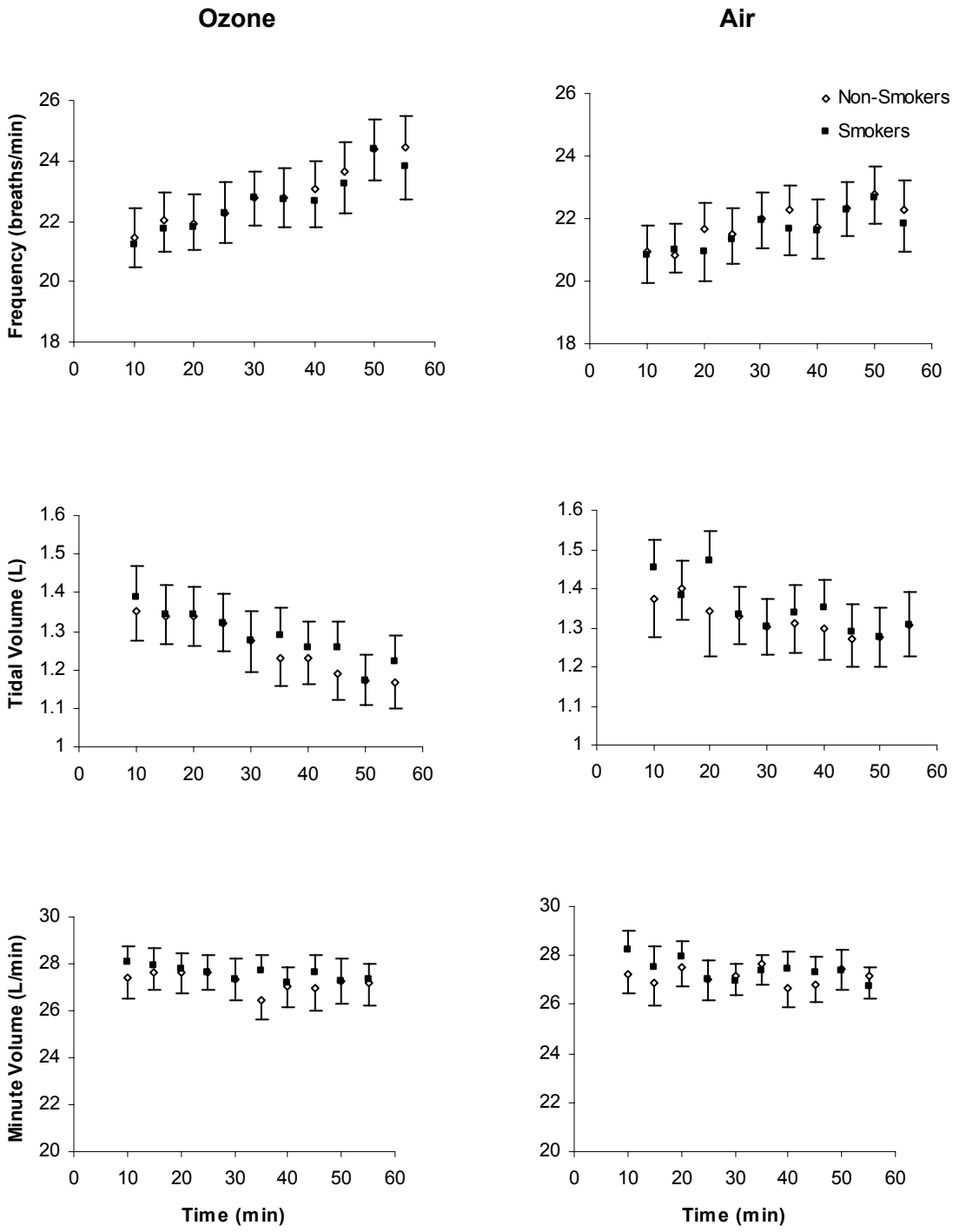


Figure 4-14: Changes in ventilatory parameters with air and ozone exposure During both the air and exposure exposures, tidal volume increases and frequency decreases similarly in the smoking and non-smoking populations. These changes occur to a greater degree with ozone exposure. Minute volume does not change as a function of time in either session.

After describing the global changes in ventilation that occur with air and O_3 exposure, we investigated any regional changes in ventilation that may occur. Figure 4-15 demonstrates the percent change in V_D/V_T with air and ozone exposure. V_D/V_T was calculated by dividing either the pre- or post-exposure V_D by V_T measured at minute 10 or 55 of exposure. Considering the pre and post data in paired t-tests, non-smokers (pre=0.12 and post=0.14, p=0.009) and smokers (pre=.12 and post=0.13, p=0.025) experienced significant increases in V_D/V_T with air exposure, respectively. With O_3 exposure, non-smokers (pre=0.12 and post=0.14, p<0.000) but not smokers (pre=0.12 and post=0.13, p-0.09) experienced significant increases in V_D/V_T. These results indicate that with air exposure, both smokers and non-smokers elevate V_D/V_T. However, with O_3 exposure non-smokers further elevate V_D/V_T while smokers do not. Therefore, with O_3 exposure, non-smokers more highly ventilate their conducting airways compared to smokers.

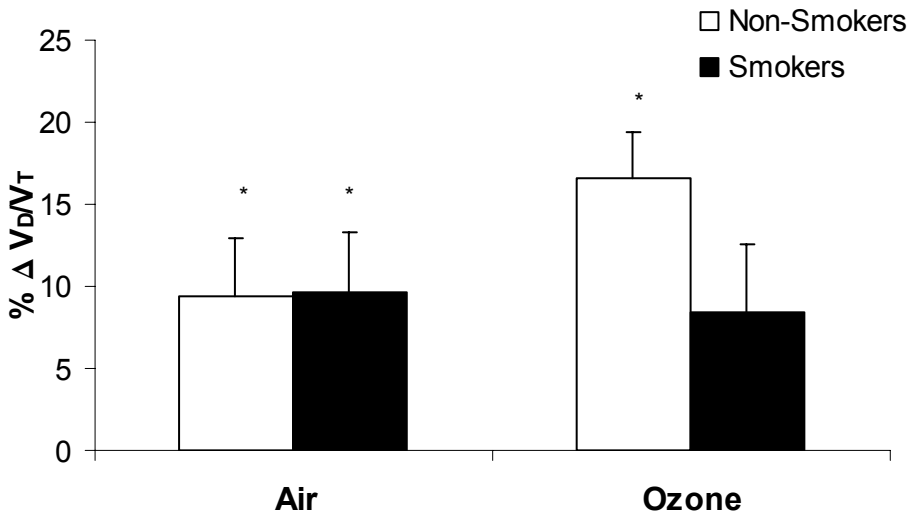


Figure 4-15: Change in the ratio of dead space to tidal volume (V_D/V_T) with Air and Ozone Exposure. V_D/V_T was calculated by dividing either the pre- or post-exposure V_D by V_T obtained in the 10^{th} or 55^{th} minute of exposure. With air exposure, both smokers ($p=0.25$) and non-smokers ($p=0.009$) increase V_D/V_T. During O_3 exposure, non-smokers ($p<0.000$) but not smokers ($p=0.087$) increase V_D/V_T.

Comparison of Inhaled Dose with Response

Figure 4-16 depicts dose parameters measured during the O_3 exposure and compares the smoking and non-smoking populations. Panel A depicts uptake rate (μg/min) as a function of time Upon visually inspecting these data, we concluded that there is a large amount of inter-period variability in the data such that there is no consistent trend over time and no clear difference between smokers and non-smokers. In order to asses if the populations were different in uptake, we calculated total dose by summing the uptake over each period (See Panel C). A two sample t-test of the data indicates that smokers and non-smokers are not different in terms of the total uptake of O_3 ($p=0.91$).

Because of the large variability in uptake rate, we also considered uptake efficiency between the two populations as a potentially less variable measurement (see Panel B). Uptake efficiency describes the fraction of O_3 removed by the lung. Because it is calculated as one minus the exhaled over inhaled dose and, thus normalizes by the inhaled dose, it accounts for differences in lung size, breath size, and inhaled amount. Upon visual inspection of these data we noted an apparent linearity in the data. ANCOVA of these data, using time as a covariate and smoking status as a fixed factor, indicates that uptake efficiency is related smoking status but not to time. At each time point the mean uptake efficiency in smokers is higher than that of non-smokers. Because of uptake efficiency proved to be a less variable parameter, we investigated the potential dose-response relationship using mean uptake efficiency over the 60 minute period as the index of dose.

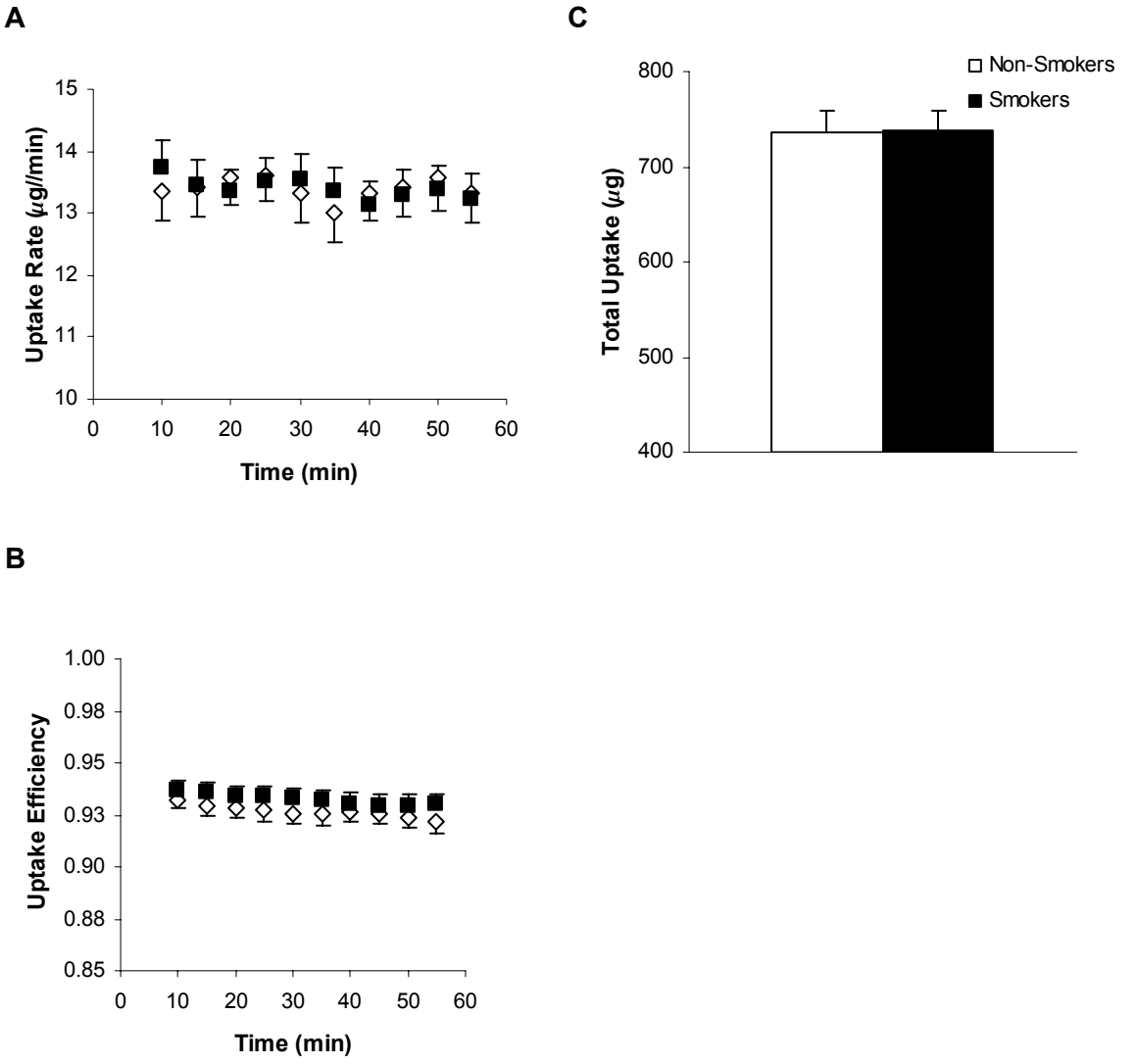


Figure 4-16: Dose parameters in non-smokers and non-smokers. Uptake efficiency (Panel B) is related to smoking status (p=0.02) but not to time. Smokers and non-smokers are not different in terms of uptake rate (Panel A) or total uptake (Panel C).

Among smokers and non-smokers, we compared the post-O_3 exposure percent change in V_D, S_N and FEV_1 with uptake efficiency. Data were analyzed using linear regression methods and smokers and non-smokers were considered as separate populations. Among non-smokers, uptake efficiency was not related to $\%\Delta V_D$ (r^2=<0.00, p=0.24), $\%\Delta S_N$ (r^2=<0.00, p=0.60) or $\%\Delta FEV_1$ (r^2<0.00, p=0.95). Among smokers, uptake

efficiency was not related to $\%\Delta V_D$ ($r^2<0.00$, $p=0.72$), $\%\Delta S_N$ ($r^2<0.00$, $p=0.22$), or $\%\Delta FEV1$ ($r^2<0.00$, $p=0.46$). The fact that in each case uptake efficiency is unrelated to the response parameters indicates that differences in total dose are not responsible for differential responsiveness.

Dependence of Response on Antioxidant Competency (Hypothesis Three)

Finally, we hypothesized that cigarette smokers experience a decreased antioxidant competency that could decrease the normal removal of O_3 in the conducting airways. To understand the antioxidant status of our populations, we measured antioxidant competency in lavage and plasma. Because decreased O_3 uptake in the conducting airways, caused by antioxidant compromise in the ELF, could alter distribution of the O_3 dose and, subsequently, response, we compared markers of antioxidants status with response.

The following section examines the antioxidant capacity of nasal lavage at the air and O_3 exposure sessions and plasma at the O_3 exposure session. It compares smokers and non-smokers and examines the effect of O_3 exposure on nasal antioxidant composition and the importance of dilution correction. Finally, it examines the predictive qualities of antioxidants in determining response.

Urea and the Dilution Correction Factor

It is not uncommon in protocols that measure antioxidants in the nasal and bronchoalveolar lavage of non-smokers to correct for dilution caused by the lavage using urea. Because urea is in equilibrium between the plasma and ELF compartments, the ration of urea in the ELF to that in plasma indicates the degree to which the lavage has diluted the ELF. We measured urea in plasma collected pre-O_3 exposure and lavage collected pre- and post-O_3 exposure and assessed the validity of using urea to correct for dilution in smokers and non-smokers.

Plasma collected pre-O_3 exposure and nasal lavage collected pre- and post-O_3 exposure were assayed for urea content. Data were analyzed using two sample t-tests. Mean values and standard errors for smokers and non-smokers are depicted graphically in Figure 4-17**.** Non-smokers and smokers are not different in terms of pre-exposure ($p=0.21$) or post-exposure ($p=0.44$) nasal urea concentration. Additionally, smokers and non-smokers are not different in terms of plasma urea concentration ($p=0.11$).

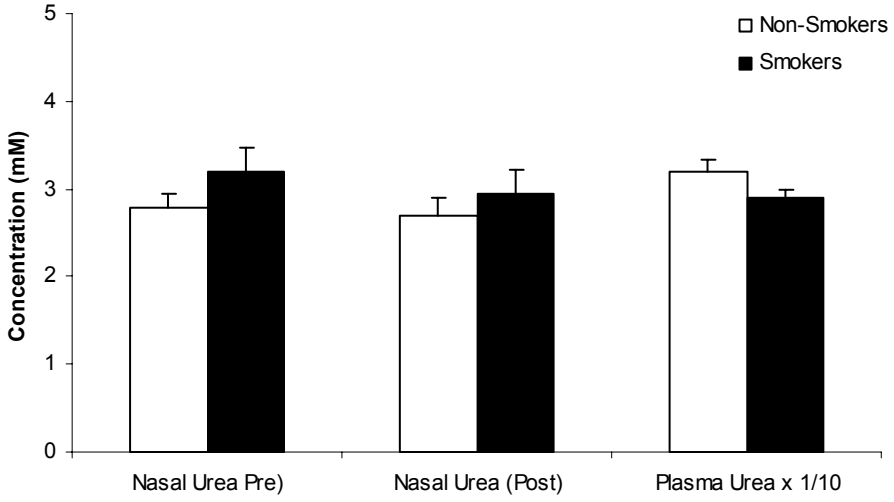


Figure 4-17: Urea concentration measured in nasal lavage obtained pre- and post-ozone exposure and plasma obtained pre-ozone exposure. Smokers and non-smokers are not different in terms of nasal or plasma urea concentration.

Figure 4-18 compares pre- and post-exposure urea concentrations in nasal lavage. Data were analyzed using multiple linear regression methods, regressing pre-exposure values against post-exposure values and using smoking status as an indicator value. Pre-exposure values are correlated with post-exposure values (r^2=0.44, $p<0.000$) but the indicator variable is not significant ($p=0.76$). This indicates that when the ELF is removed via lavage, urea is replenished to the ELF equally in smokers and non-smokers.

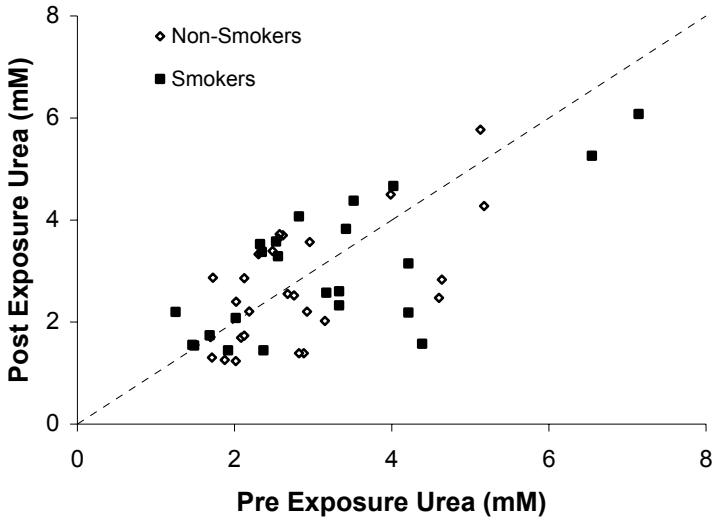


Figure 4-18: Comparison of pre- and post-exposure urea concentration in nasal lavage. In the total population, pre-exposure values are correlated with post-exposure values (r^2=0.44, p<0.000). Dotted line represents the line of identity.

For each participant, a urea correction factor was calculated by dividing the plasma urea concentration by the pre-exposure nasal urea concentration. These values were then compared based on smoking status. The correction factor computed for smokers (10.7±1.0) is not different from that computed for non-smokers (12.9±0.9) (p=0.12). This indicates that the relative abundance of urea in the ELF compared to the plasma compartment is equal in smokers and non-smokers and that the ELF of both populations is equally diluted by lavage.

Uric acid, AA, and protein concentrations, measured in nasal lavage collected pre- and post-O3 exposure were corrected for dilution be multiplying the data for any given individual by the urea correction factor calculated for that individual. An overall coefficient of variation was calculated for each antioxidant before and after dilution correction by dividing the population standard deviation by the mean and multiplying by

100. These are given in Table 4-6. In 5/10 cases, correcting by the dilution factor increased the coefficient of variation. Because correction for dilution did not improve the variability of the lavage data, and because there is no evidence to suggest that the degree of dilution is different in smokers and non-smokers, all subsequent data will be presented without the dilution correction.

Table 4-6: Variation in nasal antioxidant measurements before and after dilution correction. In 5/10 cases correcting by the dilution factor increased the coefficient of variation.

	Smokers				Non-Smokers			
	Corrected		Uncorrected		Corrected		Uncorrected	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Uric Acid	46.9	46.3	55.9	56.3	43.9	45.1	43.1	52.0
Ascorbic Acid	206.1	220.7	200.9	202.4	135.3	116.1	166.7	168.2
Protein	100.9	70.3	87.3	75.3	81.3	79.8	84.3	76.8

Nasal and Plasma Antioxidants in Smokers and Non-Smokers

We originally hypothesized that differential response in S_N might be due to reduced uptake of O_3 in the conducting airways of non-smokers. Because O_3 is taken up via reaction within the ELF with antioxidants, we sought to characterize the antioxidant competency of our populations. We did this by measuring UA and AA, two of the most abundant ELF antioxidants, in nasal lavage and blood plasma. Additionally we measured the total antioxidant capacity (or ORAC) or the nasal ELF and protein as a marker of epithelial permeability. Finally, we related these antioxidants to response.

First, we assessed the reproducibility of nasal antioxidants in order to ascertain the best method of analyzing the total data set. Figure 4-19 compares the antioxidant content of nasal lavage obtained pre-air and O_3 exposure. Data were analyzed using multiple linear regression methods, regressing data from the air session against data from the O_3

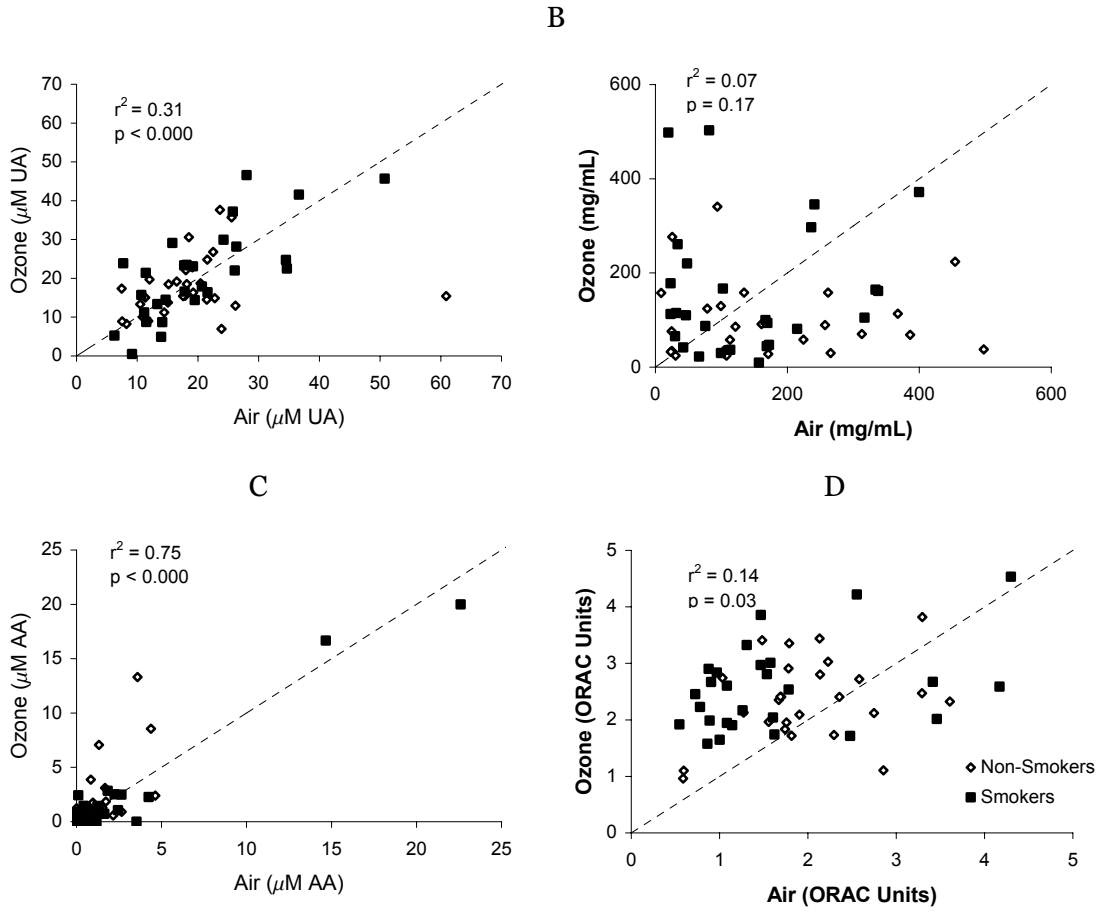


Figure 4-19: Comparison of nasal antioxidant values pre-air and ozone exposure. Nasal uric acid (Panel A), ascorbic acid (Panel C), and the antioxidant capacity (Panel D) of nasal lavage obtained pre-air exposure are related to values measured pre-ozone exposure. Nasal protein measured pre-air exposure is not related to that measured pre-ozone exposure. Individual r^2 and p-values are given above. The dotted lines represent lines of identity.

session and using smoking status as an indicator variable.

The UA contents of nasal lavages obtained pre-air and O_3 exposure were correlated (r^2=0.31, p<0.000) but the indicator value was not significant (p=0.25). The ORACs of lavages obtained pre-air and O_3 exposure were correlated (r^2=0.14, p=0.01) but the protein contents were not (r^2=0.07, p=0.75). In both cases, the indicator variable was not significant (p=0.15 and p=0.06). The AA contents of nasal lavages obtained pre-air and O_3 exposure were correlated (r^2=0.76, p<0.000) but the indicator variable was not significant (p=0.14).

However, this data set includes data for three individuals whose pre-air exposure lavage AA values are an order of magnitude larger than the population mean and a fourth individual whose pre-exposure lavage AA value is three times higher than the population mean. In order to investigate whether the data from these individuals were unduly influencing the regression, the data were removed and the regression repeated. Pre-air exposure values of lavage AA remained correlated with pre-O_3 exposure values, although the strength of the correlation decreased (r^2=0.31, p<0.000). The indicator variable remained nonsignificant (p=0.29). Because antioxidants measured on the air exposure day in both groups were similarly correlated with those measured on the O_3 exposure day, we chose to investigate potential differences between the populations by considering an average value for each antioxidant.

Figure 4-20 compares mean values of nasal UA, AA, ORAC, and protein obtained pre-exposure in smokers and non-smokers. Values represent an average of those measured pre-exposure on the continuous air and O_3 exposure days. Two-sample t-tests indicate that smokers and non-smokers are not different in terms of nasal UA (p=0.30), AA

(p=0.38), ORAC (p=0.71), or protein (p=0.58). This indicates that smokers and non-

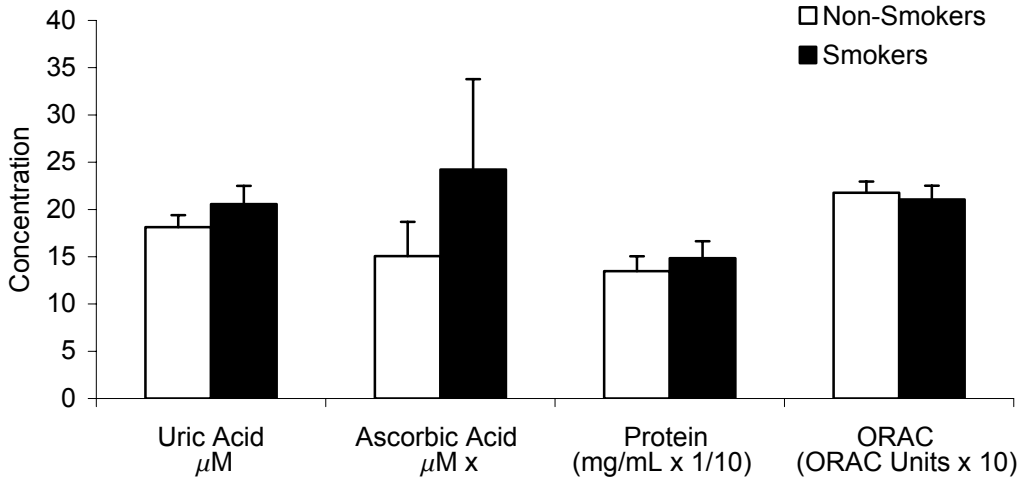


Figure 4-20: Nasal antioxidants in smokers and non-smokers. Values are an average of those measured pre-air and ozone exposure. Error bars represent the standard error about the mean. Individual antioxidants are not different in smokers and non-smokers.

smokers are similar in terms of nasal antioxidant status and epithelial permeability.

Figure 4-21 compares mean values of plasma UA and AA obtained on the pre-screening and O_3 exposure days. Two-sample t-tests indicate that smokers are not different from non-smokers in terms of plasma UA and AA concentrations at the pre-screening session (p=0.29 and p=0.62) or pre-O_3 exposure (p=0.09 and p=0.32). However, paired t-tests reveal that, in non-smokers, plasma AA was higher in samples obtained pre-O_3 exposure compared to those obtained during the pre-screening (p=0.007). In smokers, plasma UA was higher in samples obtained pre-O_3 exposure compared to those obtained during the pre-screening session (p=0.005). As with the cotinine assay, this reflects an important factor in the design of this experiment. Plasma antioxidants were sampled at two timepoints where participants had been asked to prepare differently for the session

(i.e. fasting versus being asked to eat in advance). This is probably the reason for the differences observed at the pre-screening session and pre-O_3 exposure. For this reason, only data obtained on the day of the continuous O_3 exposure was considered in subsequent analyses.

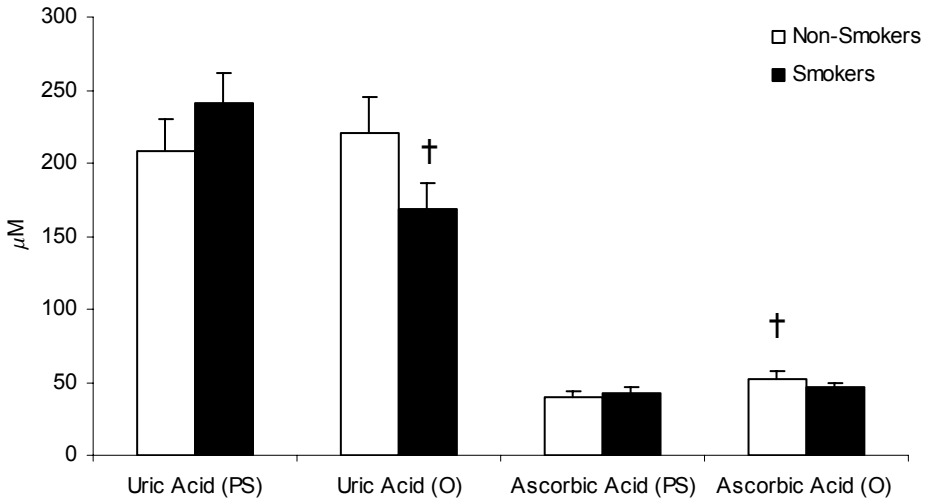


Figure 4-21: Plasma uric acid and ascorbic acid measured during the pre-screening session (PS) and pre-ozone exposure (O). At each session, smokers and non-smokers were not significantly different from each other. However, pre-ozone exposure, ascorbic acid was higher in the plasma of non-smokers, compared to pre-screening values ($p=0.007$). Pre-ozone exposure, uric acid was higher in the plasma of smokers compared to pre-screening values ($p=0.005$). (†) indicates $p<0.05$ compared to the pre-screening value.

Among smokers and non-smokers we sought to determine the factors influencing the ELF antioxidant status. As was discussed in Chapter 2, because ELF values of AA and UA have been related to values in plasma among non-smokers, we compared nasal UA and AA values obtained pre-O_3 exposure with values obtained in plasma on the same day. Data were analyzed using multiple linear regression methods, regressing plasma antioxidant values against lavage antioxidant values and using smoking status as an indicator variable. The concentration of UA in nasal lavage was not correlated with the concentration of UA in plasma ($r^2=0.04$, $p=0.49$) and the indicator variable was not

significant (p=0.17). The concentration of AA in nasal lavage was not correlated with the concentration of AA in plasma (r^2=0.02, p=0.65) and the indicator variable was not significant (p=0.63).

The ORAC, obtained from nasal lavage, was compared to the individual antioxidant components of lavage using ANCOVA. Data obtained pre-air and O_3 exposures were considered. Uric acid, ascorbic acid, and protein concentrations were used as covariates. Session and smoking status were included as fixed factors. Of the individual antioxidants, the nasal lavage ORAC was related to UA (p<0.000) but not AA (p=0.57) or protein (p=0.08). Session had a significant effect on the ORAC (p=0.000), but smoking status (p=0.23) did not. Figure 4-22 compares nasal lavage UA concentration to the ORAC pre-O_3 exposure. Regression of these data indicates that 43.8% of the variation in the ORAC is attributable to the variation in UA concentration (r^2 = 0.44, p<0.000).

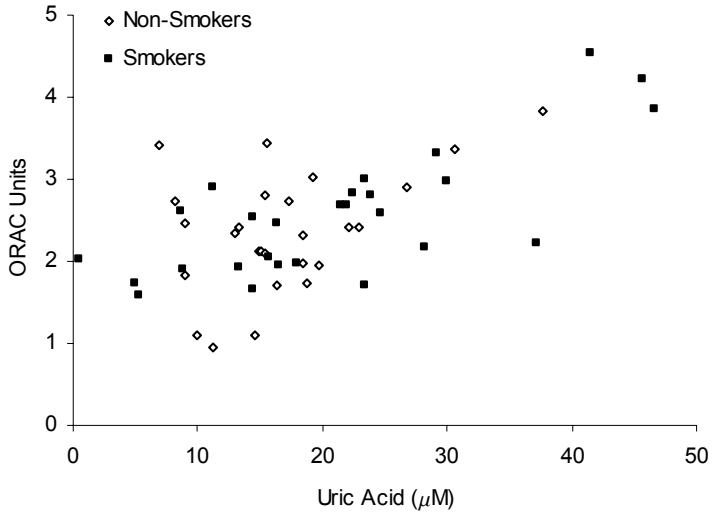


Figure 4-22: Comparison of nasal uric acid concentration and ORAC, obtained pre-ozone exposure. Uric acid concentration and the ORAC are significantly correlated (r^2=0.44, p<0.000).

Finally, because lavage was collected pre- and post-exposure, we examined the effects of pulmonary air or O_3 exposure on nasal antioxidants. Data were analyzed based on the percent change from pre-exposure and assessed using one sample t-tests. Post-air exposure, smokers experienced no change in nasal UA (p=0.06), AA (p=0.84), or protein (p=0.10) concentrations and no change in the ORAC (p=0.20). Post-O_3 exposure, smokers experienced no change in nasal UA (p=0.24), AA (p=0.97), or protein (p=0.20) concentrations and no change in the ORAC (p=0.92). Post air exposure, non-smokers experienced an increase in nasal UA concentration (p=0.03), but no change in AA (p=0.59) or protein concentrations (p=0.12), or the ORAC (p=0.72). Post-O_3 exposure, smokers experienced an increase in nasal protein concentration (p=0.02), but no change in UA (p=0.11) or AA (p=0.15) concentrations, or the ORAC (p=0.66).

Relation Between Antioxidants and Smoking History

As with the response variables, we were interested in whether, among our smokers, smoking history had any effect on antioxidant status. We assessed the effect of both short-tertm exposure to cigarette smoke, assesed by plasma cotinine, and self-reported smoking history on antioxidants and the ORAC in nasal lavage collected pre-O_3 exposure.

First, data were analyzed by ANCOVA using total years smoking and packs/week as covariates and including a term to describe an interaction between the two covariates. Second, data were analyzed by ANCOVA using cotinine as a covariate. P-values resulting from these ANCOVAs are given in Table 4-7**.** Nasal and plasma antioxidants were unrelated to total years smoking, packs/week, or the interaction term. Nasal and plasma antioxidants were unrelated to plasma cotinine. Because the ANCOVA makes an assumption of linearity, each of the response variables was plotted against each of the covariates to ensure that a non-linear model did not better describe the data. No apparent nonlinear model was a better for the data.

Table 4-7: Relation between markers of smoking history and nasal and plasma antioxidants measured before continuous ozone exposure. Values are p-values from ANCOVA analysis relating smoking parameters as covariates to individual antioxidant levels. Nasal and plasma antioxidants are unrelated to total years smoking, packs/week smoked, the interaction between the two, and plasma cotinine.

	Continuous Ozone Exposure					
	Nasal Lavage				Plasma	
	Uric Acid	Ascorbic Acid	ORAC	Protein	Uric Acid	Ascorbic Acid
Years Smoking	0.13	0.77	0.41	0.19	0.56	0.98
Packs/Week	0.34	0.14	0.72	0.08	0.91	0.57
Interaction	0.08	0.53	0.36	0.18	0.53	0.99
Cotinine	0.53	0.45	0.75	0.66	0.24	0.88

Relation Between Antioxidants and Response

This section explores the relation between nasal and plasma antioxidnts and response in terms of $\%\Delta FEV_1$, $\%\Delta S_N$, and $\%\Delta V_D$. Because there were no apparent differences between smokers and non-smokers in terms of antioxidant status, data for the entire population were compared using linear regression methods. Antioxidant measures were regressed against response parameters. When the population was considered in its entirety, no antioxidant measures were significantly related to the response parameters. Analysis was then completed using linear regression methods with smokers and non-smokers considered as serperate populations.

Table 4-8: r^2 and p-values from regresssion analyses of nasal and plasma antioxidants and percent change in response. Among non-smokers, nasal UA is related to $\%\Delta S_N$ (p=0.046).

			Nasal UA	Nasal AA	Nasal ORAC	Plasma UA	Plasma AA
Non-Smokers	$\%\Delta V_D$	r^2 (p)	<0.00 (0.82)	0.03 (0.41)	0.05 (0.27)	0.02 (0.46)	0.01 (0.61)
	$\%\Delta S_N$	r^2 (p)	**0.14 (0.046)**	2.8 (0.39)	1.1 (0.59)	0.4 (0.75)	0.4 (0.75)
	$\%\Delta FEV_1$	r^2 (p)	0.02 (0.43)	0.02 (0.50)	0.01 (0.58)	0.08 (0.14)	<0.00 (0.66)
Smokers	$\%\Delta V_D$	r^2 (p)	<0.00 (0.81)	0.03 (0.41)	<0.00 (0.84)	0.11 (0.08)	0.02 (0.43)
	$\%\Delta S_N$	r^2 (p)	0.04 (0.36)	0.04 (0.34)	<0.00 (0.73)	0.10 (0.10)	<0.00 (0.72)
	$\%\Delta FEV_1$	r^2 (p)	0.04 (0.40)	<0.00 (0.79)	<0.00 (0.63)	0.09 (0.11)	0.04 (0.29)

Data were again compared by regressing each antioxidant measure against each response parameter. The resulting r^2 and p-values are given in Table 4-8. When smokers and non-smokers were considered separately, however, $\%\Delta S_N$ in non-smokers was positively correlated with pre-O_3 exposure nasal UA concentration (see Figure 4-23).

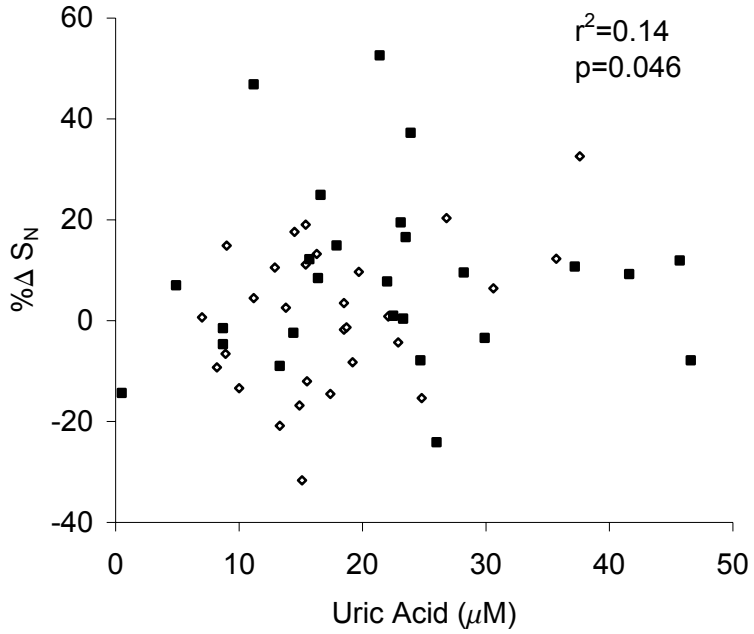


Figure 4-23: Comparison of the percent change in normalized slope (%ΔS_N) with nasal uric acid. Among non-smokers, nasal uric acid is positively correlated with %ΔS_N (r^2=0.14, p=0.046).

Chapter 5 - Discussion

This investigation was conducted with two goals in mind; first, we sought to determine if cigarette smokers are susceptible to O_3-induced changes in pulmonary function. Second, we sought to determine a mechanism by which these changes might occur. Specifically, we hypothesized that, while smokers would be insensitive to O_3 in their conducting airways, they would be more responsive than their non-smoking counterparts in their smaller, distal airways. As a result, we predicted that smokers would fail to experience changes in FEV_1 and V_D, markers of conducting airway function, but would show larger changes in S_N, a marker of distal airway function. Mechanistically, we hypothesized that these changes would be related to altered antioxidant competency within the lung's ELF. Increased mucus thickness and a lower concentration of water soluble antioxidants in the conducting airways would lead to decreased O_3 uptake in the conducting airways and a deeper penetration of O_3 into the distal airways and respiratory airspaces.

In order to test these hypotheses, we exposed smoking and non-smoking human volunteers to 0.30 ppm O_3 continuously while they performed 60 minutes of cycle ergometer exercise. Before and after exposure we measured forced spirometric parameters and obtained CO_2 expirograms in order to assess pulmonary function. We also obtained nasal lavage and blood plasma in order to assess antioxidant competency. During exposure, we made real-time measures of O_3 uptake, breathing frequency, and tidal volume in order to compare dosimetry between the two populations.

The Subject Population

Crucial to the investigation of these hypotheses is the composition of the subject population. We recruited 30 smokers and 30 non-smokers to participate in our protocol. Our population was recruited from the students and staff of The Pennsylvania State University and, as a result, its composition was generally reflective of that of the campus. We aimed to recruit equal numbers of male and female participants between cohorts such that they would be well-matched in terms of age, height, and weight. In terms of race, our population reflected the composition of the Penn State community; our populations contained an equal number of minority volunteers (1 Hispanic, 1 Black, and 5 Asian non-smokers and 1 Hispanic, 3 Black, and 3 Asian smokers).

As is reflected in Table 4-1**,** we recruited homogeneous populations with the exception of smoking history. Additionally, we sought to recruit smokers and non-smokers with clinically normal pulmonary and cardiovascular function. This was done both to ensure the safety of our participants and to isolate the effects of smoking independent of gross airway pathology. As is reflected both in Table 4-1 and Figures 4-1 and 4-2**,** our populations were indistinguishable in terms of distal and conducting airway function. This is an important consideration as it has been hypothesized that the lack of pulmonary responsiveness previously observed in smokers is the result of already compromised pulmonary function. However, because our smoking population has no apparent mechanical abnormalities of their airways and is similar to the non-smoking population in terms of other anthropometric markers, differences between the populations should be simply a result of cigarette smoking and not due to variations in the cohorts' compositions or the confounding effects of pathology.

Indicators of Smoking History

Of vital importance in the comparison of cigarette smokers and non-smokers is the verification that the individuals within the entire population had been assigned to the appropriate subpopulation. For this reason, we collected information as to their smoking status both via questionnaire and by assaying blood plasma for cotinine, the primary metabolite of nicotine. We chose cotinine as an indicator of smoking status because of its longer half-life (12-17 hours) compared to nictoine itself (2 hours). This makes it less sensitive to diurnal changes in smoking habits. The use of cotinine as a biomarker of smoking status is well established (Bramer-2003).

We collected questionnaire information both at the pre-screening session and the continuous O_3 exposure session in order to compared the reliability of the participants' responses. The responses to the question "Within the last 2 months, on average, how packs of cigarettes per week did you smoke?" are shown in Figure 4-4. Also given in Figure 4-4 are the responses to the question "How many years have you been smoking cigarettes?" Overall, we found the answers to both questions given during the pre-screening session to be well-correlated ($p<0.000$) with those given on the day of the continuous O_3 exposure.

However, answers given to question regarding the number of packs smoked per week were less well correlated ($r^2=0.61$) than answers given to the question regarding the number of years smoked ($r^2=0.97$). When asked the number of packs/week smoked, 9/30 smokers gave answers during the O_3 exposure session that were not the same as those given during the pre-screening session. This may be the result of two potential

sources of variability. Even though their smoking habits may not have actually changed, their perception of their smoking frequency may have changed or they may have been erroneous in their response to the question. In some cases, participants took as long as 12 weeks to complete the protocol. It is possible that between the pre-screening and continuous O_3 exposure, their smoking habits did change. This would be reflected in the pack/week history but not necessarily the reported total number of years. However, we were satisfied overall by the high degree of reproducibility in our participants' responses

As with the questionnaire, plasma cotinine was measured twice in order to verify the reliability of our findings. In previous studies of non-smokers, plasma cotinine values are typically less than 10 ng/mL. Use of this threshold accounts for dietary nicotine ingestion and occasional secondhand smoke exposure that non-smokers experience (Eskenazi-1992). Plasma cotinine values in smokers average 300 ng/mL (Benowitz-1996). A higher cut-off point of 32 ng/mL was chosen in this study largely because of the limited sensitivity of our assay; resolution of cotinine between 10 and 32 ng/mL proved difficult. While we appreciated that accepting a higher cut-off point might cause an increase in smokers and non-smokers falsely testing negative, our decision to use this method was based on the reported relationship between plasma cotinine and nicotine exposure. Although methods exist to measure cotinine levels in urine and saliva, a direct relationship between these measurements and nicotine exposure has not been established.

At the pre-screening session, four self-reported non-smokers tested positive for cotinine and five self-reported smokers tested negative. On the day of the continuous O_3 exposure, six non-smokers tested positive for cotinine and two smokers tested negative.

Of the non-smokers testing positive, two tested positive on both days. Of the smokers testing negative, one tested negative at both sessions. Because of the relatively limited number of smokers testing negative for cotinine, we believe the effects of accepting a higher cotinine concentration as indicative of a positive test to be minimal. The mean plasma concentration of cotinine measured in our smokers on the day of the continuous O_3 exposure was similar (400 ng/mL) to the average measured by other investigators. Because of the small number of individuals whose self-reported history was inconsistent with their plasma cotinine results on both occasions, we believe the smoking status reported by these individuals to be reliable. However, in order to verify that the data from these individuals did not bias the overall data set, response data were considered both with the inclusion and exclusion of these individuals' data. We found that excluding these data did not affect our findings and chose to leave them included in the data set.

When we compared the average cotinine concentration at both sampling times between populations we found, at the pre-screening session, the average cotinine concentration among the non-smokers was not different from that of the smokers. This is probably due to the large plasma cotinine values demonstrated by a small number of the non-smokers and the number of smokers testing negative for cotinine. On the day of the continuous O_3 exposure, however, plasma cotinine values among the smokers were higher than those of the non-smokers ($p<0.000$). This is the result of fewer smokers testing negative for cotinine.

The difference in plasma cotinine in smokers and non-smokers seen on the day of the continuous O_3 exposure that was not observed at the pre-screening session underlies the

importance of verifying plasma cotinine levels at more than one time point within a protocol. This is especially important when research sessions require that a volunteer prepare in advance for participation. We found that non-smokers were more likely to test positive on the day of the continuous O_3 exposure and smokers were more likely to test negative on the day of the pre-screening. Among non-smokers, plasma cotinine is sensitive to secondhand smoke exposure. Among smokers, plasma cotinine is sensitive to smoking abstinence. It is possible that, in asking the participants to fast in preparation for the pre-screening session, they altered their normal behavior. For example, in conversations prior to the beginning of the pre-screening session, several of the smokers reported that because of the fasting requirement, they went to bed earlier in the evening and did not participate in typical social behaviors. Thus, individuals in the smoking population may have smoked less prior to the pre-screening session than prior to the ozone exposure session.

Among smokers, we compared plasma cotinine concentrations at the pre-screening session to values measured on the O_3 exposure day and found them to be uncorrelated. This indicates that there is a large amount of day-to-day variability in this population. Again, it is possible that in fasting in preparation for the pre-screening session, or in preparation for the exercise required during the continuous O_3 exposure, participants altered their smoking behavior. Additionally, it is possible that in a population of young smokers, there is a large amount of day-to-day variability in their smoking habits. Because of the inconsistency in plasma cotinine values, only values measured on the day of the O3 exposure were related to response.

Pulmonary Responsiveness to Ozone in Smokers

In three separate investigations, Frampton *et. al.*, Foster, *et. al.,* and Kerr, *et. al.* reported that cigarette smokers did not experience O_3-induced changes in FEV_1. However, FEV_1 is an indicator of gross conducting airway function and gives no information about the efficiency of gas exchange, the distribution of ventilation, or the function of the small, distal airways. Therefore, in addition to forced spirometry we measured V_D and S_N from the capnogram to gain additional insight into the effect of O_3 on regional airway function in this population compared to a population of comparable non-smokers.

As we expected, air exposure caused no significant changes in FEV_1, S_N, and V_D in both the smoking and non-smoking populations. All participants tolerated the exercise and exposure equipment and reported no distress. The most important finding of this work, however, is that with O_3 exposure, the young smokers in this population not only retained their responsiveness to O_3 in terms of FEV_1, but demonstrate an increased responsiveness in terms of V_D and S_N compared to non-smokers. The following sub-sections will discuss how these changes are related to possible changes in airway mechanics.

The Change in Forced Expired Volume

Changes in FEV_1 that are greater than or equal to 5% within a single day are considered clinically important. Within the non-smoking population, 20/30 participants experienced decrements in FEV_1 greater than 5% during O_3 exposure. Within the

smoking population, 16/30 experienced decrements greater than 5%. Day-to-day changes in FEV_1 greater than or equal to 11% are considered clinically important. Among the non-smokers, eight participants experienced changes with O_3 exposure greater than 11%. Among the smokers, eleven participants experienced changes with O_3 exposure greater than 11%. Finally, in the total population, the average change in FEV_1 was not significantly different. These findings indicate that these smokers were equal in responsiveness to comparable non-smokers.

The mechanism by which O_3 induces decrements in FEV_1 is multifactorial and includes a combination of bronchoconstriction and inspiratory limitation. Knowing that ours is the first investigation demonstrating that smokers may be sensitive to O_3 in terms of FEV_1, we sought to determine if the mechanisms by which O_3 caused a fall in FEV_1 in smokers and non-smokers were similar by comparing the absolute change in FEV_1 with the absolute change in FVC. We found that when the change in FVC was regressed against the change in FEV_1 in both populations, the slopes of the regression lines were equal to or nearly equal to one and that the intercepts of the regression lines were not different from zero. Additionally, the change in FEV_1 was closely related to the change in FVC (r^2 = 0.73 and r^2=0.78). This suggests that, in both populations, the fall in FEV1 is caused by a limitation in inspiratory capacity.

In healthy non-smokers exposed to O_3 while performing intermittent exercise, pre- and post-exposure measurements of lung volumes established that O_3 does not alter total lung capacity or functional residual capacity (Foster-1993, Hazucha-1973). Additionally, short-term O_3 exposure does not alter pulmonary compliance (Bates-1972), nor does it cause a restrictive pathology (e.g., fibrosis) of the parenchyma. Thus, what might be

diagnosed as a restrictive defect by using standard clinical criterion (e.g., a decrement in FEV_1 with $FEV_1/FVC>70\%$) is probably an inspiratory limitation.

The effect of O_3 on inspiratory capacity has important implications in the ability to interpret post-exposure spirometry. FEV_1 and FVC are dependant on the size of the preceding inspiration. When the volume of the inspiration is decreased, FEV_1 will decrease because of both the volume limitation and the decreased elastic recoil at the lower initial lung volume. (Hyatt-1958). With decreasing inspiratory volumes, intrapleural pressures generated upon expiration may not become sufficiently high to elicit effort independent flow limitation in the first second (Altarifi-2003). In addition to the volume of the inspiration, the rate at which the volume is inspired is important. When inspiration is performed from FRC to TLC over varying periods of time, FEV_1 decreases as a function of increases in the inspiratory period. (D'Angelo-1993). This indicates the importance of both the quality and the volume of the inspiration preceding a forced expired maneuver. Because we did not collect information on the quality of the inspiratory maneuver, we cannot discern whether the decline in FEV1 was simply a result of volume limitation or a combination of volume limitation and changes in the inspiratory flow.

Spirometry is an important and valuable clinical tool. However, because the results are potentially confounded by the quality of the inspiratory maneuver, and this quality is changed with O_3 exposure, it may not be an appropriate primary endpoint for determination of the effects of O_3 on airway mechanics. In the future, when FEV1 is employed to assess the health effects of a toxin, values of the forced inspiratory capacity (FIC) and the forced inspiratory volume in one second (FIV_1) should also be reported.

Unless maneuvers can be performed using standard inspiratory maneuvers pre- and post-exposure, the interpretation of FEV_1 as a marker of bronchoconstriction may be misleading.

Changes in Dead Space and the Normalized Slope of the Alveolar Plateau

In addition to FEV_1, we measured changes in V_D and S_N derived from the capnogram and found smokers to be responsive in terms of both parameters. Non-smokers, however, were not. In investigating a potential mechanism by which O_3 caused an increase in S_N in smokers, we found that V_D was significantly correlated with S_N ($p=0.006$). Therefore, not only did individuals who experienced decreases in V_D also experience increases in S_N, but the magnitude of the changes were related. If the decrease in V_D occurs non-uniformly among different airway paths, this could explain the increase in S_N and the dependence of S_N on V_D.

In a model lung that is uniform in structure, S_N is entirely explained by the continued evolution of CO_2 a cross the alveolar epithelium during exhalation (Farmery-1995). In the normal human lung, this is largely, but not entirely, sufficient to explain S_N (Grønlund-1987). In order to completely explain S_N, the lung's innate heterogeneity must be considered. As was discussed in Chapter 2, P_{CO_2} is regionally distributed such that the lung apex experiences a lower P_{CO_2} compared to the base. The P_{CO_2} distribution is determined by the regional matching of ventilation and perfusion. In the apex of the lung, ventilation is in excess of perfusion. The reverse is true in the base.

Individual acini empty at sequentially different rates depending on their mechanical time constants (τ). Differences in regional emptying are driven by differing regional mean

values of τ. The positive value of S_N could be explained if these regionally different τ were overlain with the distribution of P_{CO_2} such that areas with low P_{CO_2} emptied first and high P_{CO_2} emptied last. If O_3 caused bronchoconstriction proximal to some acini relative to others, this could intensify the distribution of τ and explain the increase in S_N. Representative expirograms obtained from a female smoker pre- and post-O_3 exposure are shown in Figure 5-1. This figure demonstrates that, relative to the pre-exposure expirogram, O_3 exposure shifts the expirogram to the left. Because V_D is smaller, CO_2 appears earlier in the expirate.

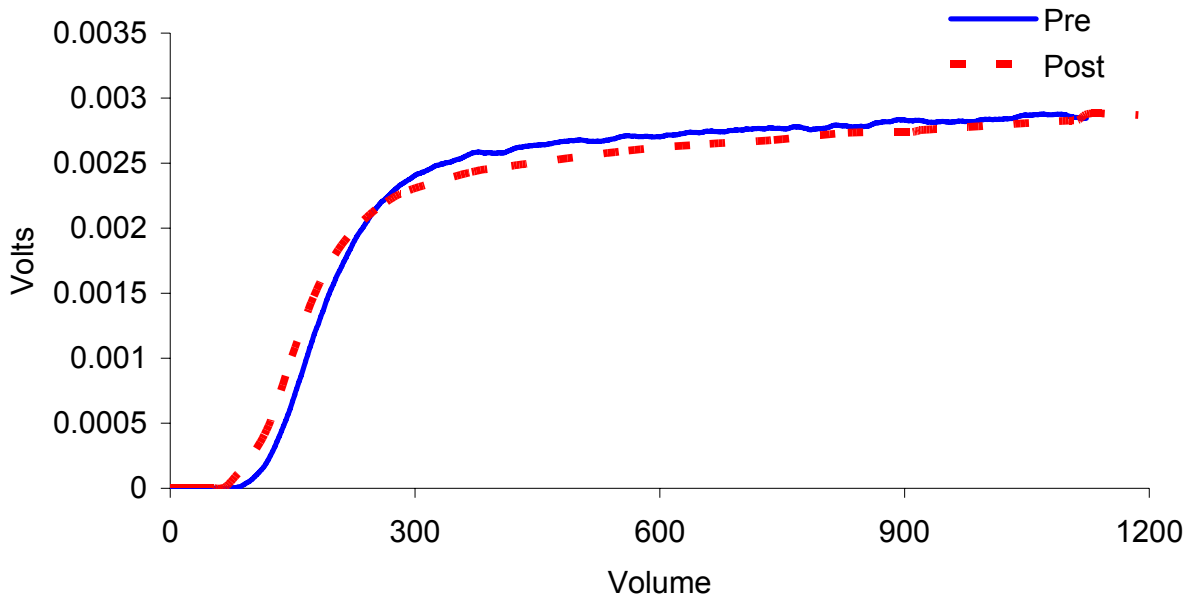


Figure 5-1: Representative pre- and post-ozone exposure expirograms obtained from a female smoker. Expired volume is given on the x-axis and the voltage output from the capnometer is given on the y-axis. Relative to pre-exposure, the post-exposure expirogram is shifted to the left, resulting in a lower deadspace. Additionally, although end-tidal CO_2 is the same, the slope of the alveolar plateau is increased.

Recovery of O_3-Induced Changes in Pulmonary Function and the Correction for Exercise-Induced Effects

As a safety precaution, participants remained in our lab for 60 minutes after the termination of the O_3 exposure. We took advantage of this opportunity to monitor the recovery of pulmonary function changes at 30 and 60 minutes post-exposure. We found that, in both smokers and non-smokers, FEV_1 fell with O_3 exposure, but tended to increase towards its baseline value in the hour following exposure. However, both smokers and non-smokers continued to experience a small but significant decline in FEV_1 that persisted at 60 minutes post-exposure. The fact that the population means for smokers and non-smokers are indistinguishable at each recovery time point supports the idea that the mechanism behind the decline in FEV_1 is similar.

With O_3 exposure, smokers experienced a significant increase in S_N. Although it tended toward recovery, S_N remained elevated at 30 and 60 minutes post exposure. This is mirrored by a fall in V_D with exposure that remained significant at 30 minutes. What was most interesting, however, is that fact that while non-smokers did not changes in V_D or S_N that were significantly different from zero immediately post-exposure, the mean change at each timepoint paralleled the mean change experienced by the smokers the smokers. Indeed, at 60 minutes post-exposure, non-smokers experienced a significant increase in V_D. This would suggest that in the non-smokers, the effect of O_3 on V_D and S_N may be masked by some additional confounder in this population. As a potential candidate we considered the exercise-induced effects on airway morphometry.

In the airways, smooth muscle tone is maintained vagally (Kondo-2000). An increase in respiratory flow, as occurs with exercise, causes a decrease in parasympathetic efferent activity and a withdrawal of vagal tone (Pichon-2005). This results in bronchodilation (Warren-1984) and an increase in airway conductance. Furthermore, this bronchodilatory response may be sufficient to overcome noxious stimuli that would normally result in mild bronchoconstriction (Kagawa-1970). Therefore, we considered whether the exercise performed had a bronchodilatory effect that masked the bronchoconstrictory effects of O_3 in the non-smokers.

In toxicological research it is common to correct exposure data for effects related specifically to the experimental design. In this case it would be appropriate to consider correcting data from the O_3 exposure session by using the air exposure data. In theory, this would allow the assessment of the effects of O_3 independent of the exercise. Correcting for the effects of exercise on airway function is physiologically reasonable.

When we compared the pre-to-post percent change in S_N observed with air exposure to that observed with O_3 exposure, we found them to be significantly correlated (r^2=0.14, p=0.047). Because of the relation between $\%\Delta S_N$ measured in the two sessions, we investigated the effect of correcting the O3 response for changes observed with air (what we called "$\%\Delta\Delta S_N$"). We found that, while the mean $\%\Delta\Delta S_N$ was positive, it was not significantly different than zero. Additionally, the use of a $\Delta\Delta$ response variable inflated the standard error. Therefore, it may not be appropriate to correct data collected on one day by collected on another. Indeed, the change in S_N with air exposure may have significant inter-day variability. In order to diminish this variability, $\%\Delta S_N$ should be

measured in a population of volunteers studied in replicated O_3 and air exposure sessions as a means of reducing the standard error in the ΔΔ variable.

Comparison to Other Populations

Non-Smokers and the Health Effects Population

This study was designed to be similar to that conducted by Ultman, *et. al.* and sponsored by the Health Effects Institute (HEI) (Ultman-2004). Participants were assessed using similar measurement techniques and exposed using a similar exposure protocol (0.25 versus 0.30 ppm). The major difference, however, is that while Ultman and colleagues sought to assess inter-subject variability, we sought to minimize it in order to assess inter-population differences. In the HEI study, 60 volunteers were exposed to O_3 while exercising at a workload sufficient to elicit a minute volume equal to 30 L/min. By fixing ventilation rate, small subjects with small lung volumes tended to breathe at a lower V_D/V_T than larger subjects. This was done in order to maximize differences between subjects in their retained O_3 dose. In the current study, the target minute volume was scaled by body surface so that the V_D/V_T should be more comparable among subjects. This was done to achieve a similar dose per unit body surface between participants. As a consequence of this important difference between the two studies, HEI non-smokers experienced a much larger population mean value of $\%\Delta S_N$ during O_3 exposure than the smokers in the current study ($\%\Delta S_N$=17.5±2.2% versus $\%\Delta S_N$=0.7±2.6%, p<0.000).

In our population, non-smokers achieved an average minute volume of 27.2 ± 0.9 L/min. As a consequence of the lower minute volume, our non-smokers received a lower, but

insignificant, total dose (735±24 μg) compared to the HEI population (807±29 μg, p=0.36). We considered whether the lower dose received by our non-smokers could account for the difference in response by comparing total uptake to $\%\Delta S_N$ in the combined population. The results are shown in Figure 5-2.

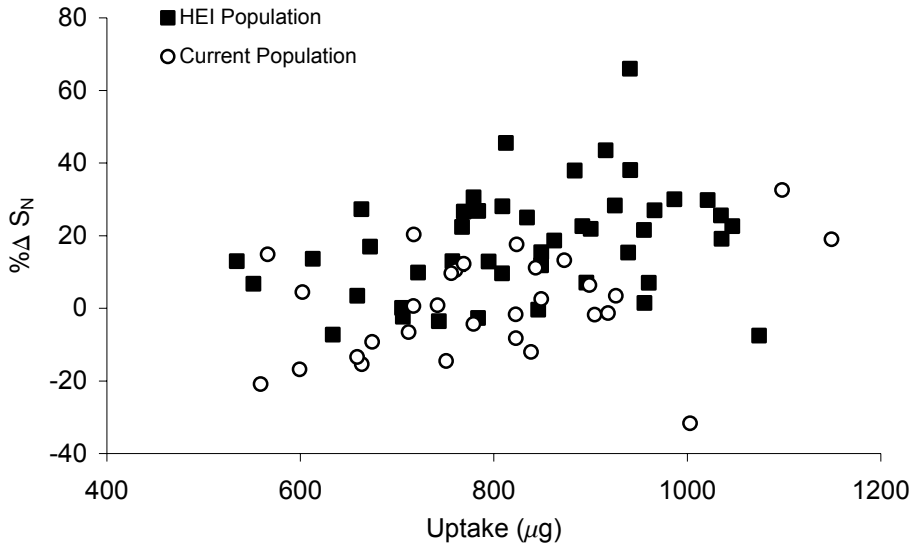


Figure 5-2: Comparison between uptake and the percent change in normalized slope ($\%\Delta S_N$) in the Health Effects and current populations. When the populations are combined, uptake is correlated with $\%\Delta S_N$ (r^2=0.14, p=0.001).

Figure 5-2 demonstrates that, when the two populations are combined, the total uptake of O_3 is significantly correlated with response in terms of $\%\Delta S_N$. This indicates that, although there is considerable inter-subject variability, the fact that our many of the individuals in our population received a lower total dose can explain their lack of responsiveness. To date, sufficient work has not been done to develop a reliable relationship between ΔS_N and dose (either in terms of μg dose or exposure concentration). However, it is possible that the dose response relationship, as it is

between exposure concentration and FEV1 or sR_{AW}, is sigmoidal. Our population and the HEI population may fall on either side the steeply rising portion of the sigmoid such that small changes in dose would have a large effect on the ability to perceive a change in S_N; this would explain the lower ΔS_N responses measured in this study as compared to the HEI investigation.

Smokers and Previous Smoking Populations

Smokers within our population were more responsive in FEV_1 than smokers in the historical populations discussed earlier. An obvious difference between our work and that done by previous investigators is the composition of our subject population. Our population was derived from the general university population and, as a result, has a relatively limited smoking history (6±4 total years smoking and 4±2 packs/week currently smoked). While it is typical to collect smoking history in terms of pack-years (or the product of the number of years smoking and the average number of packs/day smoked), we chose to report information in terms of the total number of years smoked and the average number of packs smoked per week over the previous 2 months. We refrained from describing our population in terms of a pack-year history.

In populations of any smoking history, the clinical calculation of a pack-year history is based on the number of packs/day currently smoked and the total number of year smoking. It usually overestimates the cumulative smoke exposure. In adolescence and young adulthood, the number of packs smoked per day is often low and sporadic. As nicotine dependence is established, the pack/day history becomes more regular and the number of packs smoked each day becomes more substantial (Kandel-2007). In older

smokers, the period marked by sporadic smoking and low pack/day use comprises a small portion of their overall history and, thus, the overestimation of their pack-year history is small. In younger smokers, however, the overestimation may be much larger.

Solely to compare our results to previous work, however, we estimated the upper limit of the pack-year histories of the smokers in our population to be 4±3 pack-years. In contrast, smokers described in Frampton's work had an average history of 13±9 pack-years. The smokers in Foster's population had an average history of 33±16 pack-years. Ozone-related decrements in FEV_1 are mediated, in part, by bronchial neurons that may be desensitized, or lost, with long-term cigarette smoking (Schelegle-1993, Dicipinigaitis-2003). When cigarette smokers are challenged with an oxidizing irritant like cigarette smoke, bronchial reactivity, measured in terms of the decrement in FEV_1 caused by the irritant decreases as a function of pack-year history (Jensen-1998). Therefore, our population may represent a population that has not yet experienced a degree of bronchial pathology necessary to ablate the response to O_3.

While the mean change in FEV_1 was equal between our smokers and non-smokers, we also investigated the distribution of the response and compared our smokers and our nonsmokers to the HEI nonsmokers (Figure 5-3). We did this by scrutinizing the frequency of response per 10% change in FEV_1. First, we noted that the variability in response among our non-smokers and the HEI non-smokers was virtually identical. Approximately 10% of both populations had improvements between 0% and 10% in FEV_1. On the other hand, decrements in FEV1 between 0 and 10% were found in the majority of individuals in the two populations (43% in the HEI population and 60% in our population). In both of these nonsmoking populations, decrements occur with

increasing less frequency as the size of the decrement increases. As with the non-smokers, the largest number of smokers who participated in the current study experienced declines in FEV1 between 0% and 10% (40%). However, the same tailing of the distribution as a function of increasing decline in FEV_1 is not apparent. Although 40% of smokers experienced declines between 10% and 30%, the smoking population seems to lack the extreme responders ($\%\Delta FEV_1$ >30%) observed in the non-smoking populations. Among young smokers, cigarette smoking may attenuate response in individuals who would normally be hyperresponsive to O_3 exposure.

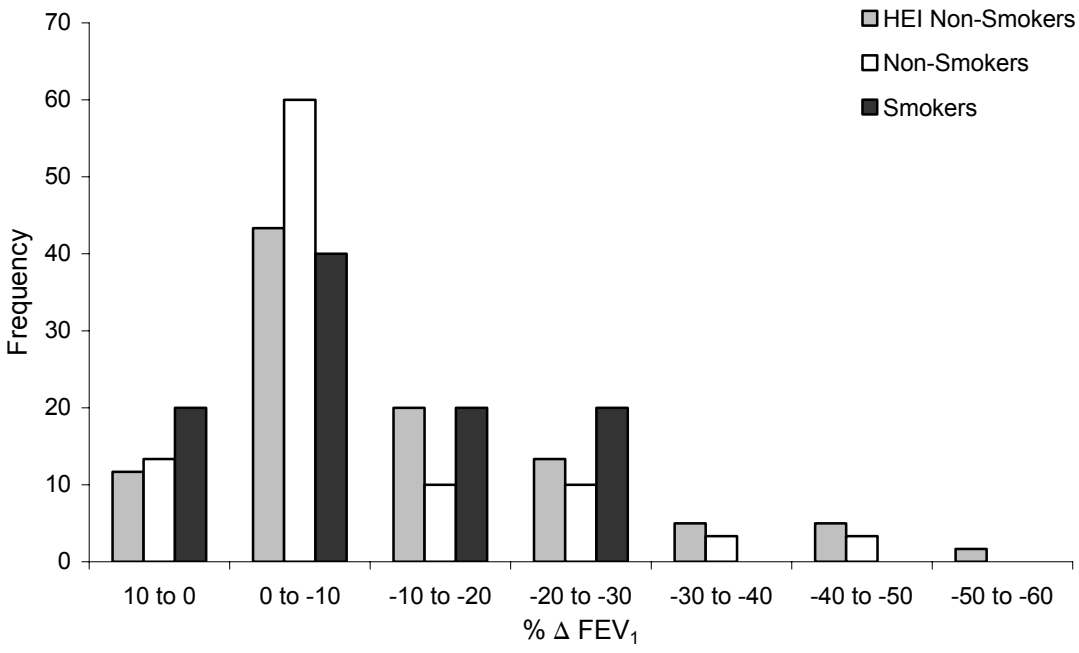


Figure 5-3: Frequency of response in FEV_1 in non-smokers and smokers. Non-smokers in the HEI population and our population demonstrate similar degrees of population variability. However, extreme responses in FEV1 seem to be attenuated in smokers.

Young smokers may represent a subpopulation that, because they experience less airway pathology than smokers with a higher pack-year history, retains sensitivity to O_3. While

we attempted to relate response to markers of smoking intensity, we found no clear relationship between response and smoking history. This is probably the result of a limited number of smokers with longer histories (in terms of total years smoking) and higher intensities (in terms of packs/week). Studies of smokers as a clinical population typically include individuals with a minimum history of 10-20 pack-years. Yet, research in men and women between the ages of 18-44 is vital since these ages comprise nearly half of cigarette smokers in the United States (CDC-2006). Indeed, in toxicological studies aimed at determining the health effects of a pollutant in smokers, it is important that these individuals comprise a representative portion of the participant population.

Perception of Ozone-Related Symptoms

As part of our data safety and monitoring program we collected information as to the symptoms experienced after air and O_3 exposure. As we considered the similar responsiveness in FEV_1 between the smokers and non-smokers, and increased responsiveness in S_N among the smokers, we became interested in whether the populations experienced the same degree of discomfort. With air exposure, smokers and non-smoker experienced symptoms approximately equally. With O_3 exposure, however, approximately twice as many non-smokers experienced shortness of breath (18 compared to 8) and chest burning (11 compared to 5). Additionally more non-smokers (12 non-smokers compared to 9 smokers) experienced post-exposure cough.

Our findings are consistent with those described by Frampton *et. al.*'s finding that smokers experience fewer symptoms with O_3 exposure (Frampton-1997). Previous

investigators have used headache and nausea, measured post air exposure, as "sham symptoms" in order to gauge a population's likelihood of reporting symptoms and the degree of severity they assign to the symptom (Hazucha-1995). In the current study, with air exposure, smokers and non-smokers reported headache and nausea with similar frequency and severity. With O_3 exposure, we found that smokers tended to report symptoms related to chest discomfort and breathing difficulty less frequently. The decrease in the number of smokers reporting symptoms is the result of fewer smokers reporting symptoms within the category indicative of the least severity ("barely perceptible"). However, a similar number of smokers and non-smokers rated their symptoms as "distinctly perceptible" or "a nuisance." Three more smokers than non-smokers reported symptoms as "offensive."

Cigarette smoking may cause desensitization to pulmonary discomfort caused by O_3 exposure. This may occur preferentially in smokers who are already minimally responsive to oxidant-induced irritation compared smokers that are more sensitive to O_3-induced symptoms. When change in FEV_1 is compared to the severity of symptoms reported in non-smokers, decline in FEV_1 is linearly related to severity of cough, wheezing, pain upon inspiration, and shortness of breath (Aris-1995). Therefore, the ability to sense discomfort may be protective in that an individual may remove themselves from the polluted environment or halt physical activity with the presentation of symptoms. The fact that smokers experience fewer symptoms than non-smokers suggests an uncoupling of the relation between response and discomfort that may be deleterious. If the sensation of discomfort is advantageous because it would cause individuals to remove themselves from a polluted environment, then the loss of the

sensation may cause individuals to remain in the environment. If this is the case, smokers may be at risk for increased pulmonary injury.

The Dose-Response Paradigm

In generating a hypothesis as to a mechanism by which O_3 caused an increase in S_N in smokers relative to non-smokers, we considered the dose-response paradigm. Paracelsus first wrote in the 16th century that "all things are poison and nothing is without poison, only the dose permits something not to be poisonous (Langman-2006)." Since, scientists and physicians have refined this idea to describe how an organ or tissue's response to a substance is determined not simply by the dose to which the organism is exposed; instead, response is determined by the local dose received by the organ or tissue. Furthermore, heterogeneity in response within an organ or tissue may be related to the regional dose.

Figure 5-4 details the dose-response paradigm as it related to O_3 exposure and the lung. Local dose to a particular region (i.e., the conducting or peripheral airways) is dependant on the dose delivered to the total lung, the regional distribution of the dose, and the local uptake. The total dose delivered is a function of the exposure concentration (which was standardized between our populations) and minute volume. The amount to which a region is exposed is dependant on the local airway anatomy and tidal volume. Finally, the amount removed within a region is dependant on the airway biochemistry, specifically the composition of antioxidants in the ELF with which O_3 reacts. There is then some inherent tissue sensitivity that determines response.

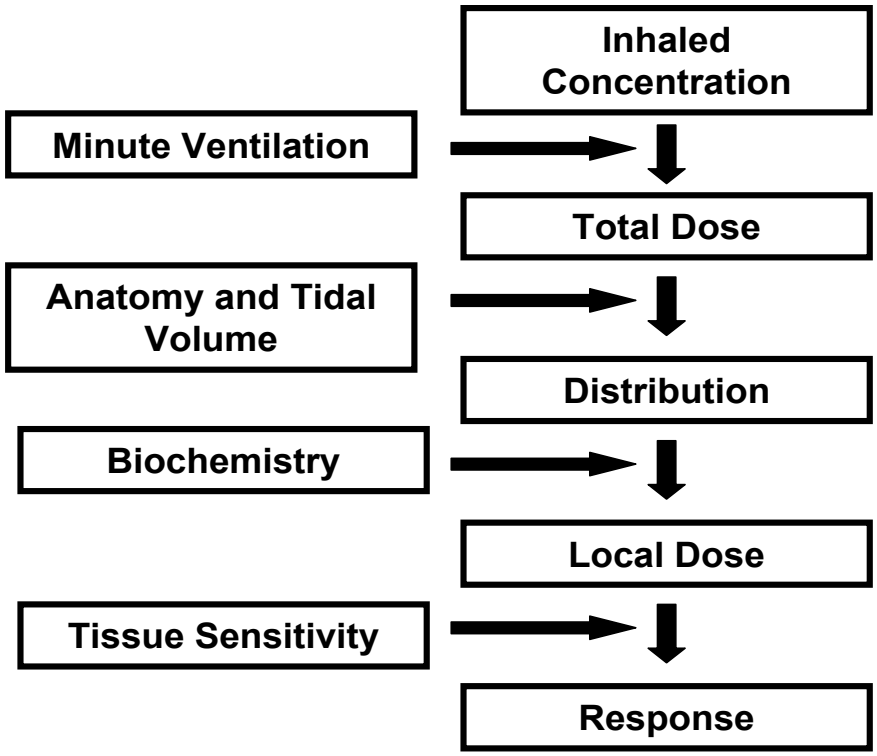


Figure 5-4: The dose-response paradigm. Physiological response is related to total inhaled dose, the distribution of the dose, the local delivered dose, and the sensitivity of the underlying tissue. These are determined by minute volume, lung anatomy and tidal volume, the biochemistry with the epithelial lining fluid, and tissue sensitivity.

We measured minute volume in both populations to ensure that both populations were exposed to equal amounts of O_3 and found them to be equal. Additionally, differences in dose were assessed by comparing uptake rate (μg/min), a fractional uptake efficiency, and total uptake (μg). We opted to use uptake efficiency as a marker of total dose in lieu of uptake rate or total dose because it appeared to be a less variable indicator of total lung dose. Although we found a small but significant increase in the uptake efficiency of smokers compared to non-smokers, intersubject differences in FEV_1, V_D, or S_N responses were not related to differences in uptake efficiency. Therefore, if there is a dose-response

relationship that unifies response in smokers and non-smokers, then differences in response between the two populations must be related to differences in local dose.

A Model of Peripheral Ozone Dose in Smokers and Non-Smokers

We hypothesized that smokers would experience changes in peripheral airway function because of differences in the longitudinal distribution of O_3; specifically we hypothesized that smokers would be exposed to a larger dose of O_3 in the peripheral airways. The following model describes peripheral airway dose in terms of two parameters measured in this study – uptake in the conducting airways and V_D/V_T.

The Bohr model subdivides the respiratory system into an anatomical deadspace compartment corresponding to the conducting airways (V_D) and an alveolar compartment corresponding to the peripheral airspaces. During a single inspiration of tidal volume V_T, the alveolar compartment is ventilated with a volume of air given by V_A (Equation 5-1).

$$V_T = V_A + V_D \qquad \text{Equation } \mathbf{5-1}$$

Multiplying each side of this equation by the frequency of breathing (f) and rearranging provides an equation for the alveolar ventilation rate (Equation 5-2).

$$fV_A = fV_T - fV_D \qquad \text{Equation } \mathbf{5-2}$$

The Bohr model is sufficient to predict the distribution of an inert gas to the conducting and peripheral airspaces. Because it is a reactive gas, a complete model for O_3 must also consider its uptake in the conducting airways during the course of inspiration.

The rate at which O_3 reaches the alveolar compartment (D_A) is the product of the concentration of O_3 reaching the compartment ($[O_3]_A$), f, and V_A (Equation 5-3).

$$D_A = f V_A [O_3]_A \qquad \text{Equation } \mathbf{5-3}$$

The dose reaching the alveolar compartment per minute can also be described in terms of the volume of O_3 that would ventilate the alveolar region were it an inert gas minus its uptake in the conducting airways (U) (Equation 5-4). Here, $[O_3]_T$ represents the concentration of O_3 in inhaled air. This is shown graphically in Figure 5-5.

$$D_A = f V_A [O_3]_T - U \qquad \text{Equation } \mathbf{5-4}$$

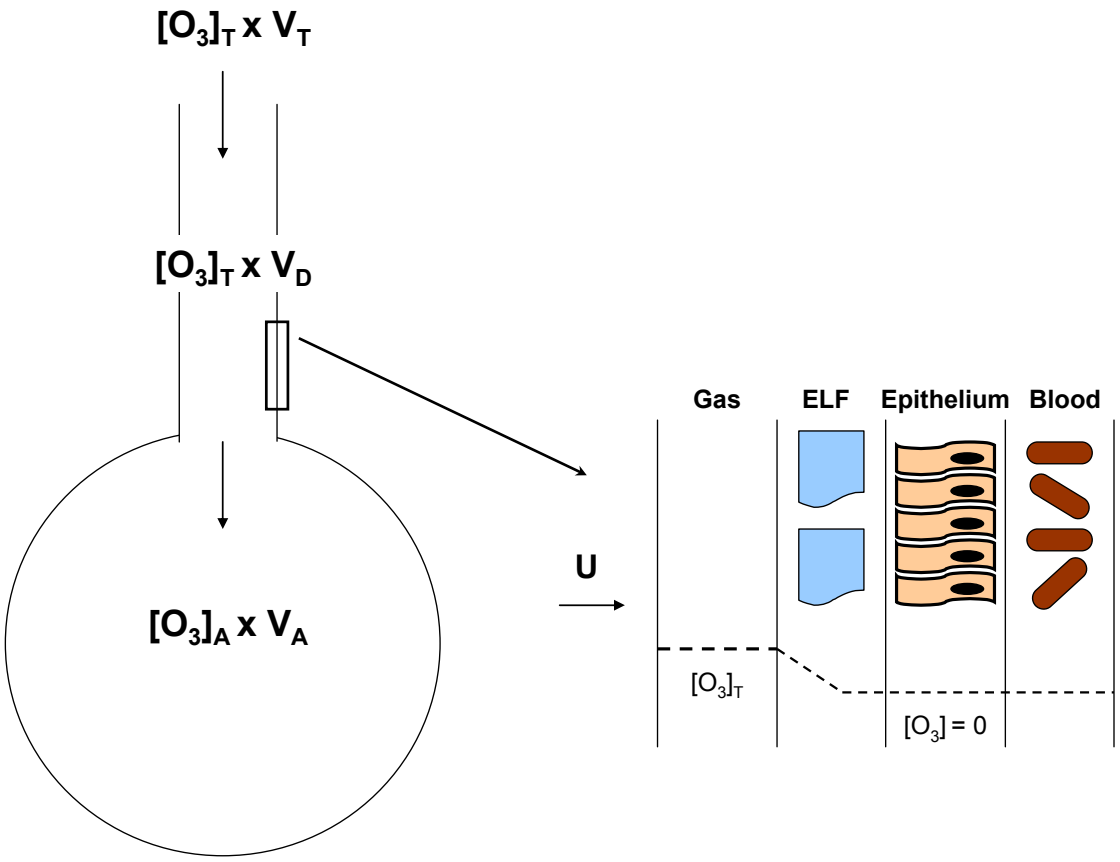


Figure 5-5: Two compartment model of the longitudinal distribution of ozone. This model contains dead space and alveolar compartments. The inhaled tidal volume (V_T) is distributed to V_D leaving a volume V_A=V_T-V_D to ventilate the alveolar compartment. The dose reaching the alveolar component is equal to the dose entering at the mouth ($[O_3]_T$ x V_T) minus the dose retained in V_D ($[O_3]_T$ x V_D) and the uptake at the conducting airway wall (U) (shown in insert). The insert shows the composition of the airway wall with the local concentration of O_3 shown as a dotted line. Ozone is taken up in the epithelial lining fluid (ELF). Because of it high reaction rate and the abundance of ELF substrates, the concentration of O_3 reaches nearly zero within the ELF. (Insert is adapted from Ultman, 1988**)**

Substituting Equation 5-2 into Equation 5-4 yields Equation 5-5.

$$D_A = [O_3]_T \, f \, (V_T - V_A) - U \qquad \text{Equation } \mathbf{5-5}$$

The uptake rate of O_3 in the conducting airways, measured in mass/min, is determined by the concentration gradient between the lumen of the airway and the ELF. Because O_3 reacts very quickly with abundant substrates in the ELF, the assumption can be made that the concentration of O_3 in the ELF is zero and that the concentration gradient is entirely determined by the conducting airway concentration, approximated as $[O_3]_T$. Additionally, uptake is determined by the surface area (S) and a mass transfer coefficient (K) that describes the rate of transport of O_3 to the ELF (Equation 5-6).

$$U = KS[O_3]_T \qquad \text{Equation } \mathbf{5-6}$$

The value of K, expressed as mass per minute per surface area per concentration, is determined by the anatomy of the airways, the air velocity and the diffusion coefficient of O_3 in air. Multiplying the right-hand side of the equation by V_D/V_D allows uptake to be described in terms of K, a surface-to-volume ratio (a), V_D, and the concentration of O_3 (Equation 5-7).

$$U = K_a V_D [O_3]_T \quad \textit{where} \quad a \equiv K \frac{S}{V_D} \qquad \text{Equation } \mathbf{5-7}$$

Substituting Equation 5-7 into Equation 5-3 and dividing both sides by V_T and rearranging yields Equation 5-8.

$$F_{O3} = 1 - \left(1 + \frac{Ka}{f}\right)\frac{V_D}{V_T} \quad \text{where} \quad F_{O3} \equiv \frac{D_A}{fV_T[O_3]_T} \qquad \text{Equation } \mathbf{5-8}$$

Here, we describe the dose of O_3 delivered to the peripheral airspaces in terms of F_{O3}, which represents the peripheral airspace dose normalized by the total inhaled dose ($fV_T[O_3]_T$). In other words, FO_3 describes the fraction of the inhaled dose penetrating to the alveolar region. Equation 5-8 demonstrates that the primary determinants of peripheral dose are V_D/V_T and Ka. While there are certainly more comprehensive (and, thus, more complicated) models of longitudinal O_3 distribution (Overton-1988), this model allows for the determination of peripheral dose using a conveniently measured parameter – V_D/V_T. We assumed that the transport coefficient Ka is a constant and equal between smokers and non-smokers. Considering that our cohorts of smokers and nonsmokers contain an approximately equal number of participants of each sex of similar ages, this is reasonable assumption when using the model to compare the two populations.

Regional Dosimetry and Changes in the Expirogram

We investigated the extent to which the difference in S_N response were due to a difference in the distribution of O_3 dose between conducting airway and alveolar regions in the two populations. Early in the O_3 exposure period, smokers and non-smokers had similar V_D and maintained a similar V_T.; there were not initial differences in V_D/V_T. Thus, according to Equation 5-8, the dose of O_3 to the periphery should be the same in the two populations.

We also measured plasma and nasal antioxidants in order to investigate if there were potential differences in airway biochemistry caused by cigarette smoke exposure that

could cause differences in regional dose. Differences in conducting airway biochemistry could result in differences in uptake between smokers and non-smokers. Uric acid, in particular, is major determinant of nasal uptake. When UA concentrations in the nose are perturbed by O_3 exposure or wash-out, uptake is reduced (Santiago-2001, Fassih-2007). We measured UA both in the nose and plasma, but no in bronchoalveolar lavage as other investigator have done (Blomberg-1999). Sampling of bronchoalveolar lavage fluid prior to exposure would alter airway biochemistry such that dosimetry measurements would have been meaningless. Because UA is similar in concentration between the nasal and pulmonary ELFs (see discussion in Chapter 2), and smokers experience a large degree of nasal cigarette smoker exposure (Benninger1999), we used the nasal ELF as a surrogate for lung ELF. Additionally, because glandular UA concentration is considered to be in equilibrium with that in blood, we measured UA in plasma as a second surrogate measurement.

Our results indicate that concentrations of UA in nasal lavage and plasma are not different between smokers and non-smokers. We expected that nasal and plasma values of UA would be correlated but found them to be unrelated in both populations. This indicates that the mucosal glands responsible for releasing US into the nose are not equilibrated with the blood that perfuses them. In fact there was reproducibility in our day-to-day measurements of UA, suggesting that transport into the nasal ELF is regulated.

We considered other components of the ELF that smoking could alter. We found the concentration of AA to be equal between smokers and non-smokers. However, the AA content was minimal and unrelated to values measured in blood plasma. We also found

no differences protein levels between the two populations. This suggests that epithelial permeability, and therefore direct diffusion of antioxidants across the epithelium, is not enhanced in our population of young smokers. Additionally, we found that the ORAC of the ELF was not different in smokers and non-smokers. Uric acid is responsible for an equal portion of the ORAC in both groups. These data confirm that, at the level of the nose, these smokers do not experience antioxidant alterations that can explain a difference in O_3 uptake between smokers and non-smokers.

The Consequences of Failing to Increase V_D/V_T

Although V_D/V_T is not different between smokers and non-smokers early in the exposure, it did change over the course of the one-hour exposure. As is predicted by Equation 5-8**,** changing V_D/V_T has important consequences in terms of the degree to which the gas exchanging regions of the lung are exposed.

Non-smokers exposed to O_3 exhibited a progressively decreasing V_T as the time of exposure increased. We have shown that, because V_D does not change under these exposure conditions, this results in an elevated V_D/V_T. Such a change in breathing pattern is thought to be protective in that it decreases the degree to which the peripheral airways, where the ELF is especially thin, are exposed to O_3. Smokers experience similar decreases in V_T over the course of exposure. However, they also experience decreases in V_D. Because smokers lower V_D and V_T by similar degrees, they fail to elevate V_D/V_T. As a consequence of failing to increase their V_D/V_T over the course of an O_3 exposure, smokers increase the degree to which the distal airways and airspaces are exposed relative to non-smokers.

The pooled data from the smokers and nonsmokers indicate that the overall uptake efficiency of the respiratory system is inversely proportional to the degree to which an individual elevated V_D/V_T during the course of O_3 exposure (see Figure 5-6). As expressed in Equation 5-7 the local uptake of O_3 is proportional to the surface-to-volume ratio (a) of an airway. Because of their smaller diameters, a is much larger in the peripheral airspaces than in the conducting airways, and as a result, local uptake efficiency is higher in the periphery. As V_D/V_T increases, less O_3 is transported to the periphery, and thus, the overall efficiency of O_3 uptake in the entire respiratory tract must decreases, explaining the negative correlation revealed in Figure 5-6.

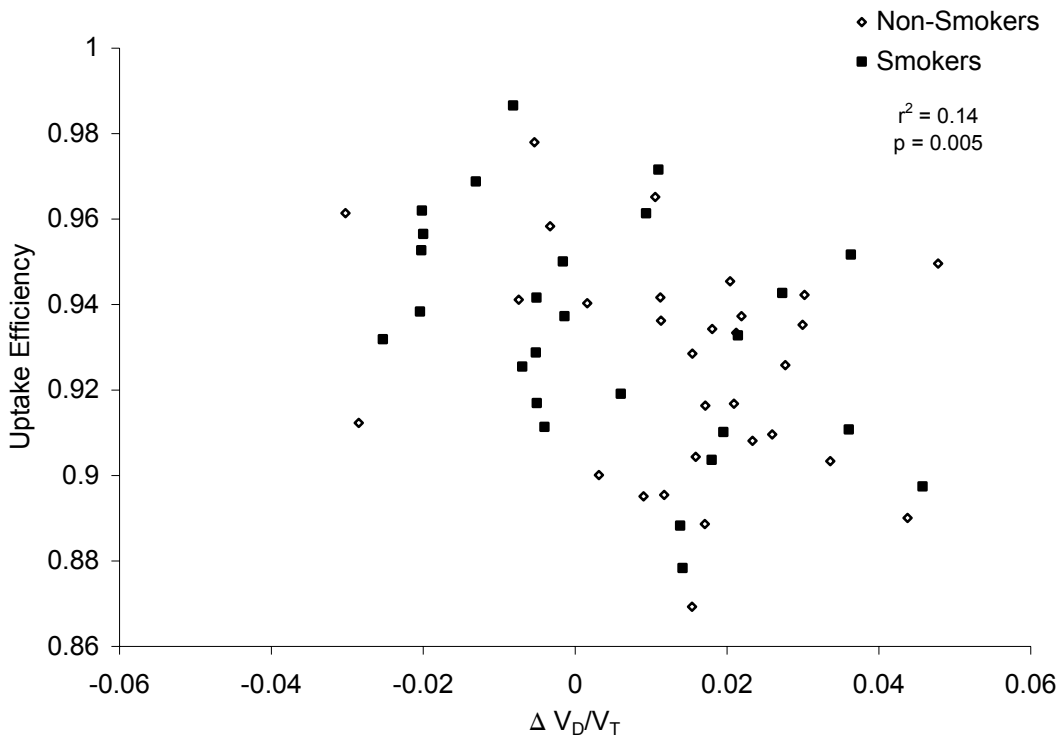


Figure 5-6: Increase in uptake efficiency with decreases in V_D/V_T. The above represents average uptake efficiency as a function of the change in V_D/V_T after 50 minutes of O_3 exposure compared to baseline (r^2=0.14, p=0.005). One outlying point (a nonsmoker with $\Delta V_D/V_T$ = 0.14 and UE = 0.94) has been removed from the analysis.

An increase in peripheral airway dose could, over both the short-term and long-term, have serious consequences for cigarette smokers. In the short-term, an increased dose delivered to the periphery where ELF antioxidants are less abundant could lead to increased inflammation relative to that experienced by non-smokers. Cigarette smokers retain their short-term inflammatory responsiveness to O_3 (Torres-1997.). The effects of increasing peripheral airway dose on local inflammation have not been studied. However, via meta-analysis of 21 investigations, Mudway *et. al.* determined that the influx of alveolar polymorphonuclear granulocytes in non-smokers, largely neutrophils, is related to cumulative dose (Mudway-2004). Given this total lung dose-response relationship, it is conceivable that there is a regional dose-response relationship such

that smokers would experience increased peripheral inflammation compared to non-smokers that is a direct result of increased peripheral dose.

In the long-term, repeated O_3 exposure alters the character of the airways and parenchyma. In both rats and bonnet monkeys exposed chronically, O_3 caused a remodeling of the peripheral airways (Fujinaka-1985, Barr-1988, Barr-1990). This remodeling was more apparent in the distal airways where O_3 caused an increase in the thickness of the connective tissue within the respiratory bronchioles and a decrease in terminal and respiratory bronchiole lumen diameters. Additionally, in young rats, O_3 caused a decrease in the formation of new respiratory bronchioles, suggesting that O_3 suppresses epithelial cell differentiation. This may have important implications in terms of the lungs ability to heal following injury.

Finally, O_3 alters airway ciliary function in humans and, with chronic exposure, decreases the surface area occupied by airway cilia in rats. This prevents the clearance of airway mucous and particulates (Foster-1987, Barry-1988). Loss or airway ciliary function and number in smokers as a result of smoking severely limits the ability to clear the airways and leads to decreased particulate clearance, cough, and expiratory flow limitation (Smaldone1993). Exposure to O_3 may exacerbate this by hindering any remaining ciliary function.

Chapter 6 – Future Directions

We have determined that young cigarette smokers retain their responsiveness to O_3 in terms of FEV_1. In both smokers and non-smokers, decrements in FEV_1 are caused by inspiratory limitation rather than by bronchoconstriction. Uniquely, smokers experience changes in V_D that lead to heterogeneity in airway morphometry. This conclusion is supported by the observed increase in S_N and the correlation between changes in V_D and S_N. We have determined that changes in the expirogram are not a result of initial differences in the dose penetrating to the peripheral airways. However, a decrease in V_D/V_T over the course of the exposure increases the dose to which the peripheral lung is exposed relative to the conducting airways. This difference in longitudinal O_3 distribution is responsible for higher uptake efficiency among smokers.

These findings demonstrate that smokers are more sensitive to the health effects of O_3 than non-smokers. Additionally, because cigarette smokers experience fewer symptoms, they may remain in a noxious environment longer than their non-smoking counterparts and be susceptible to increased lung damage caused by exposure to a higher dose of O_3. The following sections describe subsequent studies that could be conducted as a follow-up to this investigation.

Dose-Response Studies

Exposure limits are based primarily upon dose-response studies that have been conducted using FEV_1 as a marker of response in non-smoking populations exposed to varying concentrations of O_3. Combining our non-smoking population with non-smokers in the HEI population we determined that $\%\Delta S_N$ is related to cumulative uptake. Experiments exposing individuals to multiple concentrations of O_3 at different levels of exercise should be performed in order to fully understand the nature of this dose-response relationship. These measurements could be combined pulmonary ventilation and perfusion imaging and blood gas analysis to determine the effect of altering S_N on gas exchange. Understanding this second marker of O_3-induced lung injury's relationship to dose will allow for either validation or refinement of current exposure standards in non-smokers and smokers.

Although non-smokers experienced no significant change in $\%\Delta V_D$ or $\%\Delta S_N$ with O_3 exposure, values of $\%\Delta V_D$ and $\%\Delta S_N$ immediately, 30 minutes, and 60 minutes post exposure paralleled those of the smokers. We investigated the effects of correcting for the effects of exercise using the air exposure data and described a new response term ($\%\Delta\Delta S_N$). Use of this parameter did not improve the significance of the data and resulted in an inflated standard error about the mean. This is probably due to substantial day-to-day variability in the effects of exercise on airway caliber. Additional work should be done in order to quantify this variability and, indeed to determine the validity of correcting O_3-induced pulmonary function changes for changes induced by the experimental design. Ideally, in this work a single population would be exposed

repeatedly to both air and some concentration of O_3. By correcting the mean response to O_3 by the mean response to air, the standard error may be minimized.

In this work we identified a population of smokers that are more responsive in terms of $\%\Delta S_N$, and not different in terms of $\%\Delta FEV_1$, than their non-smoking counterparts. They are, however, more responsive in terms of FEV_1 than smokers with higher pack-year histories. Although we investigated the effect of smoking history on response, our study was not designed with this in mind and probably lacked sufficient power to achieve this endpoint. Therefore, additional dose-response investigations among smokers, designed specifically to investigate the effect of smoking history on response, should be conducted. Populations of smokers with varying pack-year history should be recruited and exposed to varying concentrations of O_3 and response measured in terms of $\%\Delta FEV_1$ and $\%\Delta S_N$. Dose-response curves can then be constructed for the different smoking populations. By comparing the smokers to each other, and to comparable non-smoking populations, it can be determined whether smokers with limited smoking histories are more sensitive in both FEV_1 and S_N than smokers with longer histories. Additionally, it can be determined whether current exposure limits are sufficient to protect smokers from O_3-associated health effects.

Dosimetry Studies

We determined that that nasal and plasma antioxidants were not different between smokers and non-smokers and concluded that, as a result, Ka should be similar between the populations. This assumption, however, should be validated. Hu, *et. al.* have described a method by which boluses of O_3 may be used to determine the longitudinal

distribution of O_3 and values of Ka at different levels of the conducting airways (Hu-1994). The longitudinal distribution of O_3 should be measured in a group of smokers comparable to those in our population and compared to a comparable group of non-smokers. Additionally, this method may be used to compare younger smokers to smokers with longer smoking histories to determine the effect of smoking history on the longitudinal distribution of O3.

In our study we described changes in V_D/V_T early and late in exposure by comparing pre- and post exposure values of V_D to values of V_T measured in the 10^{th} and 55^{th} minute of exposure. We then used these values to demonstrate that smokers receive a larger dose of O_3 to the peripheral airways compared to non-smokers. As a result of using pre- and post- data, we are limited in our ability to compare differences in peripheral O_3 dose between smokers and non-smokers over the course of an exposure and to calculate a meaningful value of F_{o3}. The addition of capnometry equipment to our exposure apparatus could allow for real-time monitoring of V_D/V_T. The dose at which V_D/V_T begins to change could then be calculated and the dose to the alveolar compartment quantified.

Inflammatory Markers and Symptom Scores

Our results indicate that smokers experience fewer symptoms as a result of exposure and that the relationship between symptoms and lung injury may be uncoupled in this population. However, the importance of O_3-induced symptoms has not been quantified; specifically, no one has determined if individuals that experience more severe symptoms are more likely to leave a polluted environment, thereby limiting their overall exposure.

In order to investigate this further, exposure studies should be conducted using a non-smoking population and a smoking population composed of smokers with varying smoking histories.

The study should have two experimental arms, each involving an O_3 exposure and a control air exposure. In the first arm, participants would be asked to sit at rest while breathing O_3 until they wish to terminate their exposure. As a control, this could then be replicated using as an air exposure. In the second arm, participants would be asked to perform moderate exercise while breathing O_3 until they express a desire to terminate the exposure. Again, as a control, this could be replicated using an air exposure. Before and after exposure, participants would be asked to complete symptom questionnaires. This information, combined with the average dose to which each population allowed themselves to be exposed under the different conditions could determine whether increased respiratory symptoms cause individuals to limit exposure and whether there is a difference in voluntary exposure time and dose between smokers and non-smokers.

A hallmark of O_3-induced lung damage is the biphasic nature of the injury. Alterations in mechanical function, the major subject of this work, are apparent immediately following short-term exposure. These changes, however, tend to resolve within one or two hours after the termination of the exposure. Beginning approximately six hours post-exposure, healthy non-smokers develop an inflammatory response characterized by an increase in bronchial and alveolar inflammatory cytokines, leukotrienes, and epithelial permeability. This is mirrored by an increase in airway neutrophils, lymphocytes, and macrophages (Mudway-2000) (see Figure 6-1).

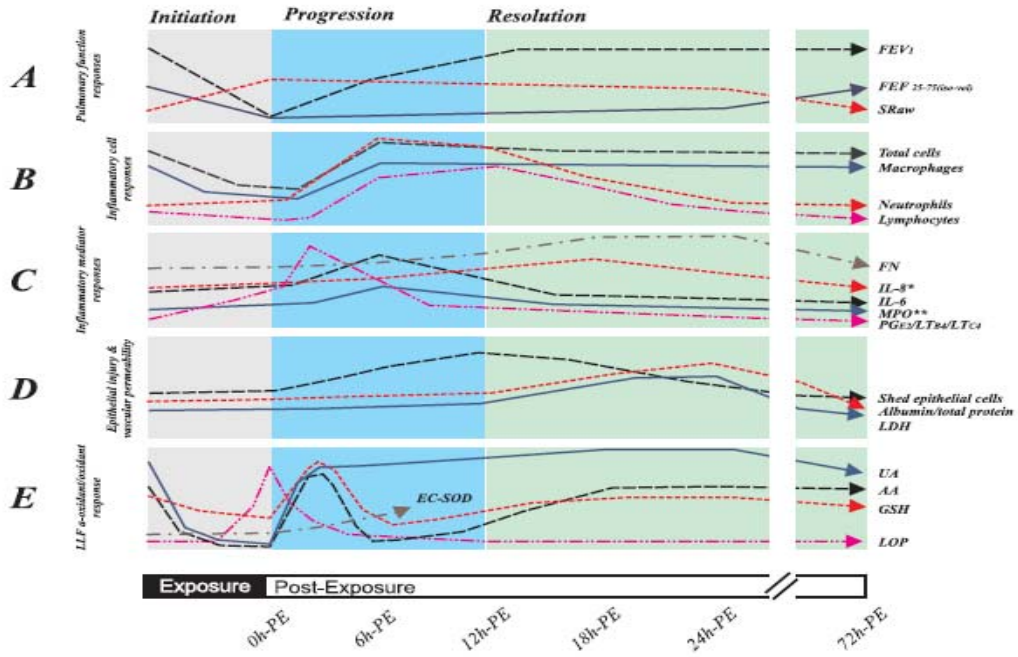


Figure 6-1: Time course of mechanical and inflammatory changes induced by ozone exposure.

In non-smokers, changes in spirometric parameters and inflammation are not necessary coupled such that changes in FEV_1 are not predictive of the magnitude of the subsequent inflammation (Balmes-1996). However, older smokers and non-smokers appear to be similarly responsive in the inflammatory phase. Torres *et. al* exposed a group of smokers with a history of 13±9 pack-years to 0.22 ppm O_3 for 4 hours and then assessed the cellular and liquid phases of bronchoalveolar lavage (BAL) measured immediately post- and 18 hours post-exposure (Torres-1997). The investigators concluded that, although the smokers in this population failed to experience decrements in FEV_1, cellular and biochemical markers of inflammation present their BAL post-exposure were similar

to those found the BAL of non-smokers. Therefore, especially in this population, differences in FEV_1 are not indicative of differences of inflammatory response.

Because FEV_1 is confounded by the effects of inspiratory limitation (see Chapter 5), its use is not appropriate in attempting to couple the mechanical changes induced by O_3 with inflammatory changes. Whether younger smokers would experience a similar inflammatory response compared to non-smokers, or an augmented inflammatory dose because of the higher alveolar dose received, is unknown. However, the inflammatory response in this population, and that of older smokers and non-smokers, should be measured and compared to an estimated alveolar dose to determine if altering mechanical function (by changing V_D/V_T) causes inflammatory changes and alveolar pathology.

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Appendix A -- Informed Consent

INFORMED CONSENT FORM FOR CLINICAL RESEARCH STUDY

The Pennsylvania State University

Title of Project: Ozone Dose to the Human Respiratory Tract: Effect of Cigarette Smoking

Principal Investigator: James S. Ultman, Ph.D.
106 Fenske Lab
Penn State University
jsu@psu.edu

Other Investigators: Abdellaziz Ben-Jebria, Ph.D., Melissa Bates, B.S., Timothy Brezna, B.S., Ali Fassih, B.S., Rebecca Bascom, M.D., Roberta Millard, MD, Steven Arnold Ph.D.

This is to certify that I (print name) _____, have been given the following information with respect to my participation as a volunteer in a program of investigation under the supervision of Dr. James Ultman.

1. Purpose of the Study

Ozone is a common air pollutant generated by the action of sunlight on automobile emissions. This study will determine how inhaled ozone is distributed and retained in different regions of the lungs of smokers as compared to nonsmokers. It will also determine whether the inhalation of ozone causes a change of lung function in smokers that is different than in nonsmokers. We hope to demonstrate that this difference in lung function response can be explained by a corresponding difference in the distribution of ozone to different parts of the lungs.

2. Procedures to be Followed

You will be included in this study only if you are a smoker and 21-40 years old or a non-smoker and 18-40 years old, have reasonably normal lung function, and have no history of cardiovascular disease or any other disease. You will be excluded from the study if you are very overweight or if you are allergic to latex rubber. You will also be excluded if you regularly take any medications including anti-histamines, decongestants, and anti-inflammatory drugs. For female subjects: You will be excluded from participation in this study if you are pregnant.

You will attend a prescreening session and a health screening session prior to your participation in three research sessions. If you don't feel well or find it necessary to take medication within 24 hours of any session, then you should notify an investigator. This may delay these sessions.

The purpose of the prescreening and health screening sessions is to obtain medical information. At the beginning of the prescreening session, you will have a small volume of blood (about 4 teaspoons) sampled from your arm. This blood test will be used to evaluate your personal risk of having heart disease. During the prescreening session, you will complete medical and smoking questionnaires, and you will undergo lung function tests that require you to breathe forcefully into test equipment for about 6 seconds. The test will continue until 2 tests that are similar have been obtained. The health information collected during the prescreening session will be evaluated to determine whether or not you are allowed to schedule the health screening session.

During the health screening session, you will be given a routine medical examination, and you will also undergo an exercise test on a stationary bicycle for assessing your heart's reaction to physical activity. The health information collected during the health screening session will be evaluated to determine whether or not you are allowed to participate in the research sessions.

If medical abnormalities are discovered during the prescreening session, the health screening session or any subsequent research sessions, you will be notified of the findings so that you can consult your physician.

During one of the research sessions, you will inhale a small puff of diluted ozone about 2 times a minute for one hour while you are at rest. This will require that you breathe through equipment that determines how these ozone puffs become distributed in your lungs. During another research session, you will continuously inhale diluted ozone while you exercise on a stationary bicycle for 1 hour. This will require that you breathe through a mask connected to an apparatus for determining the amount of ozone that is retained in your lungs. During yet another research session, you will continuously inhale room air through the mask while you perform exercise on a stationary bicycle for 1 hour. This is a sham experiment in which there should be no ozone effects. The level of exercise that you will perform on the stationary bicycle is similar to continually walking up a gradually sloping hill at a brisk pace
At the beginning of one of the three research sessions, you will have a small volume of blood (about 4 teaspoons) sampled from your arm. At the beginning and end of the prescreening session and all three research sessions, you will have each of your nostrils washed out with a small volume (about 1 teaspoon) of warm salt water. Both the blood and nasal washings will be analyzed for antioxidant compounds such as vitamin C that affect how ozone is retained in the lungs. The blood samples will also be tested for nicotine byproducts to verify your smoking history. If you are a woman, you will donate a urine specimen at the beginning of all sessions. This will be used in a 3-minute hCG assay for pregnancy. If the pregnancy test proves to be positive, you will be excluded from the session and from the remainder of the study.

You will be given lung function tests at the beginning and end of each of the three research sessions to determine the effects of ozone and/or exercise on your lungs. During the first of

these tests, you will be asked to breathe forcefully through a mouthpiece for about 6 seconds. You will continue to repeat the tests until two tests that match have been obtained. In the second test, you will be asked to breathe slowly through a mouthpiece and your exhaled breath will be measured for carbon dioxide. This will be done to determine the amount of your lung available for gas exchange. You will also complete a symptom questionnaire for recording physical sensations associated with ozone inhalation.

3. Discomforts and Risks

During one of the research sessions, you will be asked to inhale through a mouthpiece approximately 120 times. Each time you inhale, a small puff of ozone will be injected into the stream of air you are inhaling. The maximum concentration of ozone in each puff will be several times greater than what you might continuously breathe in polluted city air. However, as you are inhaling, this ozone puff mixes with the much greater amount of ordinary air that you are also inhaling. In this manner, ozone is diluted to an actual amount that less than what is present in a polluted city.

During another research session, you will continuously breathe a diluted ozone mixture while you are exercising on a stationary bicycle. The ozone concentration in this mixture will be about twice the maximum level prescribed by the US Environmental Protection Agency for an eight-hour exposure to ozone. Since you will only be exposed to ozone for one hour, however, your risk of adverse reactions is even smaller than anticipated by this EPA standard. Exposures of healthy individuals to this ozone level for a limited amount of time are often practiced in laboratories that study the health effects of ozone, including the US Environmental Protection Agency. In the past, we have safely used this ozone inhalation procedure on more than 70 subjects in our own lab.

Even limited exposures to ozone can irritate the respiratory system, causing coughing, shortness of breath, or discomfort in the nose or in the chest. These symptoms usually disappear within a short time following exposure. In the previous three years our lab has completed 55 exposure sessions, including the sham (air) exposure. In those 55 sessions mild to moderate symptoms were described 5 times. In each of those sessions, symptoms disappeared within 30 minutes of the exposure. Even so, the long term effects of ozone are not known for sure. Ozone might also injure some of the cells lining the nasal passages and breathing tubes of the lung. However, the amounts and time periods used in this study have never been proven to cause any irreversible adverse health effects. To safeguard against the possible carryover of physiological responses from one exposure to the next, sessions will be spaced by a period of at least 1 week.
During the nasal washing procedure, there is a risk that you could cough when the sterile water is placed inside your nose. This risk will be minimized by asking you to hold your breath during the procedure.

A nurse will be monitoring your vital signs during the research sessions, and a clinician will be called to the lab should you experience any unexpected, adverse symptoms. Should you develop such symptoms and the nurse or clinician asks you to return at a later date for a follow-up medical examination, you agree to comply. Should you develop such severe symptoms that the nurse or clinician decides that medical treatment or closer observation is necessary, you agree to go to the Emergency Room at the Mount Nittany Medical Center. In the unlikely event that this should happen, you agree to end your participation in this study.

A risk in these experiments is the possible transmission of infection by breathing from the breathing equipment. This risk will be minimized by using disposables and by employing a standard clinical cleaning method of nondisposables. The drawing of blood carries a small risk of bruising, infection, lightheadedness, developing a small clot, and/or fainting. This risk is similar to having blood drawn in a hospital or doctor's office. To minimize this risk, an individual trained in blood collection will draw the blood sample using a standard aseptic technique.

4. Benefits

a. Benefits to you: None.

b. Benefits to society: This research will provide information that is necessary for the government to improve regulatory standards, particularly for potentially high risk individuals such as smokers.

5. Alternative Procedures

Experiments could alternatively be carried out on laboratory animals. However, because of differences in their physiology, the data obtained from animals cannot be used directly for people. It is vital for ozone dose-response experiments to be carried out on human subjects like yourself.

6. Time Required

The prescreening session, health screening session, and the 3 research sessions each will last from 1 to 3 hours. The time period for completion of this study is 4 to 6 weeks.

7. Confidentiality

All records associated with your participation in the study will be subject to the confidential standards applicable to medical records, (e.g. such as records maintained by physicians, hospitals, etc.) and in the event of any publication resulting from the research no personally identifiable information will be disclosed. The Office of Human Research Protections in the U.S. Department of Health and Human Services, the Food and Drug administration (FDA), the Office for Research Protections at Penn State and the Biomedical Institutional Review Board may review records related to this project.

8. Right to Ask Questions

You will have been given an opportunity to ask any questions that you may have, and all such questions or inquiries will have been answered to your satisfaction. If, at a later time, you have questions regarding your rights as a research participant you should contact:

The Office for Research Protections

201 Kern Building,
University Park, PA 16802
(814) 865-1775

If you have questions about the research or your participation in the research, you should contact:

Dr. Aziz Ben-Jebria
(814) 863-8049 (Weekdays)
(814) 237-0739 (Evenings & Weekends)

In the event of a research-related injury, you should contact:
Dr. James Ultman
814-863-4802 (Weekdays)
814-237-6335 (Evenings & Weekends)

9. Compensation

You will be compensated at a rate of $15 for the prescreening session, $20 for the health screening session, $40 for the first research session, $60 for the second research session, and $60 for the third research session. You could earn a total of up to $195 for participating in the study.

If you are an employee of Penn State University, the compensation you receive for participation will be treated as taxable income and therefore taxes will be taken from the total amount. If you are not employed by Penn State University, total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS. This may require you to claim the compensation that you receive for participation in this study as taxable income.

10. Injury

Medical care is available in the event of a research-related injury, but neither financial compensation nor free medical treatment is provided. You are not waiving any rights that you may have against the university for injury resulting from negligence of the university or investigators.

11. Voluntary Participation

Your participation in this study is voluntary, and you may withdraw from this study at any time by notifying any of the investigators. Your withdrawal from this study or your refusal to participate will not in any way affect your care or access to medical services.

The investigators have the right to terminate the sessions at any time for whatever reason(s), and in that event you will not be compensated for sessions you did not complete.

Moreover, you have the right not to answer certain questions of a personal nature such as marital status, financial status, etc.

In the event that abnormal test results are obtained, you will be informed of the results and recommended to contact your private medical provider for follow-up.

This is to certify that I consent to give permission for my participation as a volunteer in this program of investigation. I understand that I will receive a signed copy of this consent form. I have read this form, and understand the content of this consent form.

_____ _____

Volunteer Date

_____ _____

Appendix B – Study Procedures, Questionnaires, and Forms

Session Procedures

Pre-Screening	Air	Ozone
◊ Subject Reports to 115 Noll	◊ Subject Reports to 115 Noll	◊ Subject Reports to 115 Noll
◊ Informed Consent Obtained	◊ Spirometry/Pulmonary Function	◊ Spirometry/Pulmonary Function
◊ Screening, History and Smoking Questionnaires Administered	◊ Pre-Session Symptom Questionnaire	◊ Pre-Session Symptom Questionnaire
◊ Subject is Directed to Nurse's Station	◊ Subject is Directed to Nurse's Station	◊ Subject is Directed to Nurse's Station
◊ ***Pulmonary Function Testing***	◊ ***hCG Pregnancy Test***	◊ ***hCG Pregnancy Test***
◊ ***Blood Draw including CBC, Cardiac Risk, and Chem12, and antioxidant sample***	◊ ***Pre-Session Medical Assessment Questionnaire and Vital Signs***	◊ ***Blood draw for antioxidant sample***
◊ ***Medical History including Cardiac Risk Assessment***	◊ CO2 Expirogram	◊ ***Pre-Session Medical Assessment Questionnaire and Vital Signs***
◊ ***Subject is Discharged from GCRC by Staff Member***	◊ Nasal Lavage	◊ CO2 Expirogram
◊ Subject Returns to Room 115, Completes Bill, and Receives Reminder Sheet and Compensation	◊ Continuous Air Inhalation (~2 hours)*	◊ Nasal Lavage
Screening	◊ CO2 Expirogram	◊ Continuous Air Inhalation (~2 hours)*
◊ Subject Reports to 115 Noll and is Directed to Nurse's Station	◊ Nasal Lavage	◊ CO2 Expirogram
◊ ***hCG Pregnancy Test (if applicable)***	◊ Spirometry	◊ Nasal Lavage
◊ ***Physical Exam with Vital Signs***	◊ Post-Session Symptom Questionnaire	◊ Spirometry
◊ ***EKG***	◊ Subject is given Reminder Sheet	◊ Post-Session Symptom Questionnaire
◊ ***Exercise Tolerance Test***	◊ Subject is Directed to Nurse's Station	◊ Subject is given Reminder Sheet
◊ ***Subject is Discharged from GCRC by Staff Member***	◊ ***Post-Session Medical Assessment Questionnaire and Vital Signs***	◊ Subject is Directed to Nurse's Station
◊ Subject Returns to Room 115, Completes Bill and Receives Reminder Sheet and Compensation	◊ ***Subject is Discharged from GCRC by Staff Member***	◊ ***Post-Session Medical Assessment Questionnaire and Vital Signs***
	◊ Subject Returns to Room 115, Completes Bill, and Receives Compensation	◊ Subject is Discharged from GCRC by Staff Member
		◊ Subject Returns to Room 115, Completes Bill, and Receives Compensation

* Nurse monitors breathing rate, heart rate, blood pressure, and pulse oximetry every 15 minutes during experimental sessions

Procedures noted in bold-italics performed by GCRC Staff

PENNSTATE
RESPIRATORY RESEARCH LABORATORY

Are you a healthy smoker?

We are studying the effects of airborne pollutants on the lungs of healthy smokers between the ages of 21 and 40 and need research volunteers.

All participants will be compensated for their time

For more information contact
Melissa Lowe Bates at mal361@psu.edu or by calling 863-9543

* This study has been approved by the Institutional Review Board at Penn State*

PENNSTATE
RESPIRATORY RESEARCH LABORATORY

Do you have extra time this semester?

We are studying the effects of airborne pollutants on the lungs of healthy people between the ages of 18 and 40 and need research volunteers. Volunteers must be in good health and take no regular medication.

All participants will be compensated for their time

For more information contact
Melissa Lowe Bates at mal361@psu.edu or by calling 863-9543.

* This study has been approved by the Institutional Review Board at Penn State*

Initial Phone Contact/Screening Questionnaire

Subject Initials _____ _____ _____			**Today's Date** _____/_____/_____ Month Day Year
Age _____	**Gender** _____	**Height** _____	**Is there a possibility you could be pregnant?** ❍Yes ❍ No ❍N/A (male subjects only)

	YES	**NO**
Do you currently smoke?	❍	❍
Do you regularly drink alcoholic of caffeinated beverages?	❍	❍
Would you be willing to withhold these beverages for 12 hours before each session?	❍	❍
Do you regularly take any nutritional supplements (e.g. vitamins)?	❍	❍
Are you currently taking any medications?	❍	❍
Do you have any respiratory or cardiovascular problems or other chronic illnesses?	❍	❍
Subject Scheduled for Screening Session	❍	❍
Date of Session: _____		

Information Collected by: _____

Symptom Questionnaire

Subject Code:___ ____ _____ ____ ____ Session # _____ Date: _____/_____/_____
(month) (day) (year)

Investigators Present: _____

_____

Please rate how you currently feel with respect to the following symptoms by circling the appropriate number:

0 = None
1 = Just Perceptible
2 = Distinctly Perceptible
3 = Nuisance
4 = Offensive
5 = Unbearable

Before Exposure	Symptom	After Exposure	Symptom
0 1 2 3 4 5	Headache	0 1 2 3 4 5	Headache
0 1 2 3 4 5	Runny Nose	0 1 2 3 4 5	Runny Nose
0 1 2 3 4 5	Shortness of Breath or Difficulty Taking a Deep Breath	0 1 2 3 4 5	Shortness of Breath or Difficulty Taking a Deep Breath
0 1 2 3 4 5	Cough or Urge to Cough	0 1 2 3 4 5	Cough or Urge to Cough
0 1 2 3 4 5	Chest Burning or Discomfort	0 1 2 3 4 5	Chest Burning or Discomfort
0 1 2 3 4 5	Dizziness or Nausea	0 1 2 3 4 5	Dizziness or Nausea

Comments

_____

_____

_____

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_____

Last Updated 2/25/2008

Pre-Session Assessment

Name	ID Number	Session		
_____	_____	B	A	O

Are you currently experiencing, or have you experienced within the last 24 hours, any of the following symptoms?

Nasal Congestion	**YES**	**NO**	Allergy Symptoms	**YES**	**NO**
Chest Congestion or Shortness of Breath	**YES**	**NO**	Sore Throat	**YES**	**NO**
Headache	**YES**	**NO**	Cough	**YES**	**NO**
Fever	**YES**	**NO**	Nausea	**YES**	**NO**

Have you taken any prescription or over-the-counter medications in the past 24 hours? If yes, were any of the following taken?

Nyquil or other cold/flu medication	❍	Antibiotics	❍	Advil/Ibuprofen/Motrin	❍
Naprosyn (Alleve)	❍	Antihistaimes	❍	Tylenol	❍
Sudafed	❍	Inhalers	❍	Benadryl	❍
Aspirin/Bayer	❍	Other _____	❍	Prednisone	❍

Signature: _____ Date: _____

When was your last cigarette? (smokers only) _____

Additional Pre-Session Assessment (for Female Subjects)

Is it possible that you are pregnant? **YES NO**

Do you have a regular menstrual cycle? **YES NO**

If yes, the cycle is about _____days long.

The last period ended about _____days ago.

An average period last approximately _____ days

I have been given a urine test for pregnancy today and I have been notified that this test indicates that I am not pregnant.

Subject Signature:_____ Date: _____

Staff Signature: _____ Date: _____

Smoking History Questionnaire

Please either <u>circle</u> the appropriate answer or fill-in a response where indicated.

1 Have you ever smoked cigarettes? (If "no" then skip to question 14) **YES** **NO**

2 Do you currently smoke cigarettes? (If "no" then skip to question 9). **YES** **NO**

What brand do you currently smoke? _____

3 How many years have you been smoking cigarettes?

Please specify: _____ years

4 Would you consider your cigarette smoking habits to be consistent? **YES** **NO**

5 Do you inhale the cigarette smoke? **YES** **NO**

6 Within the last 2 months, on average, how many packs of cigarette per week did you smoke?

Please specify: _____ packs

7 Within the last year, on average, how many packs of cigarette per week did you smoke?

Please specify: _____ packs

8 Within the following age categories please indicate, on average, how many packs of cigarette per week you smoked (Then continue to question 14).

12 and under	13 to 18	19 to 24	25 to 30	31 to 36	37 to 42
_____ packs	_____ packs	_____packs	_____packs	_____packs	_____packs

9. Did you smoke cigarettes in the past and then quit (If "no" then skip to question 14)?

10 If "YES," how many years did you smoke cigarettes?

Please specify: _____ years

11 Did you inhale the cigarette smoke? **YES** **NO**

12 When did you quit smoking cigarettes?

Please specify date: _____

13 Within the following age categories please indicate, on average, how many packs of cigarettes per day you smoked?

12 and under	13 to 18	19 to 24	25 to 30	31 to 36	37 to 42
_____ packs	_____ packs	_____packs	_____packs	_____packs	_____packs

14 Do you work in an environment where you are regularly exposed to secondhand smoke? (If not, skip to question 16) **YES** **NO**

15 How many hours a day, on average, do you work in this environment?

Please specify:_____hours

16 Do you live with someone who smokes? (If not, skip to question 19) **YES** **NO**

17 Are you regularly exposed to their smoke? (If not, skip to question 19) **YES** **NO**

18 On average, how many hours per day to you spend with this person?

Please specify:_____hours

19 Do you currently smoke cigars? (If not, skip to question 21) **YES** **NO**

20 How many cigars do you smoke per week?

Please specify: _____ cigars

21 Did you smoke cigars in the past and quit permanently? (If not, skip to question 23) **YES** **NO**

22 How many cigars did you smoke per week?

Please specify: _____ cigars

23 Do you smoke a pipe at present? (If not, skip to question 25) **YES** **NO**

24 How many pipefulls do you smoke per week? (Then **END** questionnaire)

Please specify: _____ pipefulls

25 Did you smoke a pipe in the past and quit permanently? (If not, then **END** questionnaire) **YES** **NO**

26 How many pipefulls did you smoke per week? (Then **END** questionnaire)

Please specify: _____ pipefulls

Appendix C – Standard Curves and Additional Graphs

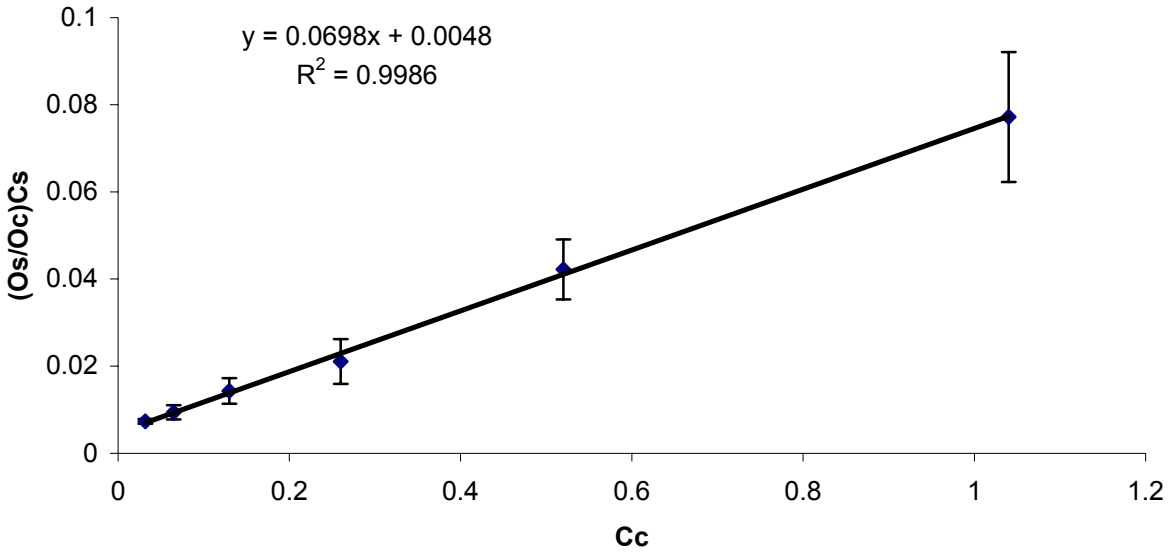


Figure C-1: Cotinine standard curve measured via HPLC. C_C is the concentration of cotinine in the sample in μg/mL, C_S is the concentration of the internal standard in the sample in μL/mL, O_C is the integrated area under the cotinine absorbance peak, O_S is the integrated area under the internal standard absorbance peak, and k_T is the sample partitioning coefficient describing the relative partitioning of cotinine and internal standard at each processing step. Bars are two times the standard error about the mean.

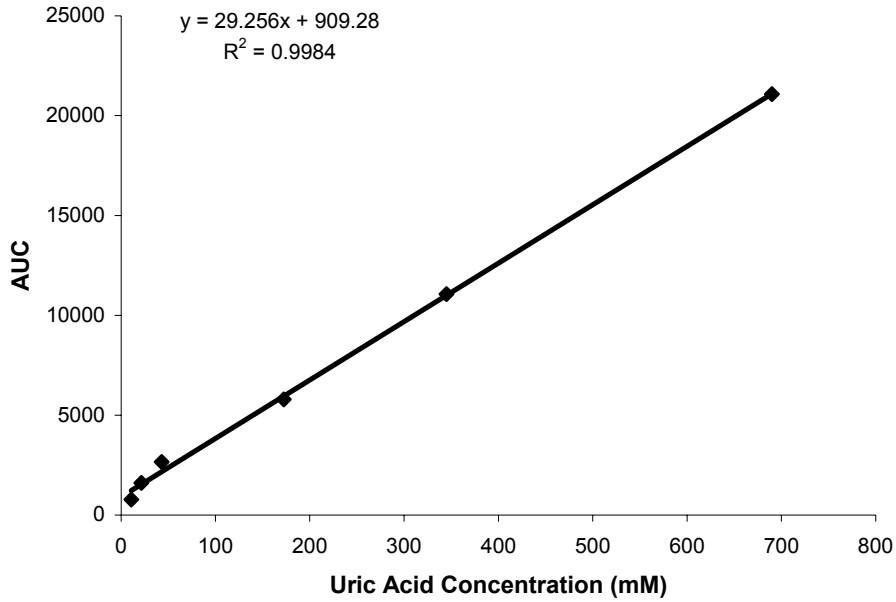


Figure C-2: Uric acid standard curve measured via HPLC. The y-axis represents the micromolar concentration of uric acid in the sample and the x-axis represents the area under the uric acid peak.

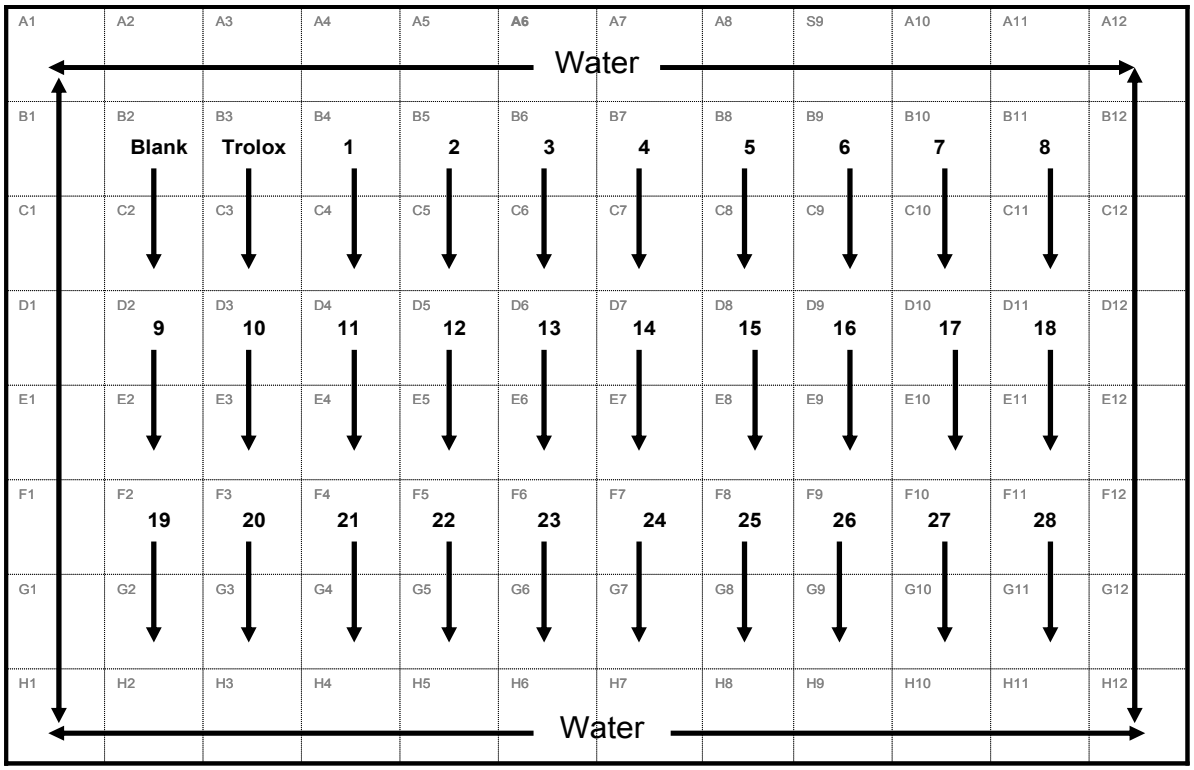
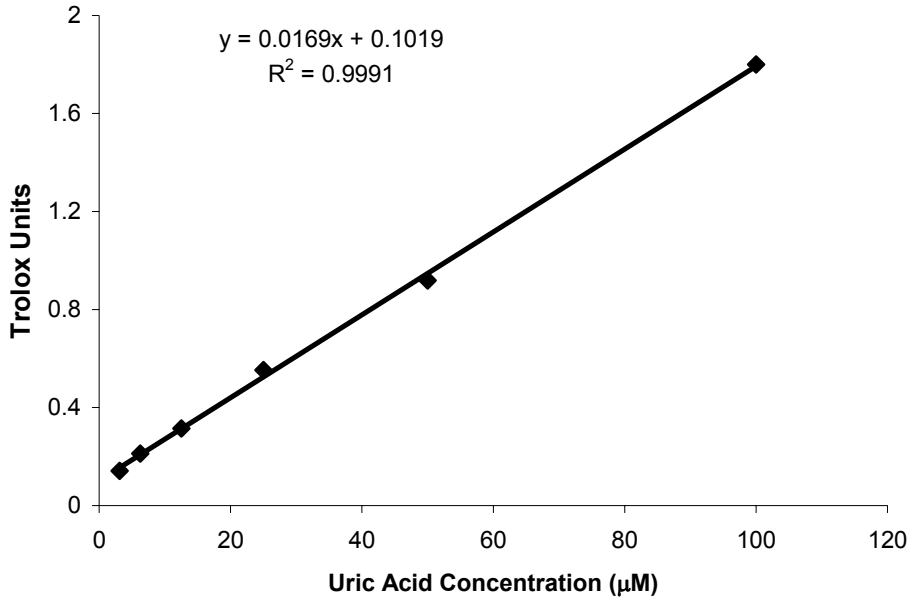


Figure C-3: 96-well microplate layout. Outer wells are filled with water. Two pairs of inner wells are filled with a buffer blank and Trolox. Remaining inner wells are filled with unknown samples 1-28.

A



B

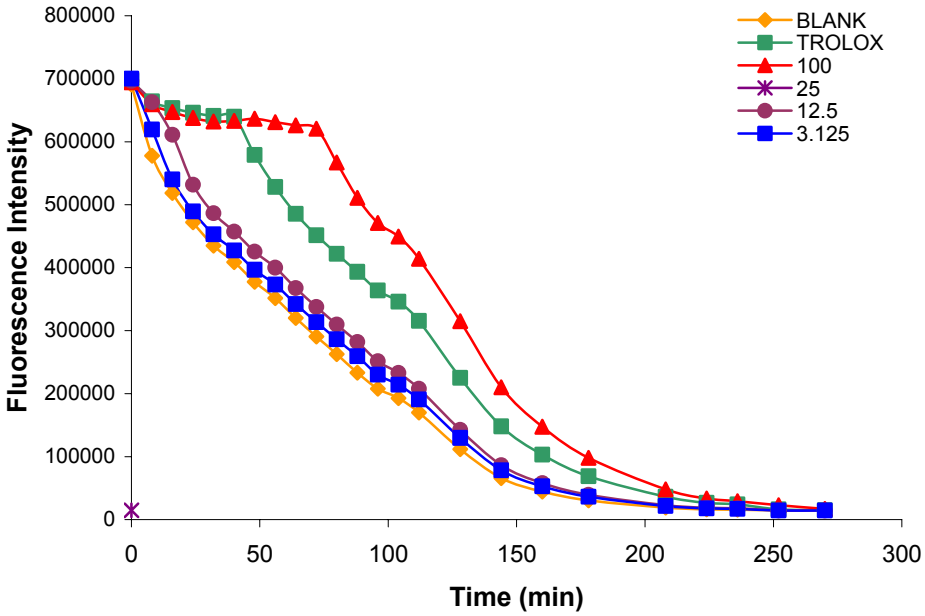


Figure C-4: Standard Curve (Panel A) and individual decay curves (Panel B) obtained via the ORAC assay using standard uric acid solutions. Concentrations, measured in μM, are given in the legend of Panel B.

Academic Vita - Melissa Lowe Bates

Educational Experience

2003-2008 Doctor of Philosophy (Anticipated), IGDP in Physiology
The Pennsylvania State University
Advisor: James S. Ultman, PhD, Distinguished Professor Emeritus of Chemical Engineering, Bioengineering, and Physiology

1997-2002 Bachelor of Science, Department of Biology
University of California, Riverside

Honor and Awards

2003 University Graduate Fellowship Award

2004-2006 Predoctoral Fellowship Award – NIH Training Grant, "Physiological Adaptations to Stress" (GM08619-09)

2006-2007 Graduate Assistantship, Ozone Dose to the Human Respiratory Tract: The Effect of Cigarette Smoking

2007 Second Place Award – Ozone-Induced Alterations in Regional Airway Function in Smokers Versus Non-Smokers ML Bates, TM Brenza, A Ben-Jebria and JS Ultman. Physiology Graduate Student Poster Exhibition. May 21, 2007

Publications

Bates, ML, Brenza, TM, Ben-Jebria, A, Bascom, R, Ultman, JS. Distribution of and response to ozone boluses in smokers and non-smokers. (In Submission).

Presentations and Abstracts

Ozone-Induced Alterations in Regional Airway Function During Exercise in Smokers Versus Non-Smokers. ML Bates, TM Brenza, A Ben-Jebria, and JS Ultman *Medicine & Science in Sports & Exercise* November 2006, Volume 38, Issue 11 Suppl1 S46. – Presented at the Integrative Physiology of Exercise Meeting, September 2006

Ozone-Induced Alterations in Regional Airway Function in Smokers Versus Non-Smokers ML Bates, TM Brenza, A Ben-Jebria and JS Ultman. -- Physiology Graduate Student Poster Exhibition. May 21, 2007 – Second Place Award.

Effect of Ozone Boluses on the Lung Function of Smokers. ML Bates, TM Brenza, A. Ben-Jebria, and JS Ultman. Experimental Biology April5, 2008.