SEX DIFFERENCES IN RESPONSE TO ACUTE DIESEL EXHAUST EXPSOURE

by

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ABSTRACT OF THE DISSERTATION

Sex Differences in Response to Acute Diesel Exhaust Exposure

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Introduction: Diesel exhaust is ubiquitous and has been shown to cause a variety of health effects related to innate immune response in the airways that leads to a cascade of local and systemic health effects. Sex is known to influence variations in immune responses and many other aspects of the internal physiological environment.

Aims: The primary aim of this dissertation research is to evaluate whether there are sex differences in the symptomatic, local and systemic inflammation and acute-phase responses that have been attributed to acute diesel exhaust exposure. The secondary aim is to see if sex differences exist in the rates of metabolism of diesel exhaust and its metabolites.

Methods: In a crossover design study, healthy subjects were exposed to 300µg/m^3 of diesel exhaust and clean air for 1 hour each in a controlled environmental exposure chamber on two different days (≥1 week washout) in random order. Nasal lavage and sputum samples were collected and analyzed for cytokines and soluble proteins in relation to local inflammation. Blood samples were collected and analyzed for cytokines, acute phase reaction proteins, neutrophils and cell blood counts in relation to systemic inflammation. Questionnaires were used to collect self-reported symptoms. Spot urine

samples were collected within 24 hours of exposure to analyze the levels of 1-aminopyrene a metabolite of 1-nitropyrene which is the most abundant nitro-polycyclic aromatic hydrocarbons in diesel exhaust.

Results: There was a statistically significant sex difference in self reported somatic and lower respiratory symptom severity ratings with females having higher symptom severity ratings than males after exposure to diesel exhaust relative to clean air. There was also a statistically significant sex difference in the concentration of tumor necrosis factor – α in nasal lavage samples and platelet count in peripheral blood with males having higher tumor necrosis factor – α concentrations than females and lower platelet counts than females after exposure to diesel exhaust relative to clean air.

Conclusion: Sex is a significant effect modifier for certain health effects of acute diesel exhaust exposure, with females tending to show greater lower respiratory and systemic effects and males tending to show greater upper respiratory effects.

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Dedication

To My Wonderful Family

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Chapter 1

Introduction

1.1 Background

Diesel exhaust is produced from the combustion of diesel fuel. The superior energy efficiency and endurance of diesel engines have led to a rapid rise in the use of diesel fuel worldwide to power vehicles and other machineries (USEPA HAD 2002). This has made diesel exhaust a significant contributor to ambient air pollution and the largest single source of airborne particulate matter (PM) in many urban areas (Riedl and Diaz-Sanchez 2005). Across populations, exposures may be acute or chronic and the exposure amounts may be either high or low depending on the temporal and spatial distribution of diesel exhaust sources. Due to the ubiquitous nature of diesel exhaust and the potential for the high exposure of certain populations like workers using diesel-powered machinery and people who live close to diesel traffic, diesel exhaust has been one of the most studied environmental pollutants in an attempt to determine its toxicological effects in humans and rodents.

Toxicology is the study of the adverse effects of a xenobiotic (Casarett and Doull's 2001) on a target population or species. This involves understanding the nature of the xenobiotic as well as the host or target factors that may make it more or less susceptible to the xenobiotic. Such factors include genetic traits, sex, age, pre-existing conditions, behavioral traits and coexisting exposures to other xenobiotics (Casarett and Doull's 2001). When making inferences from animal studies or from epidemiological studies some of these factors are usually handled as uncertainty factors and are mathematically used in the calculation of acceptable exposure levels and for policy measures.

In particular, sex is often considered to be a confounder or a modifying variable and is usually controlled for by the choice of design or by some standardization or multivariate procedure (Gochfeld 2007). This has led to the fact that in the toxicology of environmental xenobiotics, especially with regards to non-cancerous health outcomes, differences in susceptibility by sex have not received adequate attention in human and animal toxicology nor in epidemiology, and generalizations are often made about species response without data or consideration of sex differences (Gochfeld et al 2007).

Why is this important? It is important because males and females differ substantially with respect to physiology and biology and these differences may have significant effects on the toxicity of any xenobiotic and thus may affect how vulnerable males and females are to the effects of xenobiotics and other environmental stressors (Gochfeld 2007). Along the continuum, from exposure to absorption to metabolism to excretion to systemic effects and to overall health outcomes, every step may be potentially influenced by sex.

1.2 Diesel Exhaust

1.2.1 Exposure to Diesel Exhaust

Most acute exposure to very high levels of diesel exhaust and chronic exposure to low levels of diesel exhaust occur via occupational exposures, either by accident or as a routine part of the job. Toll workers, truck drivers, bridge and tunnel workers, mine workers, forklift drivers, railroad and dock drivers, and garage workers usually have the highest occupational exposure. Exposure to the general public is not as frequent as seen in the occupational setting and it is usually to lower levels of diesel exhaust. The highest

exposure to the public occurs along highways and in cities or anywhere diesel traffic is heaviest (Frumkin and Thun 2001). Children are also thought to have one of the highest opportunities for exposure to diesel exhaust fumes due to the use of diesel powered school buses.

Inhalation of diesel exhaust is the primary route of exposure; therefore, experimental studies have often been done to examine the association between diesel exhaust exposure and respiratory and cardiovascular effects.

1.2.2 Composition of Diesel Exhaust

Diesel exhaust is made up of a complex mixture of hundreds of constituents in either a gaseous or particulate form. Gaseous components of diesel exhaust include carbon dioxide, oxygen, nitrogen, water vapor, carbon monoxide, nitrogen compounds, sulfur compounds, and numerous low-molecular-weight hydrocarbons.

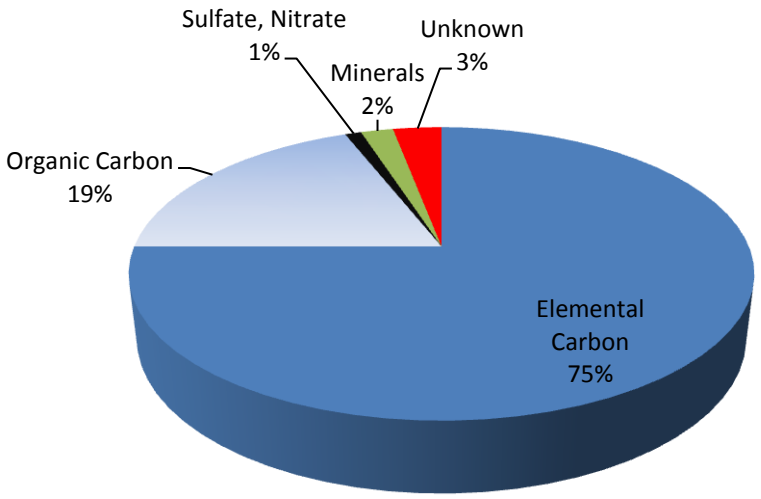

Figure 1.1. Typical Chemical Compositions for Diesel Particulate Matter $PM_{2.5}$.
Source: Health Assessment Document for Diesel Engine Exhaust, US EPA 2002.

The particles produced by diesel exhaust fuel combustion are composed of a center core of elemental carbon and absorbed organic compounds as well as small amounts of sulfate, nitrate, metals and other trace elements (Figure 1) (USEPA HAD 2002). These particles are collectively called diesel exhaust particles (DEPs) and they are primarily made up of fine (2.5 - 0.1 μm) and ultra-fine (<0.1 μm) particles. These primary DEPs can also coalesce to form aggregates of varying size ranges (Riedl and Diaz-Sanchez 2005). Both the gaseous and particulate fractions of diesel exhaust contain polycyclic aromatic hydrocarbons (PAHs), which are produced as pyrolytic products during the combustion of diesel exhaust and any fossil fuel (Frumkin and Thun 2001).

Due to these characteristics of diesel exhaust as well as its vast use in society, diesel exhaust accounts for a significant source of ambient $PM_{2.5}$ (especially in urban environments). For this reason, many experimental studies use diesel exhaust as a model for PM pollution (Torqvist et al 2007).

1.2.3 Overall Health Effects of Diesel Exhaust Exposure

In 1988, the National Institute for Occupational Safety and Health (NIOSH) proposed a potential link between occupational exposure to diesel exhaust and lung cancer based on the consistency of toxicological studies in rats and mice and the limited epidemiological evidence which was mainly from railroad workers at the time (Rogers and Davies 2005).

Overall, chronic inhalation exposure to diesel exhaust is likely to pose a lung cancer hazard to humans, as well as damage the lungs in other ways depending on the length and intensity of the exposure. While acute exposure to diesel exhaust can cause

acute irritation (e.g., eye, nose, throat, bronchial); neurophysiological symptoms (e.g., lightheadedness, nausea); inflammatory responses in the airway and the lungs; respiratory symptoms (cough, phlegm); there is also evidence for an immunologic effect (the exacerbation of allergenic responses to known allergens and asthma-like symptoms) and cardiovascular effects (USEPA HAD 2002; Donaldson et al. 2001) such as impaired vascular function and increase in blood coagulation.

Normally, the lungs are able to deal with the isolation, neutralization and removal of inhaled diesel exhaust and other xenobiotics or air pollutants via various mechanisms, but these natural defense mechanisms can often become overwhelmed for a variety of reasons including illness or high burden of pollutants and this can then lead to the adverse health effects mentioned above (Salvi and Holgate 1999).

1.2.4 Hypothesized Mechanisms of Diesel Exhaust Induced Health Effects

The mechanism by which diesel exhaust and other air pollutants cause these adverse health effects are not well known. Currently there are two leading hypotheses about the mechanisms by which diesel exhaust and other air pollutants cause the health effects that have been observed. In particular, diesel exhaust (or other particulate matter) induced effects on the cardiovascular system, lung and blood receptors are hypothesized to occur either via the direct action of DEPs or other particulate matter on these endpoints (Brook et al 2004 and Shimada et al 2006) or indirectly via mediators released in response to DEPs (or other particulate matter) initiated pulmonary oxidative stress and inflammatory responses in the airway and lungs (Brook et al 2004 and Shimada et al 2006).

The direct effect of DEPs (or other particulate matter) on cardiovascular system, lung and blood receptors are hypothesized to happen due to the translocation of ultra fine DEPs (or other ultra fine particulate matter), transition metals and gases from the lungs to systemic circulation. Under this hypothesis, the translocated DEPs or other particulate matter directly influence homeostasis and other cardiovascular endpoints (Kaewamatawong et al 2008 and Shimada et al 2006) leading to cardiovascular effects.

An alternative hypothesis upon which this dissertation research is based is that exposure to diesel exhaust (or other air pollutants) causes an inflammation reaction in the airways and lungs via oxidative stress which results in the systemic release of cytokines which influence the heart, coagulation factors and other cardiovascular endpoints and leads to cardiovascular symptoms (Kaewamatawong et al 2008 and Shimada et al 2006).

The exact mechanism (s) of the anatomical translocation of inhaled ultra fine DEPs (or other ultra fine particulate matter) across the air-blood barrier at the alveolar wall are not fully understood (Shimada et al 2006) but the alternative inflammation induced systemic effect of DEPs (or other particulate matter) is hypothesized to take place when the previously mentioned overload or hindrance of the lung's natural defenses occurs. When this happens, it sets off a variety of cascading adverse systemic reactions involving and mediated by multiple cell lines, cytokines and acute phase proteins. All of these may contribute to the overall adverse respiratory and cardiovascular health effects that have so far been linked with exposure to diesel exhaust and other air pollutants. The consensus amongst the diesel exhaust studies that have been done is that diesel exhaust is capable of causing pro-inflammatory and pro-allergenic effects which cause marked pulmonary and systemic inflammatory responses involving a variety of cell types (Diaz

and Riedl 2005; Salvi et al 1999) that include epithelial cells, macrophages, neutrophils, mast cells and lymphocytes.

As with the actions of the different components of air pollution, it is very hard to tease apart the effect of the two fractions of diesel exhaust because despite their distinct nature, it appears that both the particulate and gaseous fractions of air pollutants and diesel exhaust alike share the ability to initiate and heighten these cellular inflammation in the upper and lower airways.

1.2.5 Diesel Exhaust and Cytokine Release

It is hypothesized that the cascading events that follow diesel exhaust exposure are heavily influenced by the pro-inflammatory cytokines and chemokines released from affected cells in response to diesel exhaust induced local inflammation. Furthermore, these cytokines are also hypothesized to have an added inflammatory effect on the surrounding cells and to modify the production of acute phase proteins by the liver thereby leading to adverse cardiovascular effects.

Cytokines are intercellular signaling polypeptides produced by activated cells (Gabay C. et all 1999). Of particular interest in diesel exhaust exposure are those cytokines that are produced during inflammation and which also participate and contribute to the inflammatory process because they are the chief stimulant of the acute phase proteins. Macrophages and monocytes are the most important sources of these cytokines at the sites of inflammation, although they are also produced by a number of other different cells. These pro-inflammatory cytokines include interleukin 6 (IL-6),

interleukin 1β (IL-1β), Tumor necrosis factor - α (TNF-α), interferon γ (IFN-γ), transforming growth factor β^2, interleukin 8 (IL-8) (Gabay C. et all 1999).

1.2.6 Diesel Exhaust and Oxidative Stress

The roles of these pro-inflammatory cytokines in increasing inflammation in response to diesel exhaust exposure has been well documented (van Eeden et al. 2001), but the initial cause of the inflammatory process which stimulates the release of these cytokines is still under debate. One leading thought is that the inflammation is instigated by oxidative stress (Furuyama et al. 2006; Diaz-Sanchez and Riedl 2005; Salvi and Holgate1999) caused by the subsequent prolonged contact of the metals, PAHs and quinines contained in diesel exhaust with the epithelial and macrophage cells when the natural lung safety mechanisms become overwhelmed. The epithelial and macrophage cells are the initial target cells for particle interaction and deposition and thus are thought to be the initial site of toxicity (Salvi et all 1999). This theory has been supported by the fact that antioxidants can block the effects of DEPs in animal models and in in-vitro models (Diaz-Sanchez and Riedl 2005). Diesel exhaust has also been demonstrated to activate redox-sensitive transcription factors in the bronchial epithelium of healthy subjects consistent with oxidative stress triggering the increased synthesis of cytokines (Pourazar et al. 2005).

This oxidative stress initiated inflammation is thought to either directly or indirectly cause or trigger other mechanisms that are behind the effects that have been seen in in-vitro and in-vivo studies (Table 1).

Table 1.1. Summary of In-Vitro and In-Vivo Responses to Diesel Exhaust /Diesel Exhaust particles Exposure.

Summary of In-Vitro Cell responses		
	Summary of Responses by Cell Types	**Sources**
1.	In bronchial and nasal epithelial and endothelial cells – ▪ Increase expression of chemokines and cytokines IL-8, eotaxin, RANTES, GM-CSF and IL-6 ▪ Up regulation of the expression of histamine H-1 receptor mRNA and enhanced inducement of histamine induced increase in IL-8 and GM-CSF production.	Saxton and Diaz 2000; Riedl and Diaz-Sanchez 2005; Salvi and Holgate 1999;Salvi et al 1999; Pourazar et al 2005; Saxton and Diaz-Sanchez 2000; Diaz- Sanchez et al 1996
2.	In Eosinophils - Enhanced adhesion of to nasal epithelial cells and induced eosinophil degranulation.	
3.	In Mast cells – ▪ Enhanced IgE mediated release histamine release ▪ Increase in the production of IL-4 and IL-6	
4.	In Basophils – Induced histamine release in the absence of IgE and enhanced cytokine production of IL-4.	
5.	In PMDCs – Inducement of chemokine production of IL-8 and RANTES and combining with allergen to increase IL-8, RANTES and TNF-α production.	
6.	In B cells – Enhancement of IgE production in the presence of co-stimulatory molecules such as after IL-4 and CD40.	
7.	In Monocytes and Macrophages – ▪ Impaired Phagocytosis ▪ Induction of oxidative stress ▪ Stimulation of the release of TNF-α and IL-6 ▪ Modulation of cytokine production by inhibition of IL-12p40 production ▪ Inhibition of prostaglandin E_2 release ▪ Increase in phase 2 enzyme expression.	
Summary of In-Vivo responses		
	Summary of Responses in Animals	**Sources**
1.	Damage to alveolar macrophage membranes and depressed phagocytic activity.	Saxton and Diaz 2000; Riedl and Diaz-Sanchez 2005; Salvi and Holgate 1999;Salvi et al 1999; Pourazar et al 2005; Saxton and Diaz-Sanchez 2000; Diaz- Sanchez et al 1996 ; Salvi et al 2000 ; Nightingale et al 2000
2.	Damage to ciliated epithelial cells lining the trachea and large airways	
3.	Activation of Macrophages and release of IL-1, IL-6 and TNF-α.	
4.	Release of neutrophils into the blood from the bone marrow	
5.	Increase levels of cytoplasmic and lysosomal enzymes and total proteins in response to chronic exposure.	
6.	Increased synthesis and deposition of collagen producing thickening of the alveolar septa and blood vessels and the development of interstitial fibrosis.	
7.	Increased levels of mRNA for IL-2, IL-4 and GM-CSF in airway tissue.	
	Summary of Responses in Humans Subjects	
1.	Increase in the number of neutrophils, B cells, T cells and mast cells (inflammatory cells) in the airways.	
2.	Increase in circulating neutrophils and platelets.	
3.	Increase in histamine, fibronectin, neutrophils and B lymphocytes levels in the airway lumen.	
4.	Up regulation of the expression of adhesion molecules on the capillary endothelial cells and leucocytes cell surfaces.	
5.	Increase of gene transcription and expression of IL-8 and GRO-α protein and an accompanying trend towards an increase in IL-5 mRNA gene transcripts in the bronchial epithelium.	
6.	Decrease in macrophage function	
7.	Increase in airway resistance.	
8.	Up-regulation of redox sensitive transcription factors and cell signaling pathways in the bronchial epithelium	
9.	Enhanced local mucosal IgE production	
10.	Increase in nasal cytokine expression following DEP exposure i.e. IL-2, IL-4, IL-5, IL-6, IL-10, IL-13 and Interferon γ	

1.2.7 Respiratory and Cardiovascular Health Effects of Diesel Exhaust Exposure

The respiratory and cardiovascular effects of diesel exhaust are thought to be connected via oxidative stress initiated inflammation because inflammation is known to cause a large number of changes far from the initial site of the local inflammation and potentially involving many organ systems. More specifically changes in the levels of acute phase proteins in response to inflammation and mediated by pro-inflammatory cytokines are believed to cause the corresponding adverse cardiovascular effects that are often seen following the inhalation of diesel exhaust especially in compromised populations.

Acute phase proteins are proteins that are produced by the liver and whose plasma concentrations increase or decrease by at least 25% during an inflammatory disorder (Gabay et al. 1999). Of particular interest are fibrinogen and C-reactive proteins. Fibrinogen can cause endothelial cell adhesion, spreading and proliferation, while C-reactive proteins have been shown to be a cardiovascular risk factor in both healthy subjects and those with coronary heart disease (Peter et al. 2001). C-reactive proteins are tightly regulated and their plasma concentrations can increase by over 1000 fold during an acute phase response to inflammation or other forms of tissue damage or infection (Peter et al 2001).

Acute phase changes reflect the presence and intensity of inflammation (Gabay et al. 1999), so it is hypothesized that in response to the injury caused by diesel exhaust induced oxidative stress, the local inflammatory cells i.e. neutrophils, monocytes and macrophages, secrete a number of cytokines (IL-6, IL1β, IL-8 and TNF-α) into the blood stream. These cytokines ate thought to act as a cascade and as a network in stimulating

the production of C-reactive proteins and other acute phase proteins in the liver (Gabay et al.1999). This leads to the presentation of acute phase symptoms which include fever and lethargy (Gabay et al. 1999).

In summary, the activation and mobilization of inflammatory cells by oxidative stress, the production of pro-inflammatory cytokines, the production of acute phase proteins and the production of circulating inflammatory mediators all characterize the systemic inflammatory response (van Eeden et al 2001) that is hypothesized to cause adverse cardiovascular effects (Figure 3).

1.3 Sex

1.3.1 Definition of Sex and gender

In public health terms, sex can be thought to determine genetically based sensitivity to health determinants and gender can be thought to express some social forces that could influence exposure and responses to health determinants (Messing and Stellman 2006).

The effects of sex and gender on the adverse health effects of a xenobiotic often complement each other. Gender often has a big influence on the types and levels of a xenobiotic that men and women get exposed to especially in the occupational setting and sex often has a big effect on how men and women absorb, distribute, metabolize and excrete the xenobiotic, once the exposure has taken place (Figure 4) (Arbuckle 2006 and Dimich-Wald et al 2006).

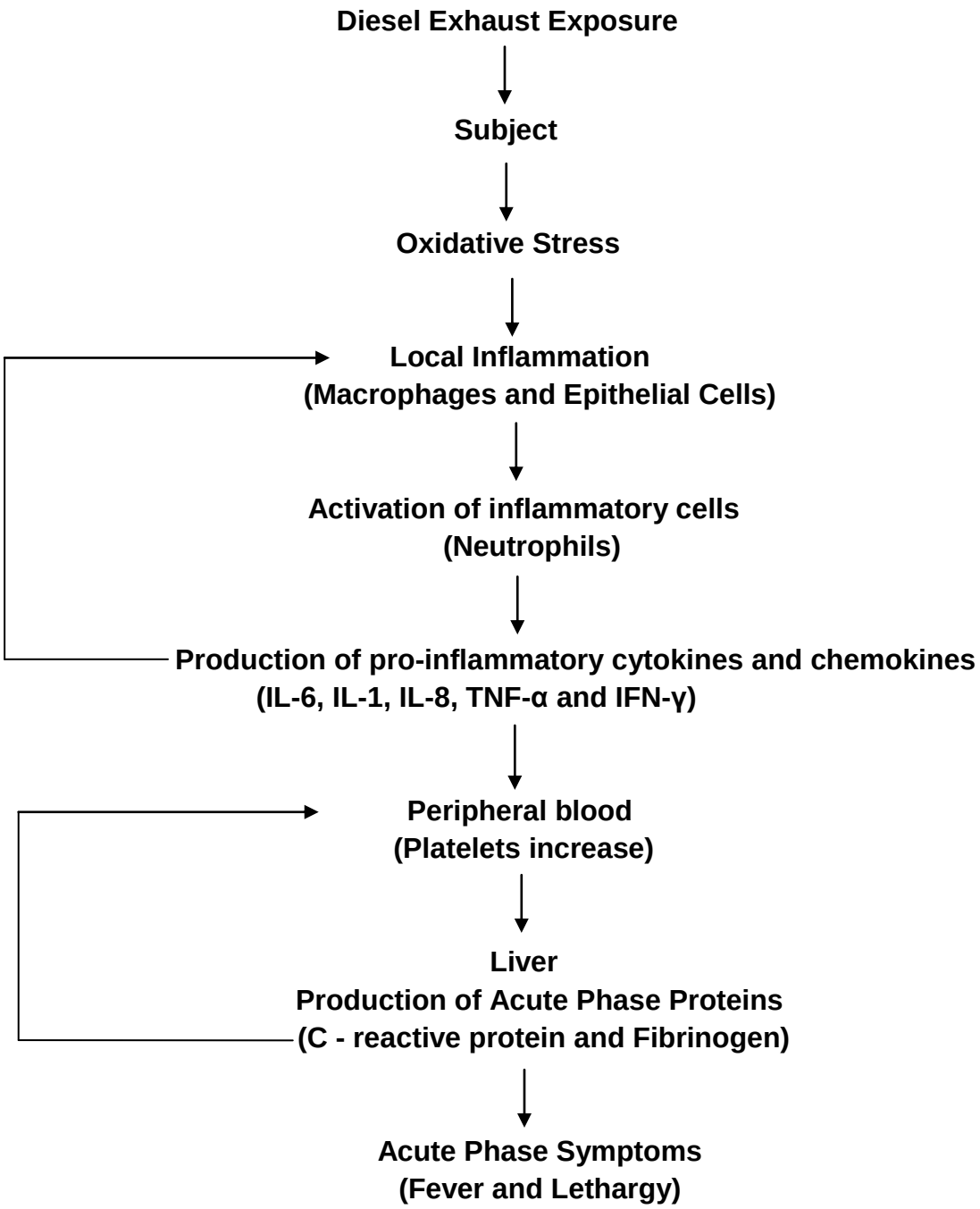

Figure 1.2. Hypothesized Diesel Exhaust Effects Model Used in this Dissertation.

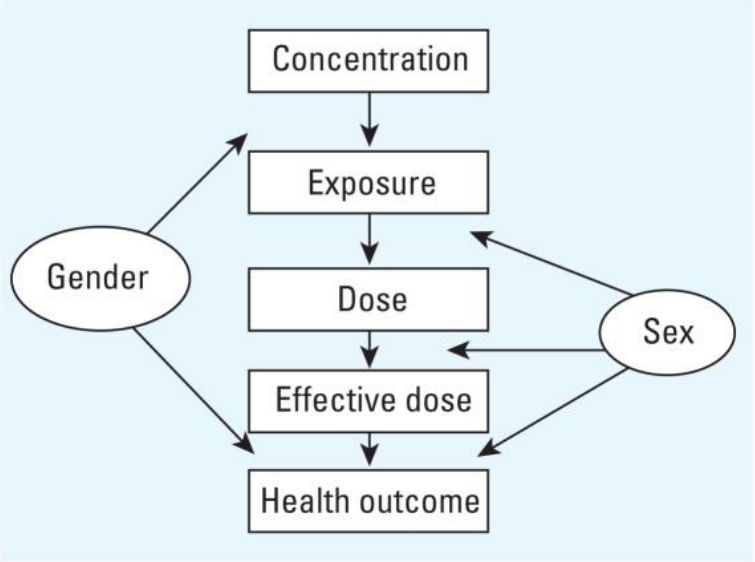

Figure 1.3. Possible roles of gender and sex in shaping observed relationships between air pollution and health.

Source: Clougherty 2010 (Reproduced with permission from Environmental Health Perspectives).

1.3.2 Sex and Toxicity

With regards to the toxicity of environmental air pollution, there is little information on environmentally related health effects that manifest differently in females compared with males and in experimental animal toxicological studies male animals have almost exclusively been used (Vahter et al. 2007). Some studies have been done to look at the differences in the reaction to different xenobiotics by sex and a lot of these studies have focused on form and structural differences between males and females rather than on functional differences (e.g. Becklake and Kauffmann 1999). These studies have looked at differences in lung capacity, ventilation rates and so on, in an attempt to identify how these morphological differences affect the rates of absorption of the inhaled xenobiotic. The fact remains that once a xenobiotic reaches the lungs, there are many local interactions involving macrophages and cytokines which can lead to inflammation

or other local pulmonary effects which are unrelated to the rates of systemic absorption (Gochfeld 2007).

Recently, some epidemiological studies have been done to examine gender and sex differences in health effects in relation to residential exposure to air pollution (Kan et al 2007, Kan et al 2008, Franklin et al, Luginaah et al 2005, Sunyer et al 2006). These studies together suggest stronger sex linked associations among women in response to air pollution such as lower forced respiratory volume in 1 second (FEV1), increased phlegm production and increased respiratory mortality (Clougherty 2010). Physiological explanations that have been suggested to explain these observed susceptibility of women to respiratory hazards include the possibility that females have differential rates of absorption, kinetics, and metabolism of xenobiotics that affect their dose for the average male or female (Dimich-Ward et al 2006).

1.3.3 Sex and the Effects of Diesel Exhaust Exposure

It is hypothesized that sex hormones (estrogens and testosterone) play an essential role in the different susceptibilities between males and females to xenobiotics. Differences in xenobiotic particle clearance, oxidative stress handling, cytokine pathway activation and cytochrome P450 enzymes activity, are all influenced by sex hormones (Fortoul et al 2005). There is also evidence that sex hormones have an influence on many aspects of airway behavior especially in females, which may be unrelated to the gender related socioeconomic determinants of exposure, for example, it has been suggested that the airways of girls (and women) may be more sensitive to the effects of tobacco smoke than those of boys and men (Becklake and Kauffmann 1999). This raises the question of

whether this increased susceptibility to tobacco smoke is unique or whether it may be applicable to other environmental exposures like diesel exhaust.

1.3.4 Sex and the Metabolism of Diesel Exhaust

Not all differences between male and female susceptibility can be attributed to hormones (Vahter et al. 2007). For example, the metabolism and excretion of a xenobiotic and its metabolites is something that is highly influenced by differences in morphology and other internal metabolic processes. The rate and type of metabolism and the rate of excretion of a xenobiotic and its subsequent metabolites can have a big effect on the differences in susceptibly of males and females. Exposure to diesel exhaust is known to increase urinary levels of oxidative stress by-products and urinary metabolites of diesel exhaust components but so far no one has examined whether a difference exists between males and females in the levels of metabolites excreted in urine. This is important because the clearance rate of diesel exhaust and other inhaled xenobiotics may differ by sex depending on whether they are metabolized via conjugation, oxidative mechanisms or reduction (Arbuckle et al 2006) in the airway, lungs and subsequently in the liver. These potential differences in clearance rates may also produce sex related differences in response to diesel exhaust exposure and other xenobiotics.

1.3.4.1 1-Nitropyrene

1-Nitropyrene (1-NP) is the most abundant nitro-PAH contained in diesel exhaust and so it along with its metabolites (Figure 5) are thought to be good specific biomarkers of diesel exhaust exposure (Toriba et al 2007).

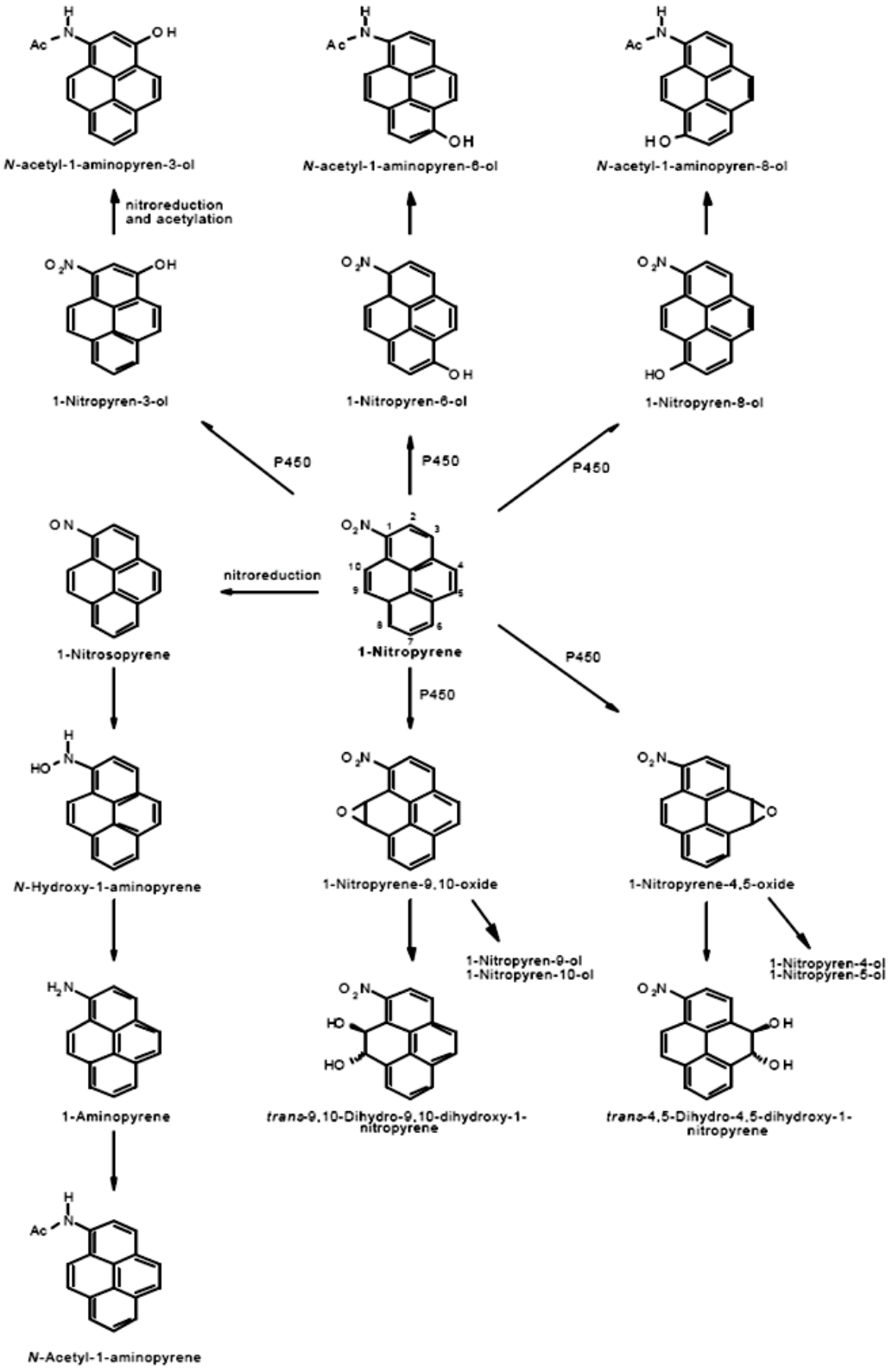

Figure 1.4. Metabolites of 1-Nitropyerene in-vivo.
Source: Howard et al 1995 (Reproduced with permission from Chemico-Biological Interactions).

1-NP is also a main contributor of the direct acting mutagenicity that has been observed in DEPs (Toriba et al 2007). The metabolism of 1-NP can occur via nitro-reduction or cytochrome P450 mediated ring oxidation (Silvers et al 1997 and Chae et al 1999) but nitro-reduction appears to be a principal pathway by which 1-NP is activated resulting in the formation of DNA adducts (Silvers et al 1997).

1.3.4.2 Sex and the Metabolism of 1-Nitropyrene

The human Cytochrome P450 family of enzymes, in particular the P450 3A3 and 3A4 isomers are the predominant enzymes involved in the oxidative metabolism of 1-NP (Silvers et al 1997 and Chae et al 1999). In the mammalian system, 1-NP is metabolized by xanthine oxidase, DT-diaphorase or aldehyde oxidase (NTP 1996).

There are known variations by sex in the function and activity of the P450 isomers, in particular CYP 3A4, with females having a higher activity than males (Anderson 2008). Also, in 1992 Relling et al showed that females had a higher activity of xanthine oxidase than males after exposure to a low dose of caffeine. Therefore there is the possibility that there may be a variation by sex in the concentration of 1-NP metabolites that are produced regardless of the metabolic pathway that is used.

1.4 Dissertation Research

1.4.1 Aims

The primary aim of this dissertation research is to investigate the potential effect of sex on local airway and systemic inflammation, acute phase responses and self reported symptomatic responses that have been attributed to acute diesel exhaust exposure. The secondary aim of this dissertation research is to examine whether there is a

sex difference in the metabolism of 1-nitropyrene, a diesel exhaust specific compound and a potent mutagen and carcinogen.

1.5 Research Design and Data

1.5.1 Data Collection

The data used for this dissertation research are part of the datasets generated from a study of diesel exhaust effects and a subsequent add-on study of diesel exhaust exposure biomarkers. The parent study was funded by the Department of Defense (DOD) through a grant to the University of Medicine and Dentistry of New Jersey (UMDNJ) (Grant # DAMD17-03-1-0537; Principal Investigator: Dr. Nancy Fiedler) and the add-on project was funded by the Environmental Protection Agency (EPA) through a STAR grant to UMDNJ (Grant # R832097; Principal Investigator: Dr. Junfeng Zhang).

The parent study and the add-on project were done using human subject protocols that were HIPAA compliant and approved by the UMDNJ Institutional Review Board.

1.5.2 Original Study Data

The main objective of the original parent study was to assess the effects of an interaction between acute psychological stress and airway inflammation due to diesel exhaust exposure. One hundred and ten healthy men and women between the ages of 18 and 50 were recruited from the community based on the established study criteria. Each subject underwent two exposure conditions; one was to diluted exhaust normalized to PM_{10} concentration of 300 $\mu g/m^3$ and the other was to filtered clean ambient air of Busch campus in Piscataway; a suburban campus of Rutgers University. Both exposure

conditions were 1 hour in duration and took place at the Environmental and Occupational Health Sciences Institutes' (EOHSI) Controlled Environmental Facility (CEF). Each exposure condition was presented with and without an acute psychological stressor to two groups of subjects high and low in self reported chemical intolerance (CI).

Study Design: 2 (CI*) X S (Stress*) x 2 (Exposure)

*** = CI and psychological stress were between subjects factors and exposure was a within subjects factor.**

The outcome variables of the original study are listed in table 2. For both the diesel exhaust and clean air exposures, the data for the outcome variables were collected as described in Table 3.

The aim of the add-on study was to test the hypothesis that exposure to diesel exhaust leads to increases in urinary levels of amino-PAHs. To test this hypothesis, urine samples were collected from 55 out of the 110 subjects and analyzed for concentrations of 1-aminopyrene, 3-aminobenzanthrone, 1-aminonaphthalene and 2-aminonaphthalene, which are metabolites of 1-nitropyrene, 3-nitrobenzanthrone, 1-nitronaphthalene and 2-nitronaphthalene respectively.

1-aminopyrene is the main metabolite produced via the nitro-reduction of 1-nitropyrene (NTP 1996) making it a potentially good biomarker of diesel exhaust exposure and susceptibility. Therefore, this dissertation research will only use the data collected on urinary levels of 1-aminopyrene from the add-on study.

Table 1.2. Controlled Environmental Exposure Study of Diesel Exhaust Outcome Variables.

Outcomes	Outcome Variables	Time Measured
Respiratory Tract Inflammation:		
Local inflammation in the nasal mucosa (upper respiratory tract)	**Nasal Lavage** Concentration of proteins, TNF-α, IL-6 and IL-8.	24 hours post exposure.
Local inflammation in the lower respiratory tract	**Induced Sputum** Concentration of proteins, TNF-α, IL-6 and IL-8.	6 hours post exposure.
Acute Phase Response:		
Peripheral Blood	Absolute number and relative percentage of neutrophils and blood cells; number of platelets and the concentration of IL-6; levels of C-reactive protein and fibrinogen.	Baseline (pre exposure), 6 and 24 hours post exposure.
Symptoms:		
Symptoms	8 Symptom questionnaires	Baseline (2), during exposure (3), post expousre (3).

Table 1.3. Original Study Data Collection Protocol.

Time	Activity
Pre Exposure	1. Complete symptom questionnaire – Qu-1
Exposure/Stress	1. Complete another symptom questionnaire – Qu-2 2. Baseline blood sample – B1 3. Baseline CO2 measurement 4. Complete another symptom questionnaire – Qu-3 5. Average Heart rate – HR-1 6. Pre stress blood – B2 7. Complete symptom questionnaire – Qu-4 8. Post stress blood – B3 9. Average Heart rate – HR-2 10. Complete symptom questionnaire – Qu-5 11. Complete symptom questionnaire – Qu-6
6 hours post exposure	1. Complete symptom questionnaire – Qu-7 2. Post exposure blood sample – B4 3. Induced sputum
24 hours post exposure	1. Complete symptom questionnaire – Qu-8 2. Post exposure blood sample – B5 3. Post exposure Nasal lavage

1.5.3 Subjects Demographic Data

The total number of subjects that completed the parent study out of the 110 subjects that were enrolled was 100 and table 4 below gives a demographic summary of the subjects.

Table 1.4. Total Subject Demographic Information

	Females					Males				
	N	**Min**	**Max**	**Mean**	**SD**	**N**	**Min**	**Max**	**Mean**	**SD**
AGE (years)	36	18	44	23.78	5.96	64	18	43	24.08	5.74
***Education (Years)**	36	12	22	15.42	2.32	64	12	25	16.00	2.71
Height (inches)	36	58	73	63.68	2.62	64	60	75	68.93	2.72
Weight(lb)	36	100	200	136.25	26.32	64	125	239	173.43	25.26
BMI* (kg/m^2)	36	17.57	37.17	23.65	4.68	64	20.80	35.86	25.66	3.46
Race	**Frequency (%)**					**Frequency (%)**				
Asian / Pacific Islander	23	35.94				15	41.67			
Black not Hispanic	5	7.81				2	5.56			
Hispanic or Latino	11	17.19				7	19.44			
Other/Unknown	1	1.56				1	2.77			
White not Hispanic	24	37.50				11	30.56			

***Education Calculated as High school (12 years) + Years of college or other. *BMI = (weight/height2) *703

1.6 Hypothesis and Research Questions

1.6.1 Overall Hypothesis

The overall hypothesis of this dissertation research is that sex has an effect on the occurrence and intensity of diesel exhaust induced local airway and systemic inflammation, acute phase responses and self reported symptomatic responses and also on the metabolism of 1-nitropyrene with females expected to be responsive to diesel exhaust exposure than males.

The proposed formula of the overall hypothesis is shown as follows:

$$\textbf{Null Hypothesis Ho} = \mu(\Delta_{Males}) = \mu(\Delta_{Females})$$

$$\textbf{Alternate Hypothesis Hi} = \mu(\Delta_{Males}) \neq \mu(\Delta_{Females})$$

Where $\mu(\Delta_{Males})$ is the mean change in the outcome variables of male subjects measured after exposure to diesel exhaust relative to clean air and $\mu(\Delta_{Females})$ is mean change in the outcome variables of female subjects measured after exposure to diesel exhaust relative to clean air.

1.6.2 Research Question 1

Do sex differences exist in the occurrence and intensity of local airway inflammation between males and females as indicated by differences in the change in the concentrations of pro-inflammatory cytokines and protein mediators in the cells of the nasal mucosa and the lower respiratory tract after exposure to diesel exhaust relative to clean air?

a. Are there sex differences in the incidence and intensity of nasal inflammation as indicated by differences in the mean changes in the concentrations of IL-6, IL-8, TNF-α and proteins measured in nasal lavage 24 hours post exposure to diesel exhaust relative to clean air between males and females?

Hypothesis 1.1: There is a difference in the expression of cytokines and protein mediators by the nasal mucosal cells of males and females after exposure to diesel exhaust relative to clean air with females showing a greater local inflammatory response in the nasal epithelium than males.

b. Are there sex differences in the incidence and intensity of inflammation in the lower respiratory tract as indicated by differences in the mean changes in the concentrations of IL-6, IL-8, TNF-α and proteins measured in induced sputum 6 hours post exposure to diesel exhaust relative to clean air between males and females?

Hypothesis 1.2: There is a difference in the expression of cytokines and protein mediators by the cells of the lower respiratory tract of males and females after exposure to diesel exhaust relative to clean air with females showing a greater local inflammatory response in the lungs than males.

1.6.3 Research Question 2

Do sex differences exist in the occurrence and intensity of systemic inflammation and acute phase responses between males and females as indicated by differences in the changes in the concentrations of pro-inflammatory cytokines, mediators and acute phase proteins in peripheral blood after exposure to diesel exhaust relative to clean air?

a. Are there sex differences in the incidence and intensity of systemic inflammation as indicated by differences in the mean changes of the levels of c-reactive proteins, fibrinogen, the absolute number and relative percentage of blood cells, number of platelets and the mean change in the concentration IL-6 in peripheral blood post exposure to diesel exhaust and relative to clean air between males and females?

Hypothesis 2.1: There is a difference in the expression of cytokines, blood cells, c-reactive protein and fibrinogen in peripheral blood after exposure to diesel exhaust relative to clean air between males and females with females showing a greater systemic inflammatory response than males.

1.6.4 Research Question 3

Do sex differences exist in self reported health symptoms collected after acute exposure to diesel exhaust relative to clean air between males and females?

a. Are there sex differences in self reported symptoms collected post exposure to diesel exhaust relative to clean air between males and females?

Hypothesis 3.1: There is a difference in self reported symptoms collected post diesel exhaust exposure relative to clean air between males and females with females reporting higher symptoms than males.

1.6.5 Research Question 4

Do sex differences exist in the metabolism of 1-nitropyrene as indicated by changes in the concentration of urinary 1-aminopyrene levels following diesel exhaust exposure relative to clean air between males and females?

a. Are there sex differences in urinary levels of 1-aminopyrene post exposure to diesel exhaust relative to clean air between males and females?

Hypothesis 4.1: There is a difference in the concentration of urinary levels of 1-aminopyrene after diesel exhaust exposure relative to clean air between males and females with females excreting a higher concentration of 1-aminopyrene than males.

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Chapter 2

Analysis of Sex Differences in Airway Inflammatory Response after Acute Diesel Exhaust Exposure

2.1 Abstract

Background: Inhalation of diesel exhaust in humans has been associated with local immune and inflammatory responses in the upper and lower respiratory airways. Studies have shown that there is a sexual dimorphism in immune response with females having a stronger immune reactivity than males.
Aims: To evaluate possible differences between females and males in local immune and inflammatory responses in the upper and lower respiratory airways after a controlled environmental exposure to diesel exhaust.
Methods: In a crossover design study, healthy human subjects were exposed to 300μg/m^3 of diesel exhaust and to clean air in random order for 1 hour each in a controlled environmental exposure chamber on two different days (≥1 week washout). Fluid from the lungs were collected from subjects via sputum induction 6 hours after each exposure and nasal mucosal cells and fluid were collected from subjects via nasal lavage 24 hours after each exposure. Cytokines (TNF-α, IL-6 and IL-8) and total protein were measured in sputum and nasal lavage. Statistical analysis was done using Mann-Whitney U Test.
Results: There was a difference between males and females in the concentrations of cytokines and total protein in the nasal lavage analyte and sputum after exposure to diesel exhaust relative to clean air exposure but except for TNF-α ($p = 0.051$) in the nasal lavage analyte, these observed sex differences were not statistically significant. In the nasal lavage analyte, males showed a diesel exhaust attributable mean increase in TNF-α concentration compared to a mean decrease in females.
Conclusion: Our results suggest that 24 hours after acute exposure to 300μg/m^3 of diesel exhaust, males have a stronger inflammatory response in the upper airway than females as indicated by a statistically significant difference in nasal TNF-α concentration between males and females; with males showing a mean increase in TNF-α concentrations compared to a mean decrease in females.

2.2 Introduction and Background

Air pollution is a major source of nasal and airway irritation. Diesel exhaust is a significant contributor to ambient air pollution and the largest single source of airborne particulate matter (PM) in many urban areas (Riedl and Diaz-Sanchez 2005). Across populations, exposures may be acute or chronic and the exposure amounts may be either high or low depending on the temporal and spatial distribution of diesel exhaust sources.

Inhalation of diesel exhaust is the primary route of exposure and has been shown to cause various health effects. Overall, chronic inhalation exposure to diesel exhaust is

likely to pose a lung cancer hazard to humans, as well as damage the lungs in other ways depending on the length and intensity of the exposure (USEPA HAD 2002; Donaldson et al. 2001). While acute exposure to diesel exhaust can cause acute irritation (e.g., eye, nose, throat, bronchial), neurophysiological symptoms (e.g., lightheadedness, nausea), inflammatory responses in the airway and the lungs; respiratory symptoms (cough, phlegm), there is also evidence for an immunologic effect (the exacerbation of allergenic responses to known allergens and asthma-like symptoms) and cardiovascular effects (USEPA HAD 2002; Donaldson et al. 2001).

2.3 Toxicokinetics of Diesel Exhaust in Human Nasal and Airway Passages

The biological effects of diesel exhaust particles (DEPs) are a function of their deposition and clearance in the airway (USEPA HAD 2002). DEPs are made up of fine particles (diameter <2.5μm), including a subgroup with a large number of ultrafine particles (diameter <0.1 μm) (USEPA HAD 2002). In humans, inhalation can occur via the nose, mouth or both and upon inhalation, the deposition of DEPs will occur throughout the respiratory tract (see figure 1 below). Primarily, the deposition pattern of DEPs in the airway is influenced by particle size but there are other factors that can alter the deposition of DEPs in humans, including sex, age, particle size and pre-existing respiratory tract disease (USEPA HAD 2002).

Following deposition, the subsequent clearance of DEPs is affected by the solubility of the particles. DEPs are poorly soluble particles and so they are primarily cleared by non absorptive process which involves the removal of the intact particles. This means that when there is an override of the clearance mechanisms of the nasal and airway

passages, this will lead to not only prolonged contact between the cells and the DEP's but also to other organic compounds that are adsorbed onto the large surface area of DEPs as well as small amounts of sulfate, nitrate, metals, and other trace elements (USEPA HAD 2002).

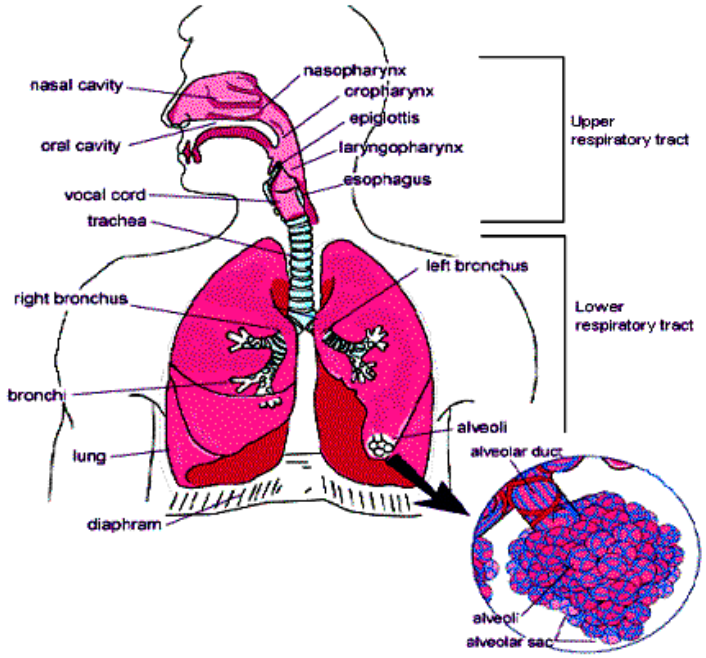


Figure 2.1. The Human Respiratory System
Source: http://www.ec.gc.ca/cleanair-airpur/default.asp?lang=En&n=FCAFC364-1 (Reproduced with permission from Environment Canada)

2.3.1 Hypothesized Mechanism of Adverse Reactions to Inhaled Diesel Exhaust

It is hypothesized that this prolonged contact between DEPs and the cells of the airway gives rise to the cascading events that follow diesel exhaust exposure and which leads to the adverse health effects that have been observed. These cascading events are thought to be heavily influenced by pro-inflammatory cytokines and chemokines which are released from affected cells in response to the immune and local inflammation response caused by diesel exhaust and that these cytokines also have an added

inflammatory effect on the surrounding cells and that they also modify the production of acute phase proteins by the liver thereby leading to adverse cardiovascular effects.

Some studies that have been done to date, have confirmed that inhalation of diesel exhaust does produce a local immune and inflammatory response (Nightingale et al 2000, Diaz et al 1996 and Nordenhall et al 2000) in the upper and lower respiratory airways. Also, the roles of pro-inflammatory cytokines in increasing inflammation in response to diesel exhaust exposure has been well documented (van Eeden et al. 2001), but the initial cause of the inflammatory process which stimulates the release of these cytokines is still under debate.

One leading thought is that the inflammation is instigated by oxidative stress (Furuyama et al. 2006; Diaz-Sanchez and Riedl 2005; Salvi and Holgate1999) caused by the subsequent prolonged contact of the metals, PAHs and quinines contained in diesel exhaust particles (DEPs) with the epithelial and macrophage cells of the nose and lungs when the natural lung safety mechanisms become overwhelmed.

Normally, the lungs are able to deal with the isolation, neutralization and removal of inhaled diesel exhaust and other xenobiotics or air pollutants via various mechanisms, but these natural defense mechanisms can often become overwhelmed for a variety of reasons including illness or high burden of pollutants and this can then lead to the adverse health effects mentioned above (Salvi and Holgate 1999). The epithelial and macrophage cells are the initial target cells for particle interaction and deposition and thus are thought to be the initial site of toxicity (Salvi et all 1999).

This breakdown in the safety mechanism then leads to the generation of reactive oxygen species (ROS) that activate and mobilize immune and inflammatory cells via

oxidative stress causing the activation of redox-sensitive transcription factors and thus changing the expression of many pro-inflammatory cytokines (Ritz et al 2005, van Eeden et al 2001). These pro-inflammatory cytokines include interleukin 6 (IL-6), interleukin 1β (IL-1β), Tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), transforming growth factor β^2, interleukin 8 (IL-8) (Gabay C. et all 1999). This oxidative stress initiated inflammation is thought to either directly cause or trigger other mechanisms that are behind the effects that have been seen in in-vitro and in-vivo studies.

Usually the cells of the airway are able to effectively reduce the cycle of ROS generation and inflammation using the cells natural defenses but the oxidative stress that leads to the immune and inflammatory response usually happens when these defenses are overwhelmed (Riedl and Diaz-Sanchez 2005) for a variety of reasons including illness or high burden of pollutants and as mentioned before, these can cause an override of the clearance mechanisms of the nasal and airway passages thus leading to inflammation and other adverse health effects (Salvi and Holgate 1999).

2.4 Nasal Lavage and Sputum Induction Studies

In humans, experimental and clinical evidence from diesel exhaust exposure studies that have been done so far to look at the ability of inhaled DEPs to initiate inflammation in the nasal and airway passages have shown varying responses. Studies done using high concentrations of DEPs have provided evidence of airway inflammation and studies done using lower concentrations of DEPs have provided less clear-cut inflammatory responses (Behndig et al 2006, Diaz-Sanchez et al 1996, Salvi et al 2000,

Nightingale et al 2000, Nordenhall et al 2000, Sydbom et al 2001 and Takizawa et al 2000).

Particularly, with regards to the mechanism of diesel exhaust induced airway inflammation, these studies have also helped to confirm that acute cell mediator and cytokine responses along with the enhanced expression of vascular adhesion molecules in the airway mucosa could represent an early stage in the inflammatory response following diesel exhaust exposure (Sydbom et al 2001). This mechanism also might be important in the development of the diesel exhaust induced airway inflammation (Sydbom et al 2001). This early stage local inflammatory response is part of the body's innate immune response to irritation or injury caused by inhaled foreign particles like DEPs.

2.5 Sex Differences in Innate Immune Response

Innate immunity is the body's non-specific first active line of defense to harmful foreign agents. It is designed to eliminate or control the injury or infection caused by these agents by phagocytosis, the release of enzymes to degrade these agents and the activation of the adaptive immune response via cytokine release and cell to cell interactions, which results in a more specific and robust immune response (Bird et al 2008 and Casarett and Doulls 2001). There is an established sexual dimorphism in immune response with females having a stronger immune reactivity than males (Da Silva 1995). This suggests that sex is an important determinant of the amplitude of the innate immune response especially since the effects of cytokines can be inhibited or enhanced by sex hormones (Gabay et al 1999).

In-vitro, sex hormones have been shown to modulate the production of different pro-inflammatory cytokines that are involved in immune responses like IL-6, thus indicating the potential role of sex hormones in the sexual dimorphism of the immune system (Da Silva 1995). While in-vivo the interactions between sex hormones and other immunomodulatory factors like glucocorticoids are thought to mediate an indirect action of sex hormones on the immune system (Da Silva 1995). Therefore they have the potential to cause variations by sex on the onset of local inflammation as part of the body's initial innate immune response that has been demonstrated to happen in response to diesel exhaust exposure. For example, in-vivo, estrogen has a rapid non-genomic effect that protects organs from damage and attenuates physiological insult induced inflammation (Bullard et al 2010).

So with regards to airway induced inflammation, either via the direct modulation (suppression or expression) of cytokines or via the indirect modulation of the immune system, it can be hypothesized that sex hormones have the potential to cause a variation in the local airway immune and inflammatory response of females and males in response to diesel exhaust exposure.

Therefore, the purpose of this study is to determine whether there are sex differences in the occurrence and intensity of local airway inflammation of females and males as indicated by the potential variation in the levels of soluble proteins and pro-inflammatory cytokines in nasal lavage and sputum induction samples collected after exposure to diesel exhaust relative to clean air.

2.6 Materials and Methods

2.6.1 Data Collection

The data that were used for this study are part of the datasets generated from a previous study of diesel exhaust effects. The study was funded by the Department of Defense (DOD) through a grant to the University of Medicine and Dentistry of New Jersey (UMDNJ) (Grant # DAMD17-03-1-0537; Principal Investigator: Dr. Nancy Fiedler). The study was done using a human subject protocol that was HIPAA compliant and approved by the UMDNJ Institutional Review Board.

2.6.2 Subjects Demographic Data

110 healthy non-smoking men and women between the ages of 18 and 50 were recruited from the local community in Piscataway, New Jersey, based on the established study criteria; 100 subjects completed the study. Out of these subjects, 18 subjects consented and provided viable nasal lavage samples and 36 subjects consented and provided viable sputum samples for analysis.

Table 2.1. Demographic and Baseline Subject Characteristics for Nasal Lavage Subjects.

	Females N = 7				Males N = 10			
	Min	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**
AGE (years)	19	43	25.71	8.24	18	33	23.90	5.02
Height (inches)	62	65	63.50	1.35	65	73	68.05	2.46
Weight(lb)	104	200	158.57	38.00	125	192	159.50	21.22
Education (years)*	12	17	15	1.83	12	20	16	2.67
Respiration	14	20	16.29	1.80	14	20	16.80	2.35
Temperature ^{o}F ^{M}	97.9	99.4	98.52	0.54	98.1	99.1	98.38	0.31
Pulse	60	88	72	9.24	60	92	69.60	10.49
Race	**Frequency (%)**				**Frequency (%)**			
Asian / Pacific Islander	1 (14.29)				5 (50)			
Black not Hispanic	1 (14.29)				1 (10)			
Other/Unknown	1 (14.29)				0 (0)			
White not Hispanic	4 (57.13)				4 (40)			

One subject had an incomplete dataset and so was excluded for the analysis, so Total N = 17.
^{M} = some subjects had missing data N = 6 for Females and N = 9 for Males.
*****Education Calculated as High school (12 years) + Years of college or other

Table 2.2. Demographic and Baseline Subject Characteristics for Sputum Induction Subjects.

	Females N = 9				Males N = 27			
	Min	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**
AGE (years)	18	44	25.89	10.07	18	41	22.93	4.60
Height (inches)	60	66	62.92	2.18	64	73	69.07	2.62
Weight(lb)	112	200	138.22	33.42	140	236	180.80	28.79
Education (years)*	12	16	14.22	1.30	12	20	15.89	2.12
Respiration [M]	14	20	16	2.0	12	40	16.74	5.03
Temperature ^{o}**F** [M]	98	99	98.43	0.64	96	99	98.00	0.81
Pulse [M]	54	76	67.43	7.89	48	84	68.30	9.49
Race	**Frequency (%)**				**Frequency (%)**			
Asian / Pacific Islander	3 (33.34)				12 (44.44)			
Black not Hispanic	1 (11.11)				1 (3.70)			
Hispanic or Latino	1 (11.11)				5 (18.52)			
Other/Unknown	0 (0)				1 (3.70)			
White not Hispanic	4 (44.44)				8 (29.63)			

[M] = some subjects had missing data: For Temperature N = 6 for Females and N = 26 for Males; For Pulse and Respiration N = 6 for Females.

*****Education Calculated as High school (12 years) + Years of college or other

2.6.3 Exposure Conditions and Experimental Design

A crossover study design was used in which each subject acted as their own controls and underwent two exposure conditions at two separate times in random order with a minimum one week wash out period between exposures; one was to diluted diesel exhaust normalized to PM_{10} concentration of 300µg/m^3 and the other was to filtered clean ambient air of Busch campus in Piscataway, a suburban campus of Rutgers University. Both exposure conditions were 1 hour in duration and took place at the Environmental and Occupational Health Sciences Institutes' (EOHSI) Controlled Environmental Facility (CEF).

There were no significant differences in the exposure conditions for all subjects. Overall, the concentration of PM_{10} that the subjects were exposed to was 276±13µg/m^3 which was close to the target concentration of 300µg/m^3.

Table 2.3. Mean Air Characteristics and Composition.

	Diesel Exhaust	**Clean Air**
PM_{10} (µg/m^3)	276 ± 13	6 ± 5
NO_X (ppm)	3.63 ± 0.90	0.02 ± 0.02
CO (ppm)	3.79 ± 0.76	0.91 ± 0.21
1 Nitropyrene (ng/m^3)	2.68 ± 0.51	<0.06

2.6.4 Nasal Lavage Sample Collection

The nasal lavage procedure was adapted from Koren & Devlin (1992) and was performed 24 hours after each exposure. While seated, the subjects were asked to tilt their heads backwards at an approximate 45 degree angle. Using a sterile, needle-less syringe with a blunt plastic tip, 5 ml of warmed (body temperature, 37 degree centigrade), sterile, normal saline was instilled into one nostril. Subjects were asked to hold their breath and perform a partial swallow in order to raise the soft palate and hold the fluid in the nasal cavity. After retaining the saline for 10 seconds, the subjects were then asked to tilt their heads forward and allow the fluid to drain passively into a sterile collection cup for 30 seconds. This procedure was repeated in the other nostril. Each nostril was washed two times.

2.6.5 Nasal Lavage Sample Analysis

After collection, a portion of the sample was run through syringe-mounted filters to remove mucous and cell debris. This resulting filtrate was frozen immediately on dry ice in aliquots and then stored at -80 C. for subsequent soluble protein and cytokine analysis using a commercially available enzyme-linked immunosorbent assay (ELISA) kits. This was done consistent with the work of Diaz-Sanchez et al in 1996.

2.6.6 Sputum Sample Collection

Sputum induction was performed at about 6 hours post-exposure. Subjects were pre-medicated with an inhaled salbutamol (200 µg) to inhibit possible airway constriction during the sputum induction. Using the method described by Pin et al. (1992) and modified by Pizzichini (1996), subjects inhaled increasing concentrations of saline (3, 4,

and 5%) for 7 minutes, each through a mouthpiece without a valve or nose clip. The aerosol was generated by an Ultraneb 99 ultrasonic nebulizer with an output of 0.87 ml/min and aerodynamic mass median diameter of 5.58 um. After each period of inhalation, a forced expiratory volume after 1 second (FEV_1) was measured for safety. Then subjects were asked to rinse their mouths, swallow some water and blow their nose in order to minimize contamination by saliva or post nasal drip. Subjects were then asked to cough sputum into a sterile container for sample collection.

2.6.7 Sputum Sample Analysis

Sputum samples were processed as soon as possible, usually within 2 hours after expectoration. Sputum was poured into a petri dish. Using blunt forceps, all portions that appeared to be free of salivary contamination (up to 1 gm) were placed in a tared 15 ml polystyrene tube and weighed. Four times the volume of 0.1% dithiotheitol (DTT) in distilled water was added to dissociate disulfide bonds in the mucous. The mixture was vortexed for 15 sec, and then rocked on a platform for 15 minutes, to ensure mixing. To terminate the reaction, 4 volumes of phosphate buffered saline (PBS) were added and the mixture was rocked for 5 additional minutes. The suspension was filtered through a 48 µm nylon gauze to remove cell debris and mucus. The resulting clear suspension was then centrifuged at 790 x g for 10 min and the supernatant was aspirated and stored in six Eppindorf tubes at –70 C for later assay.

The pellet was re-suspended in a volume of PBS, 200 to 600 µl depending on macroscopic size, and the total cell counts were determined using a hemocytometer. Cell viability was determined with the trypan blue exclusion method. The total and absolute

number of cells per milligram processed sputum was calculated. Three samples of 75 µL of the cell suspension adjusted to 1 x 10^6 /ml were cytospun at 150 x g for 6 min. Slides were air dried and stained with Wright's Giemsa stain. Nonsquamous cells (n = 400) were counted. The results were expressed as percentage of total nonsquamous cells. The levels of soluble proteins, IL-6, IL-8, and TNF-α in the induced sputum were measured using commercially available ELISA plates (R&D Systems, UK).

2.6.8 Airway Markers of Inflammation

Only 17 out of the 18 subjects that consented for nasal lavage had a complete set of samples that were collected 24 hours after both exposures. Therefore the subject with the missing sample was excluded from the analysis. All 36 subjects that consented for sputum induction had a complete set of samples and so they were all included in the analysis. Total protein and cytokine (IL-6, IL-8 and TNF-α) concentrations as measured from the nasal lavage and sputum induction samples were used as markers of local upper and lower airway inflammation.

In an attempt to control for the possible dilution of the nasal lavage and sputum samples, the cytokine concentrations were normalized using the total protein concentration. Since this is not yet an established method, all analysis was done using the actual concentrations of cytokines measured in nasal lavage and sputum analytes and the normalized concentrations.

2.7 Statistical Methods

Descriptive statistical analysis, histograms and box plots were used to evaluate the distribution of the airway inflammatory marker variables (soluble proteins and cytokines) and the results indicated that the variables were not normally distributed so non parametric statistical methods were used for all further analysis.

2.7.1 Calculation of Response Measures

The main objective of this study is to look at sex differences in the occurrence and intensity of local airway inflammation as indicated by the potential variation in the levels of upper and lower airway markers of inflammation (soluble proteins and cytokines) after exposure to diesel exhaust relative to clean air. Therefore, for the protein and cytokines, the response measure was the difference between the concentrations measured in nasal lavage and sputum samples post diesel exhaust exposure and post clean air exposure.

Response Measure = Protein/Cytokine $_{DE-CA}$ = Protein/Cytokine $_{DE}$ - Protein/Cytokine $_{CA}$

The inflammatory response measures were not normally distributed. Therefore to address the aim of the paper, the non parametric Mann-Whitney U Test was performed to see if there was a significant difference in the inflammatory response measures between sex groups (male, females) in nasal lavage and sputum induction samples.

All statistical analysis for this paper were done using SPSS software, Version 17.0 (SPSS inc., Chicago IL) and SAS software, Version 9.1 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA). Also, some graphs were created using Microsoft Excel 2007. P-values less than or equal to 0.05 were considered to be significant.

Table 2.4. Difference in Soluble Proteins and Cytokines Measured in the Nasal Lavage Analyte 24 Hours after Exposure to Clean Air and Diesel Exhaust.

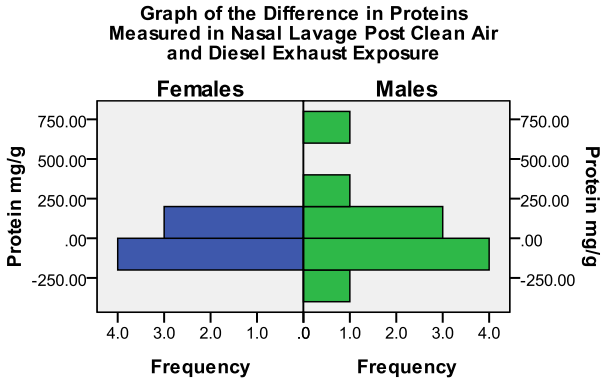
	Females N = 7				Males N = 10			
Variables	**Min.**	**Max.**	**Mean**	**S.D.**	**Min.**	**Max.**	**Mean**	**S.D.**
Protein	-138.79	82.21	-19.42	71.23	-327.77	689.70	58.29	268.32
TNF-α	-55.35	48.96	-3.80	33.16	-5.82	120.96	34.13	34.96
IL-6	-8.85	1.03	-1.65	3.48	-7.10	98.80	9.00	31.72
IL-8	-44.75	51.69	8.45	32.61	-56.02	136.53	30.93	58.28
TNF-α/Protein	0.15	1.96	0.685	0.61	0.11	0.62	0.39	0.20
IL-6/Protein	-0.08	0.02	-0.024	0.04	-0.06	0.28	0.02	0.10
IL-8/Protein	-0.31	0.44	-0.04	0.29	-0.86	0.26	-0.11	0.38

Data as presented = Concentrations measured after DE – Concentrations measured after CA. Protein concentrations are presented as µg/ml. The cytokines concentrations are presented as pg/ml and Normalized cytokine concentrations are presented as mg/g of total protein.

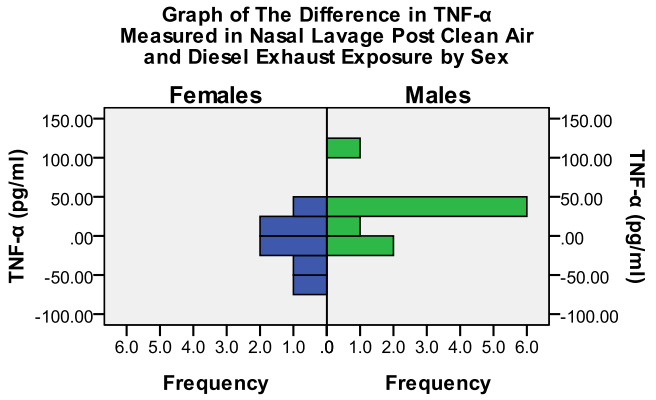
Table 2.5. Difference in Soluble Proteins and Cytokines Measured in Sputum 6 Hours after Exposure to Clean Air and Diesel Exhaust.

	Females N = 9				Males N = 27			
Variables	**Min.**	**Max.**	**Mean**	**S.D.**	**Min.**	**Max.**	**Mean**	**S.D.**
Protein	-7143.00	2070.73	-880.86	2614.21	-8166.92	49743.00	1334.33	10487.81
TNF-α	-152.47	439.34	52.49	165.16	-554.88	333.18	14.20	154.64
IL-6	-71.95	78.77	24.77	44.70	-62.01	169.53	3.07	45.14
IL-8	-28.03	242.68	93.61	81.00	-345.74	469.12	13.29	179.52
TNF-α/Protein	0.00	0.05	0.01	0.02	-0.07	0.04	0.00	0.02
IL-6/Protein	0.00	0.01	0.00	0.00	0.00	0.03	0.00	0.01
IL-8/Protein	0.00	0.02	0.01	0.01	-0.03	0.05	0.00	0.02

Data as presented = Concentrations measured after DE – Concentrations measured after CA. Protein concentrations are presented as µg/ml. The cytokines concentrations are presented as pg/ml and Normalized cytokine concentrations are presented as mg/g of total protein.



Graph 2.1.



Graph 2.2.

2.8 Results

2.8.1 Study Aim: Interaction Effect of Diesel Exhaust Exposure and Sex.

Nasal Lavage Analytes

With the exception of TNF-$\alpha_{DE\text{-}CA}$, the Mann-Whitney test of the effect of diesel exhaust exposure by sex (males vs females) revealed no statistically significant two way interaction effect (exposure*sex) on the concentrations of the upper airway inflammatory response measures (see table 6a below). There was a statistically significant two way interaction (exposure*sex) effect on the concentration of TNF-$\alpha_{DE\text{-}CA}$ ($p = 0.051$) between females (Md = -1.20, N = 7) and males (Md = 31.93, N = 10). The results indicated that diesel exhaust caused a mean decrease in TNF-α concentrations in females (TNF-$\alpha_{DE\text{-}CA}$ = -1.27) and a mean increase in TNF-α concentrations in males (TNF-$\alpha_{DE\text{-}CA}$ = 31.93) relative to clean air.

Table 2.6a. Two Way Interaction Significance Test Results and Nasal Analyte Concentrations for the Difference in the Concentrations of Proteins and Cytokines Measured in Nasal Lavage Samples 24 Hours after Exposure to Diesel Exhaust and Clean Air.

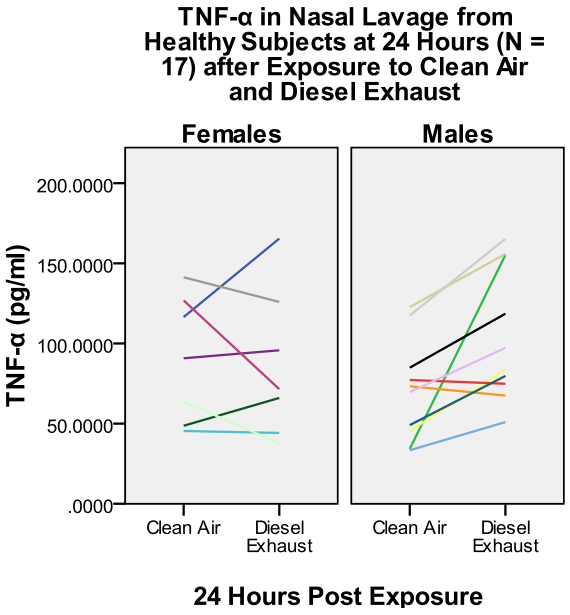
	Females		Males				
Variables	**Mean**	**Median**	**Mean**	**Median**	**Mann-Whitney U**	**p-value**	**r#**
Protein	-19.42	-2.14	58.29	29.89	28.00	0.50	-0.17
TNF-α	-3.80	-1.20	34.13	31.93	15.00	0.051	-0.47
IL-6	-1.65	0.00	9.00	1.14	22.50	0.22	-0.30
IL-8	8.45	9.60	30.93	26.67	30.00	0.63	-0.12
TNF-α/Protein	0.685	0.62	0.39	0.41	22.00	0.21	-0.31
IL-6/Protein	-0.024	0.00	0.02	0.00	24.00	0.28	-0.26
IL-8/Protein	-0.04	-0.10	-0.11	-0.00	35.00	1.00	0.00

Protein concentrations are presented as µg/ml. The Cytokines concentrations are presented as pg/ml and normalized cytokine concentrations are presented as mg/g of total protein. # r = Effect size, calculated as z score / square root of N (Cohen 1988). DE = diesel exhaust and CA = Clean air.

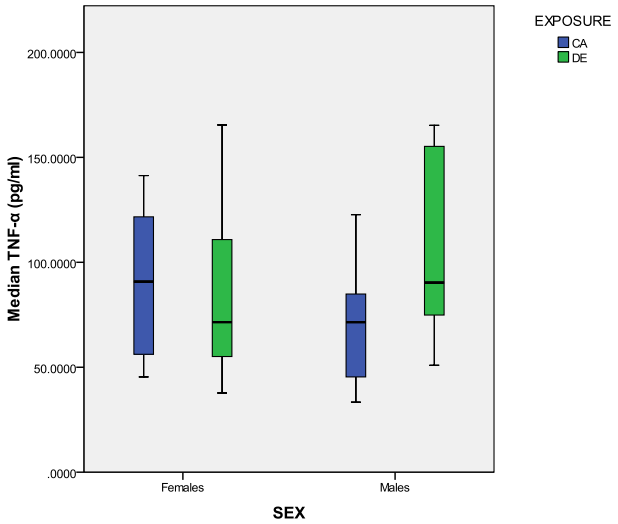
Table 2.6b. Mean Ranks for TNF-α $_{DE-CA}$ Measured in Nasal Lavage Samples 24 Hours after Exposure to Diesel Exhaust and Clean Air.

	Sex	**Mean Rank**	**Sum of Ranks**
TNF-α	Females	6.14	43.00
	Males	11.00	110.00
	Total		

Mean ranks calculated using Mann U Whitney statistical procedure.



Graph 2.3.
The horizontal Lines connect TNF-α concentrations from Nasal Lavage samples collected from each subject after both exposures by sex (Females N = 7 and Males N = 10).



Graph 2.4. Boxplot of Median TNF-α Concentrations by Sex and Exposure.

For most of the remaining cytokines and the total protein, although not statistically significant, the mean difference (e.g. IL-8_{DE-CA}) was lower for females than in males after diesel exhaust exposure relative to clean air.

Sputum Analytes

The Mann-Whitney test of the effect of diesel exhaust exposure by sex (males vs females) showed no statistically significant two way interaction effect (exposure*sex) on the concentrations of the lower airway inflammatory response measures.

Table 2.7. Two Way Interaction Significance Test Results and Sputum Analyte Concentrations for the Difference in the Concentrations of Proteins and Cytokines Measured in Sputum Induction Samples 6 Hours after Exposure to Diesel Exhaust and Clean Air.

	Females		Males					
Variables	**Mean**	**Median**	**Mean**	**Median**	**Mann-Whitney U**	**Z score**	**p-value**	**r#**
Protein	-880.86	-170.65	1334.33	-481.08	118.00	-0.13	0.90	-0.02
TNF-α	52.49	6.96	14.20	3.71	112.00	-0.35	0.73	-0.06
IL-6	24.77	29.34	3.07	4.80	78.00	-1.59	0.11	-0.26
IL-8	93.61	86.87	13.29	3.48	71.00	-1.85	0.065	-0.31
TNF-α/Protein	0.01	0.00	0.00	0.00	107.00	-0.53	0.60	-0.09
IL-6/Protein	0.00	0.00	0.00	0.00	78.00	-1.59	0.11	-0.26
IL-8/Protein	0.01	0.01	0.00	0.00	88.00	-1.22	0.22	-0.20

Protein concentrations are presented as µg/ml. The Cytokines concentrations are presented as pg/ml and normalized cytokine concentrations are presented as mg/g of total protein. # r = Effect size, calculated as z score / square root of N (Cohen 1988). DE = diesel exhaust and CA = Clean air.

Although not statistically significant, for almost all of the cytokines and the total protein measured in sputum, the mean difference (e.g. IL-8_{DE-CA}) was higher in females than in males after diesel exhaust exposure relative to clean air.

2.9 Discussion

Inflammation is a complex biological response to numerous stimuli like xenobiotics and is marked by vasodilatation and increased capillary permeability (first phase of inflammation); leukocytic and phagocytic cell infiltration (second phase of inflammation); and tissue degeneration and fibrosis (third phase of inflammation), all of which serve as a mechanism to initiate the elimination of the xenobiotic and of any damaged tissue (Suzer 2007).

Airway epithelial cells which are made up of nasal and bronchial epithelium are the first cells that come into contact with a variety of xenobiotics such as DEP's. Apart from acting as a physicochemical barrier they also play an important role in initiating and enhancing host defense mechanisms by producing and releasing a variety of inflammatory mediators like pro-inflammatory cytokines, chemokines and other inflammatory mediators that are involved in airway inflammatory responses (Purokivi et al 2001 and Takizawa et al 2000).

Cytokines are soluble glycoproteins that are released by living cells to help regulate cell functions (’Stvrtinová et al 1995). The central role of cytokines is to regulate immune response by controlling the direction, amplitude and duration of immune responses and they also help to control the remodeling of tissues (’Stvrtinová et al 1995). TNF-α and IL-1ß are the principal first order pro-inflammatory mediators as they initiate the amplification and release of other cytokines and they are also involved in the repair of processes in the nasal mucosa (Ertan et al 2007 and Purokivi et al 2001).

Cytokines are mostly produced by activated macrophages and can cause a cascade of secondary cytokines like chemokines that attract leukocytes like neutrophils and other white blood cells to areas of inflammation or immune response as part of the second

phase of inflammation (Ganong 1999 and Suzer 2007). There are two main groups of chemokines i.e. α and β and the α chemokines e.g IL-8 act to attract and activate neutrophils and are mainly involved in the acute inflammatory response (Suzer 2007 and ’Stvrtinová et al 1995) such as has been shown to occur in the airway after exposure to diesel exhaust.

This diesel exhaust induced airway inflammation is time dependent (Nordenhall et al 2000), meaning that in a controlled environmental study, the levels of these cytokines and mediators will depend on when the sampling is done in relation to the exposure event. One thing that could potentially have an effect on the time kinetics of diesel exhaust induced airway inflammation is sex. This is because sex hormones are known to regulate immunity creating a dichotomy in immune response to injury between the sexes leading to differences in immune cell activation, infiltration, and cytokine production during and after injury (Bird et al 2008).

Female sex hormones especially estrogen, stimulate immune response and male sex hormones especially testosterone are immunosuppressive (Ertan et al 2007 and Kovacs et al 2002) thus implying that sex is an important determinant in the amplitude and intensity of the innate immune response (Imahara et al 2005) that is involved in the initiation of inflammation in response to diesel exhaust exposure. This means that in addition to seeing a variation in the expression of the inflammatory mediators and markers by time, one could also expect to see a variation in them by sex as well.

The lung is the main target organ of diesel exhaust and so it can then be hypothesized that in response to diesel exhaust exposure (depending on the concentration and duration of the exposure), upon initiation of the cells of the lungs, females will show

a stronger and faster up-regulation of the creation and secretion of pro-inflammatory cytokines. This is turn will stimulate the synthesis and release of chemokines leading to the infiltration of leukocytes and hopefully the isolation, removal or degradation of the DEPs in order to prevent tissue damage. So in a controlled environmental study, this stronger and faster response in females would most likely enable them to move quickly from one phase of inflammation to the other ahead of males possibly leading to the variation in the amplitude and intensity of inflammatory markers by sex that was seen in this study.

After subtracting the concentration of the pro-inflammatory markers measured in sputum 6 hours post clean air exposure from those measured 6 hours after diesel exhaust exposure, there was a mean increase in inflammatory cytokines (TNF-α and IL-6) and chemokines (IL-8) for both females and males with females having higher mean difference levels of TNF-$\alpha_{\text{DE-CA}}$ ($p = 0.73$), IL-$6_{\text{DE-CA}}$ ($p = 0.11$) and IL-$8_{\text{DE-CA}}$ ($p = 0.065$) than males although these observations were not statistically significant. Despite this lack of statistical significance, the fact that all three of these cytokines were observed to be much higher in females than in males (e.g. IL-$8_{\text{DE-CA}}$ was approximately seven times higher in females) could reflect a stronger and faster response of females in response to diesel exhaust exposure relative to males. However, due to the fact that sputum samples were only measured at one time point (6 hours after exposure) we cannot accurately speculate about whether the observed differences are a reflection of kinetic differences in response to diesel exhaust exposure between males and females.

Diesel exhaust particles range from fine to ultrafine particles (UFPs) and upon inhalation, it is the UFPs that are thought to penetrate the lung and elicit the effects

observed above in the lungs while the larger particles are thought to induce upper respiratory effects. Therefore the observed higher increase in cytokine concentrations in females relative to males after diesel exhaust exposure relative to clean air could also indicate that females are more sensitive to UFPs of diesel exhaust than males. This is similar to what was observed by Franklin et al in 2007. They looked at the association between UFPs in the environment and both all-cause and specific cause mortality in 27 US communities between 1997 and 2002 and found suggestive evidence that women may be more susceptible to UFPs effects than men (Franklin et al 2007).

On the other hand, the significant increase in TNF-α in nasal fluid and the observed higher increase in the other cytokines in males compared to females (which were not statistically significant) 24 hours after diesel exhaust exposure relative to clean air suggests that males may be more sensitive than females to the fine particles of diesel exhaust in the upper airways. This is probably due to a greater diesel exhaust induced oxidative stress damage to the nasal epithelial cells of males relative to females owing to the potential protective effects of estrogen in females reducing leukocyte activity and exerting antioxidant effects on the nasal epithelial cells (Fortoul et al 2004). This idea is plausible because in 2004 Fortoul et al observed genotoxic damage to nasal epithelium and leukocytes which was more severe in males compared with females exposed to air pollution that was high in ozone. The potential antioxidant effect of estrogen in the upper airway needs to be studied further in order to better elucidate its mechanism of action especially with regards to the airway's epithelium.

2.9.1 Study limitations

The observations of this study need to be studied with larger populations of females and males especially since the sample size used for this study was relatively small and uneven. As an example, a power analysis using the observed mean and standard deviations of this study indicates that with the study sample size (9 for females and 27 for males) the likelihood to detect a 5% sex difference in IL-6 in sputum is approximately 0.10. This means that at the observed effect sizes a total sample size of 368 subjects will be needed to achieve an acceptable power of 0.70 to detect sex differences in IL-6 concentrations in sputum at an alpha level of 0.05.

In addition, a potential source of error that was not investigated was the possible additional exposure of subjects to DEPs outside of the exposure chamber during their diverse daily activities.

Although these findings need to be further investigated for confirmation they do have an important implication for the selection of subjects for diesel exhaust controlled environmental studies. Raising the question of whether sex should be controlled for in such studies. Combining the sexes and not controlling for their potential effect could be one of the reasons why as previously mentioned there have been some contrasting results in some studies that have been done so far especially to lower exposures levels of diesel exhaust. So when interpreting the data from these studies, the influence of sex needs to be taken into account.

2.10 Conclusions

In healthy volunteers, inhalation of 300μg/m^3 of diesel exhaust for an hour in a controlled environmental study showed a variation by sex in the occurrence and intensity of local airway inflammation as indicated by differences in the concentrations of airway inflammatory markers measured in nasal lavage fluid and induced sputum after diesel exhaust exposure compared to clean air. Six hours after the exposure to diesel exhaust relative to clean air, females tended to show a higher increase in lower airway inflammatory markers compared to males. Twenty four hours after exposure to diesel exhaust relative to clean air, males showed a significant increase in TNF-α concentration in nasal lavage compared to a decrease in females. Males also tended to have higher concentrations of other upper airway inflammatory markers in nasal lavage compared to females twenty four hours after exposure to diesel exhaust relative to clean air.

Although not statistically significant (except for TNF-α) the observations of this study suggests a sex difference in the initial site of diesel exhaust toxicity (upper airway in males versus lower airway in females) that may be due to the possibility that males may be more sensitive to effects of the fine particles of diesel exhaust which tend to deposit in the upper airways while females may be more sensitive to the effects of the ultra fine particles of diesel exhaust which tend to deposit in the lower airways.

Appendix A

Table A1. Nasal Analyte Concentration of Soluble Proteins and Cytokines 24 Hours after Exposure to Clean Air and Diesel Exhaust.

	Clean Air				Diesel Exhaust			
	Females N = 7							
Variables	**Min.**	**Max.**	**Mean**	**S.D.**	**Min.**	**Max.**	**Mean**	**S.D.**
Protein	31.48	509.78	182.78	165.33	61.37	370.99	163.36	102.94
TNF-α	45.39	141.32	90.45	38.91	37.73	165.43	86.65	45.98
IL-6	0.00	8.85	4.44	3.71	0.00	9.49	2.79	3.37
IL-8	35.65	161.48	86.28	48.10	45.25	201.45	94.73	65.89
TNF-α/Protein	0.10	4.49	1.132	1.50	0.18	2.05	0.73	0.63
IL6 Protein	0.00	0.10	0.04	0.04	0.00	0.05	0.02	0.02
IL8/Protein	0.32	1.28	0.65	0.33	0.31	0.97	0.61	0.26
	Males N = 10							
Variables	**Min.**	**Max.**	**Mean**	**S.D.**	**Min.**	**Max.**	**Mean**	**S.D.**
Protein	96.61	581.31	250.78	140.20	132.91	842.89	309.07	201.38
TNF-α	33.37	122.70	70.73	31.57	50.95	165.27	104.86	41.28
IL-6	0.09	11.14	4.18	4.07	0.00	102.34	13.18	31.42
IL-8	31.86	179.89	105.27	52.64	50.41	284.02	136.20	79.26
TNF-α/Protein	0.19	0.47	0.30	0.10	0.18	0.64	0.41	0.19
IL6/Protein	0.00	0.07	0.02	0.03	0.00	0.32	0.04	0.20
IL 8/Protein	0.12	1.53	0.58	0.51	0.25	0.89	0.47	0.22

Protein concentrations are presented as µg/ml. Cytokines concentrations presented as pg/ml and Soluble Protein concentrations presented as mg/g of total protein.

Table A2. Sputum Concentration of Soluble Proteins and Cytokines 6 Hours after Exposure to Clean Air and Diesel Exhaust.

	Clean Air				Diesel Exhaust			
	Females N = 9							
Variables	**Min.**	**Max.**	**Mean**	**S.D.**	**Min.**	**Max.**	**Mean**	**S.D.**
Protein	10475	19178	14018.24	3089.524	9925	16813	13137.38	2394.89
TNF-α	129.62	770.02	261.74	211.17	140.81	673.32	314.22	231.91
IL-6	85.84	360.03	141.72	87.47	109.77	389.38	166.49	87.57
IL-8	420.88	1013.14	623.64	0.02	433.84	1066.49	717.25	0.02
TNF-α/Protein	0.01	0.06	0.02	0.02	0.01	0.07	0.03	0.02
IL-6/Protein	0.007	0.03	0.01	0.01	0.01	0.03	0.01	0.01
IL-8/Protein	0.02	0.07	0.05	0.01	0.04	0.08	0.05	0.02
	Males N = 27							
Variables	**Min.**	**Max.**	**Mean**	**S.D.**	**Min.**	**Max.**	**Mean**	**S.D.**
Protein	8126	26428	13315.92	7917	76171	14650.25	12764.17	4417.169
TNF-α	137.89	1039.14	388.79	115.34	717.90	402.99	197.26	201.16
IL-6	85.20	186.72	127.62	82.48	298.32	130.68	40.21	30.41
IL-8	139.36	1260.06	590.52	236.61	1196.85	603.82	0.24	0.03
TNF-α/Protein	0.01	0.10	0.03	0.01	0.07	0.03	0.02	0.02
IL-6/Protein	0.00	0.02	0.01	0.00	0.04	0.01	0.01	0.00
IL-8/Protein	0.01	0.11	0.05	0.00	0.10	0.05	0.02	0.02

Protein concentrations are presented as µg/ml. Cytokines concentrations presented as pg/ml and Soluble Protein concentrations presented as mg/g of total protein.

Table A3. Nasal Lavage Median and Percentiles for Diesel Exhaust and Clean Air for All Subjects.

	Clean Air			Diesel Exhaust		
Variables	**25th**	**Median**	**75th**	**25th**	**Median**	**75th**
Protein	119.03	179.88	291.98	140.91	219.38	301.59
TNF-α	47.02	73.32	116.96	66.76	83.34	140.58
il_6	0.77	3.54	7.60	0.64	3.18	5.33
il_8	46.07	90.64	140.81	57.26	110.44	165.71
TNF-α/Protein	0.23	0.35	0.63	0.22	0.45	0.63
IL-6/Protein	0.00	0.02	0.06	0.00	0.01	0.02
IL-8/Protein	0.30	0.43	0.98	0.37	0.43	0.75

Protein concentrations are presented as µg/ml. Cytokines concentrations presented as pg/ml and Soluble Protein concentrations presented as mg/g of total protein.

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Table A4. Nasal Lavage Median and Percentiles for Diesel Exhaust and Clean Air for Females.

	Clean Air			Diesel Exhaust		
Variables	**25th**	**Median**	**75th**	**25th**	**Median**	**75th**
Protein	65.64	141.45	254.62	70.98	147.85	190.20
TNF-α	48.66	90.76	126.85	44.19	71.51	125.92
IL6	0.00	4.05	8.46	0.00	3.18	3.34
IL8	40.23	76.51	126.66	46.57	65.39	178.35
TNF-α/Protein	0.46	0.64	0.97	0.26	0.62	0.87
IL-6/Protein	0.00	0.03	0.09	0.00	0.02	0.05
IL-8/Protein	0.37	0.54	0.79	0.40	0.54	0.94

Protein concentrations are presented as µg/ml. Cytokines concentrations presented as pg/ml and Soluble Protein concentrations presented as mg/g of total protein.

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TableA5. Nasal Lavage Median and Percentiles for Diesel Exhaust and Clean Air for Males.

	Clean Air			Diesel Exhaust		
Variables	**25th**	**Median**	**75th**	**25th**	**Median**	**75th**
Protein	150.25	217.25	316.01	200.17	259.95	333.89
TNF-α	42.61	71.49	93.02	73.03	90.29	155.40
IL6	0.84	3.39	7.72	1.32	3.10	6.13
IL8	62.40	104.07	152.84	88.43	111.70	181.44
TNF-α/Protein	0.22	0.28	0.38	0.18	0.45	0.60
IL-6/Protein	0.00	0.01	0.04	0.01	0.01	0.02
IL-8/Protein	0.18	0.39	1.18	0.34	0.40	0.55

Protein concentrations are presented as µg/ml. Cytokines concentrations presented as pg/ml and Soluble Protein concentrations presented as mg/g of total protein.

Table A6. Sputum Median and Percentiles for Diesel Exhaust and Clean Air for All Subjects.

	Clean Air			Diesel Exhaust		
Variables	**25th**	**Median**	**75th**	**25th**	**Median**	**75th**
Protein	10683.17	12372.17	15240.77	10421.72	11687.79	14430.32
TNF-α	184.57	360.54	476.76	166.87	402.99	567.81
IL6	99.31	119.18	141.61	108.91	123.57	152.73
IL8	444.61	498.21	858.65	455.68	524.52	811.16
TNF-α/Protein	0.01	0.03	0.04	0.01	0.03	0.05
IL-6/Protein	0.01	0.01	0.01	0.01	0.01	0.01
IL-8/Protein	0.03	0.04	0.05	0.04	0.05	0.06

Protein concentrations are presented as µg/ml. Cytokines concentrations presented as pg/ml and Soluble Protein concentrations presented as mg/g of total protein.

Table A7. Sputum Median and Percentiles for Diesel Exhaust and Clean Air for Females.

	Clean Air			Diesel Exhaust		
Variables	**25th**	**Median**	**75th**	**25th**	**Median**	**75th**
Protein	10897.57	13459.83	16752.41	11412.63	12547.05	15691.93
TNF-α	132.32	176.91	327.67	161.88	166.50	595.49
IL6	93.06	104.99	160.45	116.16	135.76	177.48
IL8	452.30	551.81	826.23	481.38	762.01	953.03
TNF-α/Protein	0.01	0.01	0.03	0.01	0.01	0.05
IL-6/Protein	0.01	0.01	0.01	0.01	0.01	0.02
IL-8/Protein	0.04	0.04	0.06	0.04	0.05	0.07

Protein concentrations are presented as µg/ml. Cytokines concentrations presented as pg/ml and Soluble Protein concentrations presented as mg/g of total protein.

Table A8. Sputum Median and Percentiles for Diesel Exhaust and Clean Air for Males.

	Clean Air			Diesel Exhaust		
Variables	**25th**	**Median**	**75th**	**25th**	**Median**	**75th**
Protein	10573.36	12254.51	13961.03	10196.87	11401.24	14229.19
TNF-α	203.31	388.23	502.76	185.68	465.73	550.92
IL6	102.13	120.77	141.78	104.98	122.90	143.88
IL8	443.57	497.53	875.53	446.87	522.97	805.74
TNF-α/Protein	0.01	0.03	0.05	0.01	0.03	0.05
IL-6/Protein	0.01	0.01	0.01	0.01	0.01	0.01
IL-8/Protein	0.03	0.04	0.05	0.04	0.05	0.06

Protein concentrations are presented as µg/ml. Cytokines concentrations presented as pg/ml and Soluble Protein concentrations presented as mg/g of total protein.

Table A9. Nasal Lavage Median and Percentiles for the the Difference in the Concentrations of Proteins and Cytokines For Females and Males Measured in Nasal Lavage Samples.

	Females			Males		
Variables	**25th**	**Median**	**75th**	**25th**	**Median**	**75th**
Protein	-64.42	-2.14	29.89	-95.82	29.89	135.86
TNF-α	-25.95	-1.20	17.36	12.59	31.93	40.41
IL6	-3.40	0.00	0.39	-3.74	1.14	1.92
IL8	-11.11	9.60	39.97	-18.19	26.68	84.96
TNF-α/Protein	0.18	0.62	0.84	0.17	0.41	0.60
IL-6/Protein	-0.06	0.00	0.00	-0.02	0.00	0.01
IL-8/Protein	-0.31	-0.10	0.23	-0.42	-0.00	0.24

Variables = Concentrations measured after DE – Concentrations measured after CA. Protein concentrations are presented as µg/ml The Cytokines concentrations are presented as pg/ml and normalized cytokine concentrations are presented as mg/g of total protein.

Table A10. Sputum Lavage Median and Percentiles for the the Difference in the Concentrations of Proteins and Cytokines For Females and Males Measured in Nasal Lavage Samples.

	Females			Males		
Variables	**25th**	**Median**	**75th**	**25th**	**Median**	**75th**
Protein	-1610.56	-170.65	426.63	-2342.63	-481.08	1526.90
TNF-α	-20.18	6.96	93.90	-40.55	3.71	66.54
IL6	4.48	29.34	61.92	-28.50	4.80	29.97
IL8	34.54	86.87	150.40	-56.82	3.48	104.21
TNF-α/Protein	-0.00	0.00	0.01	-0.00	0.00	0.01
IL-6/Protein	-0.00	0.00	0.01	-0.00	0.00	0.00
IL-8/Protein	0.00	0.01	0.02	-0.01	0.00	0.01

Variables = Concentrations measured after DE – Concentrations measured after CA. Protein concentrations are presented as µg/ml The Cytokines concentrations are presented as pg/ml and normalized cytokine concentrations are presented as mg/g of total protein.

Table A11. Mann-Whitney U Test Results Showing the Ranks of the Difference in the Concentrations of Proteins and Cytokines For Females and Males Measured in Nasal Lavage Samples.

	Females N = 7		Males N = 10	
Variables	**Mean Rank**	**Sum of Ranks**	**Mean Rank**	**Sum of Ranks**
Protein	8.00	56.00	9.70	97.00
TNF-α	6.14	43.00	11.00	110.00
IL6	7.21	50.50	10.25	102.50
IL8	8.29	58.00	9.50	95.00
TNF-α/Protein	10.86	76.00	7.70	77.00
IL-6/Protein	7.43	52.00	10.10	101.00
IL-8/Protein	9.00	63.00	9.00	90.00

Variables = Concentrations measured after DE – Concentrations measured after CA. Protein concentrations are presented as µg/ml The Cytokines concentrations are presented as pg/ml and normalized cytokine concentrations are presented as mg/g of total protein.

Table A12. Mann-Whitney U Test Results Showing the Ranks of the Difference in the Concentrations of Proteins and Cytokines For Females and Males Measured in Sputum Induction Samples.

	Females N = 9		Males N = 27	
Variables	**Mean Rank**	**Sum of Ranks**	**Mean Rank**	**Sum of Ranks**
Protein	18.11	163.00	18.63	503.00
TNF-α	19.56	176.00	18.15	490.00
IL6	23.33	210.00	16.89	456.00
IL8	24.11	217.00	16.63	449.00
TNF-α/Protein	20.11	181.00	17.96	485.00
IL-6/Protein	23.33	210.00	16.89	456.00
IL-8/Protein	22.22	200.00	17.26	466.00

Variables = Concentrations measured after DE – Concentrations measured after CA. Protein concentrations are presented as µg/ml The Cytokines concentrations are presented as pg/ml and normalized cytokine concentrations are presented as mg/g of total protein.

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Chapter 3

Analysis of Sex Differences in Systemic Inflammation and Acute Phase Response Markers after Acute Diesel Exhaust Exposure

3.1 Abstract

Background: Epidemiological studies have linked ambient levels of particulate matter to adverse cardiovascular outcomes but the mechanisms that govern this link are poorly understood. There is a general consensus that one potential mechanism for this link is inflammation. Inflammation is part of the body's innate (nonspecific) immune response and there is an established sex dimorphism in immune response.

Aims: To evaluate possible sex differences in the occurrence and intensity of systemic inflammation and acute phase response in females and males after a controlled environmental exposure to diesel exhaust.

Methods: In a crossover design study, healthy human subjects were exposed to 300μg/m^3 of diesel exhaust and clean air in random order for 1 hour each on two different days (≥1 week washout). Blood cell counts, platelets, pro-inflammatory cytokines, mediators and acute phase proteins were measured in peripheral blood that was collected from subjects three times during the study at baseline, six hours and twenty four hours post exposure. Statistical analysis was done using a linear mixed model with repeated measures and contrast to investigate the three way interaction effect (exposure type*sampling time*sex) on the measured variables.

Results: Overall, there was a statistically significant three way interaction effect on platelet count ($p = 0.050$). The contrast analysis showed that between males and females, after controlling for baseline, there was a statistically significant difference in platelet counts ($p = 0.0145$) six hours after diesel exhaust exposure relative to clean air with females having a mean higher platelet count than males. Within males, the contrast analysis showed a statistically significant decrease in platelet counts ($p = 0.045$) twenty four hours after diesel exhaust exposure relative to clean air. Within the other markers of inflammation and acute phase reaction proteins, mixed results were observed but none of them were statistically significant.

Conclusion: Our results suggest that there was a statistically significant effect of sex on platelet counts after a controlled human environmental exposure to a known concentration of diesel exhaust (300μg/m^3) relative to clean air. Females had a stronger systemic inflammatory response than males, as there was a statistically significant increase in platelet count in females 6 hours after diesel exhaust exposure compared to a decrease in males.

3.2 Introduction

Air pollutants such as diesel exhaust have been shown to cause adverse health events and to be associated with increased cardiovascular morbidity but the mechanisms by which they do so are not well known. It has been hypothesized that the inhalation of

these pollutants over a period of time, causes an overload or hindrance of the lungs natural defenses, which then sets off a variety of cascading adverse systemic reactions involving and mediated by multiple cell lines (epithelial cells, macrophages, neutrophils, mast cells and lymphocytes), cytokines and acute phase proteins all of which may contribute to the overall adverse respiratory and cardiovascular health effects that have so far been linked with exposure to diesel exhaust and other air pollutants.

3.2.1 Cytokines

Cytokines are intercellular signaling polypeptides produced by activated cells (Gabay C. et all 1999) in response to local inflammation caused by xenobiotics like diesel exhaust. They have an added inflammatory effect on the surrounding cells and they also modify the production of acute phase proteins by the liver thereby leading to adverse cardiovascular outcomes. Of particular interest in diesel exhaust exposure are those cytokines that are produced during inflammation and which also participate and contribute to the inflammatory process because they are the chief stimulant of the acute phase proteins. These pro-inflammatory cytokines include interleukin 6 (IL-6), interleukin 1β (IL-1β), Tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), transforming growth factor β^2, interleukin 8 (IL-8) (Gabay C. et all 1999). Macrophages and monocytes are the most important sources of these cytokines at the sites of inflammation, although they are also produced by a number of other different cells.

It is hypothesized that diesel exhaust induced inflammation is instigated by oxidative stress (Furuyama et al. 2006; Diaz-Sanchez and Riedl 2005; Salvi and Holgate1999) caused by the subsequent prolonged contact of the metals, PAHs and

quinines contained in diesel exhaust with the epithelial and macrophage cells when the natural lung safety mechanisms become overwhelmed. This oxidative stress initiated inflammation is thought to either directly cause or trigger other mechanisms that are behind the effects that have been seen in in-vitro and in-vivo studies.

3.2.2 Acute Phase Response

The respiratory and cardiovascular effects of diesel exhaust are thus thought to be connected via an oxidative-stress initiated inflammation because inflammation is known to cause a large number of changes far from the initial site of the local inflammation and potentially involving many organ systems. More specifically, changes in the levels of acute phase proteins in response to inflammation and mediated by pro-inflammatory cytokines are believed to be one of the possible causes of the corresponding adverse cardiovascular effects that are often seen following the inhalation of diesel exhaust.

Acute phase proteins are proteins that are produced by the liver and whose plasma concentrations increase or decrease by at least 25% during an inflammatory disorder (Gabay et al. 1999). Of particular interest are fibrinogen and c-reactive proteins. Fibrinogen can cause endothelial cell adhesion, spreading and proliferation and is also a factor of the coagulation cascade when clotting occurs, while c-reactive proteins have been shown to be a cardiovascular risk factor in both healthy subjects and those with coronary heart disease (Peter et al. 2001).

3.2.3 Platelets and Other Blood Cells

Cascading adverse systemic reactions such as changes in acute phase reaction proteins mentioned above are mediated by multiple cell lines in the blood like platelets.

Platelets are unique anucleate mammalian blood cells that are involved in haemostasis and in the innate inflammatory response to xenobiotics. They act both directly and indirectly via the secretion of chemokines and they help to attract other blood cells like leukocytes to sites of inflammation as part of the innate inflammatory response (Weyrich et al 2004). Activated platelets also secrete key components of coagulation (Weyrich et al 2004) and so may also be involved in the adverse cardiovascular effects that have been seen following diesel exhaust exposure.

To summarize, the activation and mobilization of inflammatory cells and platelets by oxidative stress, the production of pro-inflammatory cytokines, the production of acute phase proteins and the production of circulating inflammatory mediators all characterize the systemic inflammatory response (van Eeden et al 2001) to diesel exhaust that is hypothesized to cause adverse cardiovascular effects.

3.2.4 Sex Differences in Immune Response

This systemic inflammatory response to inhaled diesel exhaust particles is part of the body's innate (nonspecific) immune response which is the body's first line of defense against potentially harmful foreign agents that have entered the body through the respiratory system or other routes such as the gut or genitourinary tract (Casarett and Doulls 2001). There is an established sex dimorphism in immune response with females having a stronger immune reactivity (humoral and cellular mediated immune) than males (Da Silva 1995).

The effects of cytokines can be inhibited or enhanced by hormones (Gabay et al 1999). In-vitro, sex hormones have been shown to modulate the production of different

cytokines that are involved in immune responses like IL-6 thus indicating the potential role of sex hormones in the sexual dimorphism of the immune system (Da Silva 1995). While in-vivo, the interactions between sex hormones and other immunomodulatory factors like glucocorticoids are thought to mediate an indirect action of sex hormones on the immune system (Da Silva 1995) and therefore have the potential to cause variations by sex in the systemic inflammation and acute phase responses that have been demonstrated in response to diesel exhaust exposure.

Therefore, the purpose of this study is to determine whether there are sex differences in the occurrence and intensity of systemic inflammation and acute phase response in males and females as indicated by the potential variation in the levels of blood cells, platelets, pro-inflammatory cytokines, mediators and acute phase proteins in peripheral blood after acute exposure to diesel exhaust relative to clean air.

3.3 Materials and Methods

3.3.1 Data Collection

The data that were used for this study are part of the datasets generated from a previous study of diesel exhaust effects. The study was funded by the Department of Defense (DOD) through a grant to the University of Medicine and Dentistry of New Jersey (UMDNJ) (Grant # DAMD17-03-1-0537; Principal Investigator: Dr. Nancy Fiedler). The parent study was done using a human subject protocol that was HIPAA compliant and approved by the UMDNJ Institutional Review Board.

3.3.2 Subjects Demographic Data

110 healthy non-smoking men and women between the ages of 18 and 50 were recruited from the local community in Piscataway, New Jersey, based on the established study criteria; 100 subjects completed the study. Due to problems with blood sample collection at various study time points, all the subjects did not have complete sets of samples collected at the three sampling time points. Therefore depending on the sampling time and the measure being analyzed the total analysis sample size was different (see tables 1a to 1d below).

Table 3.1a. CBC Subjects Demographic Information.

	Females N = 35				Males N = 60			
	Min	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**
AGE (years)	18	44	23.89	6.01	18	43	23.82	5.81
Education (years)	12	22	15.46	2.34	12	23	15.67	2.36
Race	**Frequency (%)**				**Frequency (%)**			
Asian / Pacific Islander	14	(40)			22	(36.67)		
Black not Hispanic	2	(5.71)			5	(8.33)		
Hispanic or Latino	7	(20)			10	(16.67)		
Other/Unknown	1	(2.86)			1	(1.66)		
White not Hispanic	11	(31.43)			22	(36.67)		

Table 3.1b. IL-6 Subjects Demographic Information.

	Females N = 27				Males N = 53			
	Min	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**
AGE (years)	18	44	23.93	6.58	18	43	24.49	6.04
Education (years)	12	20	15.22	1.76	12	23	16.04	2.59
Race	**Frequency (%)**				**Frequency (%)**			
Asian / Pacific Islander	12	(44.44)			21	(39.62)		
Black not Hispanic	1	(3.70)			4	(7.55)		
Hispanic or Latino	3	(11.11)			8	(15.09)		
Other/Unknown	1	(3.70)			0	(0)		
White not Hispanic	10	(37.05)			20	(37.74)		

Table 3.1c. C - reactive protein Subjects Demographic Information.

	Females N = 32				Males N = 57			
	Min	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**
AGE (years)	18	44	24.12	6.21	18	43	23.98	5.57
Education (years)	12	22	15.63	2.32	12	23	15.96	2.52
Race	**Frequency (%)**				**Frequency (%)**			
Asian / Pacific Islander	15	(46.87)			22	(38.60)		
Black not Hispanic	2	(6.25)			4	(7.02)		
Hispanic or Latino	5	(15.63)			10	(17.54)		
Other/Unknown	1	(3.12)			0	(0)		
White not Hispanic	9	(28.13)			21	(36.84)		

Table 3.1d. Fibrinogen Subject Demographics.

	Females N = 31				Males N = 61			
	Min	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**
AGE (years)	18	44	24.00	6.10	18	43	24.07	5.78
Education (years)	12	22	15.45	2.36	12	23	15.89	2.51
Race	**Frequency (%)**				**Frequency (%)**			
Asian / Pacific Islander	12	(38.71)			23	(37.70)		
Black not Hispanic	2	(6.45)			5	(8.20)		
Hispanic or Latino	5	(16.13)			10	(16.39)		
Other/Unknown	1	(3.23)			1	(1.64)		
White not Hispanic	11	(35.48)			22	(36.07)		

3.3.3 Exposure Conditions and Experimental Design

A crossover design was used in which each subject underwent two exposure conditions, in random order on two separate days; one was to a diluted diesel exhaust normalized to PM_{10} concentration of 300µg/m^3 and the other was to filtered clean ambient air of Busch campus in Piscataway, a suburban campus of Rutgers University. Both exposure conditions were 1 hour in duration and took place at the Environmental and Occupational Health Sciences Institutes' (EOHSI) Controlled Environmental Facility (CEF). Each exposure condition was presented with and without an acute psychological stressor to two groups of subjects high and low in self reported chemical intolerance (CI). Subjects were also asked to fast overnight the day before the study until after each exposure session and to also abstain from any exercise.

There were no significant differences in the exposure conditions for all subjects. Overall, the concentration of PM_{10} that the subjects were exposed to was 276±13µg/m^3 which was close to the target concentration of 300µg/m^3.

Table 3.2. Mean Air Characteristics and Composition.

	Diesel Exhaust	**Clean Air**
PM_{10} (µg/m^3)	276 ± 13	6 ± 5
NO_X (ppm)	3.63 ± 0.90	0.02 ± 0.02
CO (ppm)	3.79 ± 0.76	0.91 ± 0.21
1 Nitropyrene (ng/m^3)	2.68 ± 0.51	<0.06

3.3.4 Systemic Inflammation and Acute Phase Response Markers

The following variables were measured in peripheral blood as systemic inflammation and acute phase response markers based on similar studies that have been done so far: white blood cell count (WBC), red blood cell count (RBC), platelet count, cell blood count measures (neutrophils, lymphocytes, monocytes, eosinophils and basophils), c-reactive protein, IL-6 and fibrinogen concentrations.

3.3.5 Blood Sample Collection

Peripheral venous blood was collected from subjects at about the same time of day, 3 times during the study; before exposure, six hours after exposure and 24 hours after exposure.

3.3.6 Blood Sample Analysis

Blood was sent to Quest diagnostics laboratories for neutrophil count, platelet count and cell blood count (CBC) measurements. Peripheral blood IL-6 and c-reactive protein concentrations were analyzed using commercially available ELISAs while the peripheral blood fibrinogen content was measured using functional assay at the Robert Wood Johnson University hematology laboratory.

3.4 Statistical Methods

Descriptive statistical analysis, histograms and box plots were used to evaluate the distribution of all the variables. Based on the results of the descriptive analysis, all the values of the raw systematic inflammation and acute phase response outcome variables

were log transformed in order to normalize their distribution before use in further analysis.

A potential confounder which could have skewed the results of the analysis was body mass index (BMI). Therefore BMI was calculated for all the study subjects using height and weight and was then stratified by sex in order to evaluate the mean BMI by sex. The mean BMI for females (Mean = 23.65 and SD = 4.68) and the mean BMI for males (Mean = 25.66 and SD = 3.40) were about the same for the study population so there was no need to control for BMI in this analysis.

To address the aim of the paper, a linear mixed model with repeated analysis was used to examine if there was a significant three way interaction among exposure type (diesel exhaust and clean air), sampling time (Baseline, 6 hours and 24 hours) and sex (males and females) on the log transformed outcome variables.

Since some subjects were also exposed to an acute psychological stressor (a potential effect modifier), an initial stress stratified linear mixed model with repeated analysis was done on those subjects who were exposed to stress and those subjects who were not exposed to stress in order to determine if there was a stress effect on the concentrations of the outcome variables. The effect sizes of the stratified analyses were compared and it was determined that there was no stress effect. Therefore, the final model contained sex as a between subjects variable, exposure and time as within subjects variables with the log transformed outcome variables as the dependent variables. The ‘exposure*time*sex’ three way interaction was examined to assess the hypothesis of interest.

Tests of the three interactions were conducted using Type 3 score tests (Liang and Zeger 1986) of the interaction between exposure, time and sex. Contrasts were used to test whether males and females differed with respect to the effect of exposure on changes in the concentration of systemic inflammation markers from baseline to each subsequent time point. Contrasts were also used to test whether within males and within females, changes in the concentration of systemic inflammation markers from baseline to each subsequent time point differed between exposures.

All statistical analysis for this paper were done using SAS software, Version 9.1 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA) and SPSS software, Version 17.0 (SPSS inc., Chicago IL). Also, some graphs were created using Microsoft Excel 2007. P-values less than or equal to 0.05 were considered to be significant.

3.5 Results

Three Way Interaction Effect of Exposure, Time and Sex on Systemic Inflammation and Acute Phase Response Markers:

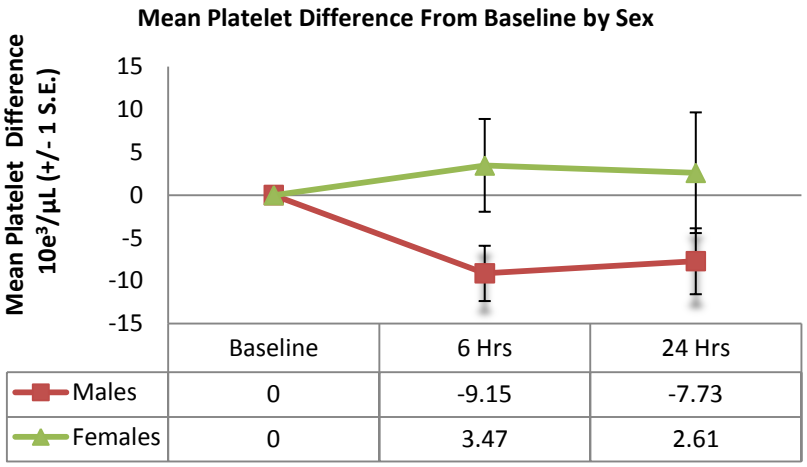
With the exception of platelets, the linear mixed model with repeated analysis of the ‘exposure*time*sex’ three way interaction revealed no statistically significant three way interaction effect on the concentrations of the systemic inflammation and acute phase reaction markers (see table 3).

Table 3.3. Three Way Interaction Results.

	E x T x S	
Variables	**F Value**	**P Value**
Platelet	3.02	0.050
WBC	0.17	0.85
RBC	1.81	0.17
Absolute CBC Values		
Neutrophils	0.47	0.62
Lymphocytes	0.28	0.75
Monocytes	0.35	0.71
Eosinophils	0.27	0.77
Basophils	0.05	0.95
Percent CBC Values		
Neutrophils	0.77	0.46
Lymphocytes	0.97	0.38
Monocytes	0.90	0.41
Eosinophils	0.31	0.74
Basophils	0.04	0.96
Mediators		
CRP	1.47	0.23
Il6	0.07	0.93
Coagulation Factors		
Fibrinogen	0.45	0.50

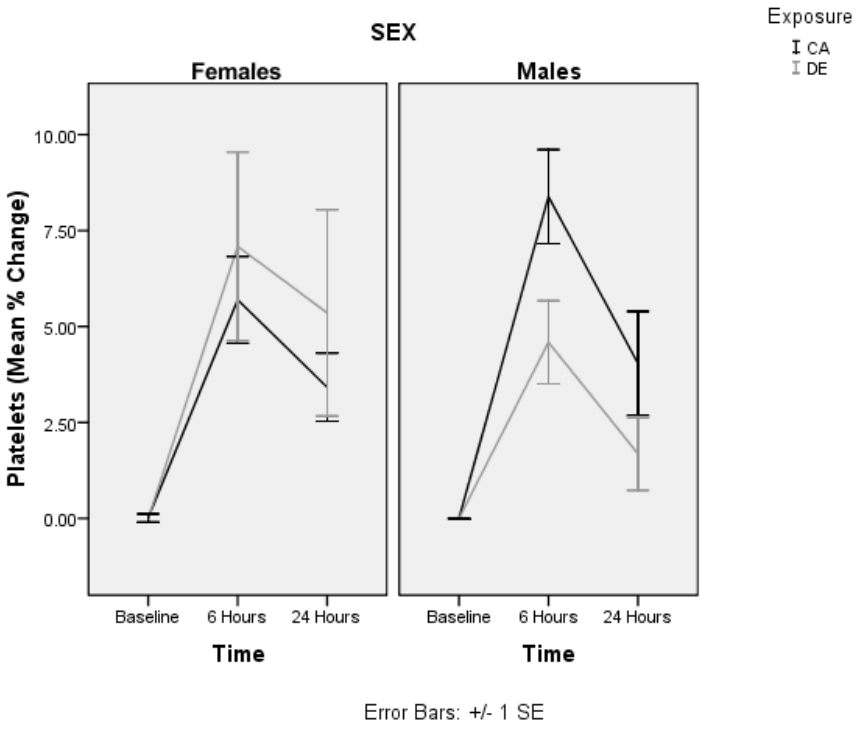
E = Exposure (Diesel Exhaust and Clean Air); T = Time (6 hrs and 24 hrs);
S = Sex (Females and Males). WBC = White Blood Cells; RBC = Red Blood Cells;
CRP = C - reactive protein; IL6 = Interleukin 6 and CBC = Cell Blood Count.
Units of WBC and Platelets = $10e^3/\mu L$; Units of RBC = $10e^6/\mu L$; Units of absolute CBC variables $10e^3/\mu L$; Units of percent CBC variables = %; Unit of CRP = mg/l; Unit of IL6 = pg/ml and Unit of Fibrinogen = mg/dl. $p \leq 0.05$ is significant.

There was a statistically significant three way interaction effect on platelet count (p = 0.050 for exposure*time*sex). The contrast analysis showed that males differed from females with respect to the effect of diesel exhaust on changes in platelet counts from baseline to 6 hours (p = 0.0145) with males showing a mean decrease in platelet count compared to a mean increase in females (see table 4a and graph 1).



Graph 3.1: Plot of Mean Platelet Count Difference by Exposure by Sex and Time.

Within males, the contrast analysis showed a statistically significant effect of diesel exhaust on the change in platelet counts at 24 hours ($p = 0.045$); while a significant effect at 24 hours was not seen within females (see table 4a and graph 2). However, these effects were also not significantly different between males and females at 24 hours.



Graph 3.2. Graph of Mean Percent Change in Platelet Counts Measured in Diesel Exhaust Relative to Clean Air by Sex.

Table 3.4a. Mean Log Blood Cell Values (Absolute) and Contrast Test Results.

			Baseline	6hr	24hr	6hr Δ	24hr Δ	Exposure Main Effect On 6hr Δ	Exposure Main Effect On 24hr Δ	Exposure by Sex Effect On 6hr Δ	Exposure by Sex Effect On 24hr Δ
Variables	**Sex**	**Exp**	**Mean (SD) N**	**Mean (SD) N**	**Mean (SD) N**	**Mean (SD) N**	**Mean (SD) N**	**t-Value (SE) p-Value**	**t-Value (SE) p-Value**	**t-Value (SE) p-Value**	**t-Value (SE) p-Value**
Log Platelet (x10e^3/µl)	**Males**	DE	5.50 (0.21) 60	5.56 (0.21) 60	5.52 (0.21) 60	0.06 (0.08) 60	0.02 (0.07) 60	-1.74 (0.01) 0.082**	-2.01 (0.01) 0.045*	-2.45 (0.02) 0.0145*	-1.42 (0.02) 0.16
		CA	5.49 (0.20) 59	5.57 (0.20) 56	5.53 (0.20) 60	0.08 (0.08) 55	0.04 (0.09) 59				
	Females	DE	5.50 (0.24) 32	5.57 (0.21) 33	5.53 (0.24) 34	0.09 (0.11) 31	0.05 (0.12) 31	1.75 (0.02) 0.081**	0.27 (0.02) 0.78		
		CA	5.55 (0.22) 35	5.60 (0.20) 35	5.60 (0.22) 33	0.05 (0.06) 35	0.03 (0.05) 33				
Log WBC (x10e^3/µl)	**Males**	DE	1.73 (0.22) 60	1.86 (0.24) 60	1.69 (0.26) 60	0.13 (0.18) 60	-0.04 (0.13) 60	-0.14 (0.03) 0.89	0.32 (0.03) 0.75	-0.58 (0.05) 0.56	-0.31 (0.05) 0.76
		CA	1.73 (0.24) 58	1.87 (0.22) 55	1.68 (0.22) 60	0.12 (0.16) 54	-0.04 (0.14) 58				
	Females	DE	1.67 (0.29) 32	1.82 (0.27) 33	1.61 (0.25) 34	0.15 (0.22) 31	-0.05 (0.14) 31	0.62 (0.04) 0.54	0.63 (0.04) 0.53		
		CA	1.72 (0.28) 35	1.84 (0.26) 34	1.64 (0.25) 33	0.12 (0.18) 34	-0.09 (0.14) 33				
Log RBC (x10e^6/µl)	**Males**	DE	1.60 (0.07) 60	1.59 (0.07) 60	1.60 (0.07) 60	-0.00 (0.03) 60	0.01 (0.03) 60	-0.15 (0.01) 0.88	-0.79 (0.01) 0.43	-1.89 (0.02) 0.060**	-0.73 (0.02) 0.47
		CA	1.59 (0.06) 59	1.59 (0.07) 56	1.60 (0.07) 60	0.00 (0.03) 55	0.01 (0.03) 59				
	Females	DE	1.39 (0.15) 32	1.42 (0.09) 33	1.42 (0.09) 34	0.03 (0.14) 31	0.04 (0.13) 31	2.25 (0.01) 0.024*	0.32 (0.01) 0.75		
		CA	1.41 (0.08) 35	1.42 (0.08) 35	1.44 (0.08) 33	0.01 (0.04) 35	0.03 (0.04) 33				

Exp = Exposure; DE = Diesel Exhaust; CA = Clean Air; Δ = Change from baseline; SD = Standard deviation and SE = Standard Error. WBC = White Blood Cells and RBC = Red Blood Cells. Signt = Significant. * = statistically significant at p = 0.05.
** = borderline statistically significant at p = 0.05

Table 3.4b. Mean Log Blood Cell Blood Count Values (Absolute) and Contrast Test Results (continued).

			Baseline	6hr	24hr	6hr Δ	24hr Δ	Exposure Main Effect On 6hr Δ	Exposure Main Effect On 24hr Δ	Exposure by Sex Effect On 6hr Δ	Exposure by Sex Effect On 24hr Δ
Variables	**Sex**	**Exp**	**Mean (SD) N**	**Mean (SD) N**	**Mean (SD) N**	**Mean (SD) N**	**Mean (SD) N**	**t-Value (SE) p-Value**	**t-Value (SE) p-Value**	**t-Value (SE) p-Value**	**t-Value (SE) p-Value**
Log Neutrophils (cells/µl)	**Males**	DE	8.07 (0.30) 60	8.22 (0.31) 60	8.03 (0.34) 60	0.16 (0.22) 60	-0.04 (0.17) 60	-0.23 (0.04) 0.82	0.13 (0.04) 0.89	-0.96 (0.07) 0.34	-0.59 (0.07) 0.55
		CA	8.06 (0.34) 59	8.22 (0.32) 56	8.01 (0.29) 60	0.14 (0.22) 55	-0.04 (0.21) 59				
	Females	DE	8.01 (0.37) 32	8.15 (0.39) 33	7.93 (0.35) 34	0.13 (0.29) 31	-0.07 (0.21) 31	1.03 (0.06) 0.30	0.84 (0.06) 0.40		
		CA	8.07 (0.40) 35	8.14 (0.39) 35	7.95 (0.34) 33	0.07 (0.24) 35	-0.13 (0.22) 33				
Log Lymphocytes (cells/µl)	**Males**	DE	7.47 (0.25) 60	7.57 (0.26) 60	7.45 (0.29) 60	0.10 (0.21) 60	-0.03 (0.17) 60	0.01 (0.03) 0.99	-0.18 (0.03) 0.85	0.71 (0.06) 0.48	0.57 (0.06) 0.57
		CA	7.47 (0.18) 59	7.56 (0.22) 56	7.45 (0.25) 60	0.10 (0.17) 55	-0.02 (0.18) 59				
	Females	DE	7.45 (0.32) 32	7.60 (0.30) 33	7.41 (0.30) 34	0.16 (0.20) 31	-0.03 (0.16) 31	-0.88 (0.04) 0.38	-0.85 (0.05) 0.40		
		CA	7.44 (0.28) 35	7.64 (0.27) 35	7.44 (0.29) 33	0.19 (0.16) 35	-0.00 (0.13) 33				
Log Monocytes (cells/µl)	**Males**	DE	6.04 (0.32) 60	6.21 (0.26) 60	6.02 (0.32) 60	0.17 (0.27) 60	-0.03 (0.27) 60	0.82 (0.04) 0.41	1.62 (0.04) 0.10	0.44 (0.07) 0.66	0.84 (0.07) 0.40
		CA	6.09 (0.31) 59	6.21 (0.24) 56	5.99 (0.30) 60	0.13 (0.26) 55	-0.10 (0.21) 59				
	Females	DE	5.87 (0.28) 32	6.06 (0.29) 33	5.81 (0.29) 34	-0.19 (0.26) 31	-0.08 (0.29) 31	0.07 (0.06) 0.95	0.19 (0.06) 0.85		
		CA	5.93 (0.28) 35	6.10 (0.29) 35	5.86 (0.26) 33	0.18 (0.24) 35	-0.08 (0.18) 33				
Log Eosinophils (cells/µl)	**Males**	DE	4.89 (0.69) 60	4.82 (0.76) 60	4.76 (0.76) 60	-0.08 (0.47) 60	-0.14 (0.28) 60	0.59 (0.08) 0.56	0.26 (0.07) 0.80	0.08 (0.13) 0.94	-0.59 (0.13) 0.56
		CA	4.98 (0.60) 59	4.82 (0.89) 56	4.79 (0.74) 60	-0.10 (0.45) 55	-0.16 (0.35) 59				
	Females	DE	4.60 (0.82) 32	4.55 (0.87) 33	4.51 (0.80) 34	-0.02 (0.36) 31	-0.03 (0.32) 31	0.35 (0.10) 0.73	0.93 (0.10) 0.36		
		CA	4.65 (0.73) 35	4.58 (0.85) 35	4.51 (0.75) 33	-0.07 (0.52) 35	-0.13 (0.26) 33				
Log Basophils (cells/µl)	**Males**	DE	3.05 (0.52) 60	3.29 (0.45) 60	3.04 (0.46) 60	0.23 (0.59) 60	-0.04 (0.55) 59	-1.47 (0.09) 0.14	-1.10 (0.09) 0.27	-0.12 (0.16) 0.91	0.19 (0.16) 0.85
		CA	3.01 (0.52) 59	3.37 (0.39) 56	3.09 (0.45) 60	0.38 (0.54) 55	0.10 (0.46) 59				
	Females	DE	3.07 (0.53) 32	3.31 (0.35) 33	3.01 (0.48) 33	0.30 (0.56) 31	-0.08 (0.48) 30	-0.96 (0.13) 0.34	-1.06 (0.13) 0.29		
		CA	3.09 (0.53) 35	3.51 (0.43) 34	3.18 (0.55) 33	0.40 (0.45) 35	0.34 (0.59) 33				

Exp = Exposure; DE = Diesel Exhaust; CA = Clean Air; Δ = Change from baseline; SD = Standard deviation and SE = Standard Error. Signt = Significant. $p \leq 0.05$ is significant.

Table 3.4c. Mean Log Cell Blood Count Values (Percentage) and Contrast Test Results.

			Baseline	6hr	24hr	6hr Δ	24hr Δ	Exposure Main Effect On 6hr Δ	Exposure Main Effect On 24hr Δ	Exposure by Sex Effect On 6hr Δ	Exposure by Sex Effect On 24hr Δ
			Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	t-Value (SE)	t-Value (SE)	t-Value (SE)	t-Value (SE)
Variables	**Sex**	**Exp**	**N**	**N**	**N**	**N**	**N**	**p-Value**	**p-Value**	**p-Value**	**p-Value**
Log Neutrophils (%)	**Males**	DE	4.04	4.06	4.03	0.02	-0.01	-0.46	-0.23	-1.23	-0.78
			(0.13)	(0.13)	(0.14)	(0.10)	(0.08)	(0.02)	(0.02)	(0.03)	(0.03)
			60	60	60	60	60	0.65	0.81	0.22	0.44
		CA	4.03	4.06	4.03	0.02	0.00				
			(0.14)	(0.13)	(0.14)	(0.10)	(0.13)				
			58	55	60	54	58				
	Females	DE	4.03	4.02	4.02	-0.02	-0.02	1.20	0.80		
			(0.15)	(0.18)	(0.18)	(0.11)	(0.11)	(0.03)	(0.03)		
			32	33	34	31	31	0.23	0.43		
		CA	4.05	4.00	4.01	-0.05	-0.04				
			(0.17)	(0.18)	(0.16)	(0.10)	(0.10)				
			35	34	33	34	33				
Log Lymphocytes (%)	**Males**	DE	3.44	3.40	3.45	-0.04	0.01	0.15	-0.55	1.37	0.90
			(0.22)	(0.23)	(0.25)	(0.16)	(0.15)	(0.03)	(0.03)	(0.05)	(0.05)
			60	60	60	60	60	0.88	0.58	0.17	0.37
		CA	3.44	3.40	3.46	-0.03	0.02				
			(0.21)	(0.21)	(0.21)	(0.15)	(0.17)				
			58	55	60	54	58				
	Females	DE	3.48	3.47	3.50	0.01	0.02	-1.60	-1.53		
			(0.24)	(0.28)	(2.26)	(0.15)	(0.15)	(0.04)	(0.04)		
			32	33	34	31	31	0.11	0.13		
		CA	3.42	3.50	3.50	0.08	0.09				
			(0.29)	(0.27)	(0.26)	(0.16)	(0.16)				
			35	34	33	34	33				
Log Monocytes (%)	**Males**	DE	2.01	2.05	2.02	0.04	0.01	1.10	1.71	1.02	1.27
			(0.26)	(0.21)	(0.23)	(0.18)	(0.21)	(0.04)	(0.04)	(0.06)	(0.06)
			60	60	60	60	60	0.27	0.09**	0.31	0.21
		CA	2.06	2.05	2.00	0.01	-0.05				
			(0.27)	(0.21)	(0.22)	(0.21)	(0.21)				
			58	55	60	54	58				
	Females	DE	1.90	1.93	1.90	0.05	-0.02	-0.45	-0.31		
			(0.21)	(0.24)	(0.26)	(0.20)	(0.22)	(0.05)	(0.05)		
			32	33	34	31	31	0.65	0.76		
		CA	1.91	1.96	1.92	0.06	0.01				
			(0.27)	(0.29)	(0.25)	(0.18)	(0.15)				
			35	34	33	34	33				
Log Eosinophils (%)	**Males**	DE	0.86	0.65	0.76	-0.21	-0.10	0.69	0.12	0.34	-0.44
			(0.63)	(0.69)	(0.67)	(0.46)	(0.27)	(0.08)	(0.08)	(0.13)	(0.13)
			60	60	60	60	60	0.49	0.90	0.73	0.66
		CA	0.93	0.63	0.81	-0.23	-0.11				
			(0.58)	(0.86)	(0.67)	(0.47)	(0.38)				
			58	55	60	54	58				
	Females	DE	0.63	0.43	0.59	-0.17	0.02	0.09	0.64		
			(0.75)	(0.84)	(0.75)	(0.32)	(0.27)	(0.10)	(0.10)		
			32	33	34	31	31	0.93	0.52		
		CA	0.63	0.43	0.56	-0.19	-0.05				
			(0.70)	(0.82)	(0.69)	(0.54)	(0.25)				
			35	34	33	34	33				
Log Basophils (%)	**Males**	DE	-0.98	-0.88	-0.96	0.10	-0.00	-1.41	-1.19	0.15	0.28
			(0.46)	(0.44)	(0.47)	(0.56)	(0.54)	(0.09)	(0.09)	(0.15)	(0.15)
			60	60	59	60	59	0.16	0.23	0.88	0.78
		CA	-1.03	-0.80	-0.90	0.26	0.14				
			(0.56)	(0.37)	(0.44)	(0.54)	(0.47)				
			58	55	60	54	58				
	Females	DE	-0.91	-0.77	-0.89	0.14	-0.03	-1.25	-1.24		
			(0.46)	(0.37)	(0.46)	(0.53)	(0.46)	(0.12)	(0.12)		
			32	33	33	31	30	0.21	0.22		
		CA	-0.93	-0.63	-0.76	0.30	0.13				
			(0.46)	(0.39)	(0.50)	(0.44)	(0.57)				
			35	33	33	33	33				

Exp = Exposure; DE = Diesel Exhaust; CA = Clean Air; Δ = Change from baseline; SD = Standard deviation and SE = Standard Error. Signt = * = statistically significant at p = 0.05 ** = borderline statistically significant at p = 0.05

Table 3.4d. Mean Log Mediators Values and Contrast Test Results.

			Baseline	6hr	24hr	6hr Δ	24hr Δ	Exposure Main Effect On 6hr Δ	Exposure Main Effect On 24hr Δ	Exposure by Sex Effect On 6hr Δ	Exposure by Sex Effect On 24hr Δ
Variables	**Sex**	**Exp**	**Mean (SD) N**	**Mean (SD) N**	**Mean (SD) N**	**Mean (SD) N**	**Mean (SD) N**	**t-Value (SE) p-Value**	**t-Value (SE) p-Value**	**t-Value (SE) p-Value**	**t-Value (SE) p-Value**
Log CRP (mg/l)	**Males**	DE	-0.32	-0.40	-0.42	0.02	-0.15	-0.76	1.20	-1.26	-1.63
			(1.29)	(1.27)	(1.49)	(0.44)	(0.52)	(0.10)	(0.09)	(0.16)	(0.15)
			55	49	55	48	53	0.45	0.23	0.21	0.10
		CA	-0.46	-0.47	-0.47	0.09	-0.02				
			(1.36)	(1.45)	(1.35)	(0.43)	(0.55)				
			56	48	57	48	56				
	Females	DE	-0.30	-0.26	-0.20	0.08	0.10	1.00	1.14		
			(1.64)	(1.53)	(1.55)	(0.39)	(0.55)	(0.12)	(0.12)		
			32	30	32	30	32	0.32	0.25		
		CA	-0.06	-0.22	-0.19	-0.04	-0.04				
			(1.34)	(1.47)	(1.53)	(0.39)	(0.34)				
			31	30	31	29	30				
Log IL-6 (pg/ml)	**Males**	DE	2.41	2.32	2.94	-0.02	0.26	0.49	0.74	0.36	0.03
			(1.73)	(1.78)	(1.85)	(0.89)	(1.05)	(0.23)	(0.26)	(0.41)	(0.46)
			49	48	26	45	25	0.62	0.46	0.72	0.97
		CA	2.53	2.41	3.00	-0.07	0.23				
			(1.84)	(1.64)	(1.90)	(1.18)	(1.52)				
			48	45	27	44	25				
	Females	DE	1.70	1.39	1.44	-0.30	-0.19	-0.11	0.47		
			(0.94)	(1.03)	(1.18)	(0.83)	(1.12)	(0.35)	(0.38)		
			21	21	13	19	11	0.91	0.64		
		CA	1.60	1.06	1.08	-0.12	-0.11				
			(1.35)	(1.23)	(0.86)	(0.54)	(1.50)				
			20	20	14	18	10				

Exp = Exposure; DE = Diesel Exhaust; CA = Clean Air; Δ = Change from baseline; SD = Standard deviation and SE = Standard Error. CRP = C - reactive protein and IL-6 = Interleukin 6. Signt = Significant. * = statistically significant at p = 0.05
** = borderline statistically significant at p = 0.05

Table 3.4e. Mean Log Coagulation Factor Values and Contrast Test Results.

			Baseline	24hr	24hr Δ	Exposure Main Effect On 24hr Δ	Exposure by Sex Effect On 24hr Δ
Variables	**Sex**	**Exp**	**Mean (SD) N**	**Mean (SD) N**	**Mean (SD) N**	**t-Value (SE) p-Value**	**t-Value (SE) p-Value**
Log Fibrinogen (mg/dl)	**Males**	DE	5.49	5.50	0.01	0.08	-0.67
			(0.23)	(0.25)	(0.20)	(0.04)	(0.07)
			57	58	55	0.94	0.50
		CA	5.48	5.48	0.02		
			(0.24)	(0.22)	(0.20)		
			53	54	49		
	Females	DE	5.62	5.61	0.03	0.84	
			(0.20)	(0.20)	(0.18)	(0.06)	
			24	22	20	0.40	
		CA	5.64	5.62	-0.03		
			(0.21)	(0.23)	(0.26)		
			22	27	22		

Exp = Exposure; DE = Diesel Exhaust; CA = Clean Air; Δ = Change from baseline; SD = Standard deviation and SE = Standard Error. Signt = Significant. p≤ 0.05 is significant.

3.6 Discussion

The results showed that there was a statistically significant exposure*time*sex three way interaction effect on platelet count after a 1-hour controlled human environmental exposure to a known concentration of diesel exhaust (300µg/m^3). This

means that for platelets, sex was a significant modifier of the effect of diesel exhaust exposure relative to clean air depending on the time that the platelet sample was measured.

For the other systemic inflammatory and acute phase response markers that were analyzed, no other statistically significant differences in concentration were observed between males and females. These results are similar to results seen in other controlled exposure studies that have been done (Lucking et al 2008, Carlsten et al 2007, Mills et al 2007, Tornqvist et al 2007 and Mills et al 2005) except that these studies did not examine sex differences and they also had subjects engaging in some form of exercise during exposure (Lucking et al 2008, Mills et al 2007, Tornqvist et al 2007 and Mills et al 2005).

Between males and females there was a statistically significant diesel exhaust attributable difference in platelet count from baseline to 6 hours with females having a mean higher platelet count than males. Platelets are hypothesized to have an early role in systemic surveillance and information transfer similar to macrophages (Weyrich et al 2009). Activated platelets are rapidly deployed through neutrophils to sites of inflammation and can modulate inflammatory processes by the secretion of chemokines which interact with leukocytes causing the secretion of cytokines and other inflammatory mediators (Weyrich et al 2004 and Semple et al 2010). Therefore the observed significant difference in platelet counts between males and females could be an indication of a diesel exhaust induced systemic inflammatory response in females. This is somewhat supported by the fact that within females there was also an observed mean increase in platelet count at 24 hours after exposure to diesel exhaust relative to clean air although this observation was not statistically significant. While within males, there was an observed mean

decrease at 6 hours and 24 hours after exposure to diesel exhaust relative to clean air although only the decrease at 24 hours was statistically significant.

Table 3.5. Difference in Platelet Counts by Exposure and Sex

Sex	Exposure	6 hr Δ	24 hr Δ
Males	DEΔ – CAΔ	-9.15	-7.73
	SE	3.23	3.85
Females	DEΔ – CAΔ	3.47	2.61
	SE	5.42	7.05

Δ = Change from baseline.

This observed mean decrease in platelet count within males could be due to the potential suppressive effect of male hormones on the innate immune inflammatory response to diesel exhaust exposure thereby causing a reduction in platelet count in an attempt to hinder any ensuing inflammatory response to the DEPs in diesel exhaust.

Another factor that could have contributed to the increase in platelets that was observed in females is psychological stress which some subjects were exposed to simultaneously with diesel exhaust. This is because psychological stress has been shown to modify hemostasis and thrombosis by causing a decrease in plasma volume thereby leading to hemoconcentration (Deepa et al 2001) and an increase in the plasma concentrations of blood cells like platelets and blood lipids like cholesterol.

In contrast to this, there is also the possibility that the mean decrease in platelet count that was observed in males could be due to platelet aggregation in response to oxidative stress induced injury. Since the mechanism by which testosterone inhibits the immune system is poorly understood and due to the fact that platelet aggregation, oxidative stress and hemoconcentration were not measured in this study there is no way to directly evaluate either of the three possible alternate explanations for the observations seen in males and females.

Regardless, the fact that there was a statistically significant mean decrease in platelet count within males 24 hours after exposure to diesel exhaust relative to clean air does help to further highlight the possibility that there is an underlying mechanism that could be protective in males (hormonal suppression of inflammation) or harmful (platelet aggregation) that needs to be further investigated for clarification.

Epidemiological studies (Diez Roux et al 2006, Zeka et al 2006, Peters et al 2001, Samet et al 2000 and Schwartz 2001) have linked ambient levels of particulate matter to adverse cardiovascular outcomes but the mechanisms that govern this link are poorly understood (Riedl and Diaz Sanchez 2005). There is a general consensus that one potential mechanism for this link is inflammation.

Diesel exhaust is the largest single source of airborne combustion derived particulate matter in many urban areas (Mills et al 2005, Riedl and Diaz Sanchez 2005) and so it is one of the most studied environmental pollutants. Epidemiological, animal and in-vitro studies that have examined the effects of exposure to diesel exhaust particles have been able to demonstrate its pro-inflammatory nature via oxidative stress (Tornqvist et al 2007) but the inflammation process itself is complex and remains incompletely understood.

Based on this, for all subjects we expected to see diesel exhaust attributable changes from baseline that exceeded those seen after exposure to clean air but this was not observed. In some instances, there were equivalent or greater clean air attributable changes than those seen after exposure to diesel exhaust. Overall, we observed a mixed response trend in all the outcome variables between the sex groups which is different

from what has been observed in other studies where subject responses have often been in one direction.

Since both the diesel exhaust and clean air exposure conditions were monitored and found to be similar for all subjects and also due to the fact that there was no significant difference in baseline values between the two sex groups, the observed results could imply the following:

- that the study exposure was not long or intense enough or
- that the observations could be due to the fact that the subjects were relatively young and healthy or
- the possibility of the effect of potential exposure to ambient DEPs to subjects after the controlled exposures or
- that the observations could be due to an interaction effect of one or more study interventions which included a stress exercise for some subjects, a prolonged fast and repeated blood sampling (Tornqvist et al 2007).

Consequently, the results do point to the possible presence of an underlying factor or factors that needs to be investigated further for clarity.

With the exception of platelets, even though this study did not find a significant difference in systemic inflammation and acute phase response markers between males and females, it still warrants further investigation because of the established link between particulate matter exposure and cardiovascular disease and also due to the pro-inflammatory nature of diesel exhaust. This is because recent studies have emerged to show inflammation as a strong independent risk indicator for cardiovascular disease (Kritchevsky et al 2005) so this particulate matter induced inflammation poses a threat to

certain vulnerable populations and may increase the likelihood of adverse cardiovascular outcomes.

Also, there are gender specific differences in the burden of cardiovascular disease, the prevalence of cardiovascular disease risk factors and the clinical presentation of cardiovascular disease (Roberts 2005) which all point to a potential sex driven mechanism that may be modifying the gender related differences that have been observed. For example, in the United States, cardiovascular disease is the leading cause of death in adult women and also a leading cause of disability (Bedinghaus et al 2001 and American Heart Association 2009). The observed increase in platelet concentration following diesel exhaust exposure relative to clean air is one of the first steps that is thought to give rise to the ambient air pollution inflammation induced cardiovascular effects (Brooks et al 2004) that have been observed. This observed platelet increase in females whether in response to diesel exhaust induced systemic inflammation or due to hemoconcentration both represent a potential risk factor and a potential modification by sex which needs to be further investigated.

3.6.1 Study Limitations

Due to issues with blood collection, not all subjects had blood samples for all time points and there was an imbalance in the number of males and females that were analyzed with a higher ratio of males to females for all the outcome variables that were analyzed. Both of these could potentially have influenced the results that were seen through the potential increase in variability between the groups (males and females) being analyzed.

In addition, a potential source of error that was not investigated was the possible additional exposure of subjects to DEPs and other types of pollution outside of the exposure chamber during their diverse daily activities. This might be a potential critical confounder as indicated by the mixed response trend that was seen after exposure to diesel exhaust and especially after exposure to clean air.

Lastly, since stress is a potential modifier of the response by sex due to the effect of glucocorticoids on the cell mediated immune response, the acute psychological stressor that was used in this study may have masked any potential sex differences. Consequently further investigation using a larger, more balanced study, excluding any additional acute stressors (psychological or physiological) may help to better elucidate the potential modifying effect of sex on the systemic inflammation and acute phase response to acute diesel exhaust exposure.

3.7 Conclusions

Using a controlled human environmental exposure to a known concentration of DE (300μg/m^3), with the exception of platelet count, we observed a mixed response trend in identified systemic inflammation and acute phase response markers by sex that was not statistically significant. Platelet count was statistically significantly different between males and females at 6 hours after diesel exhaust exposure relative to clean air with females having a mean higher platelet count than males. There was also a statistically significant decrease in mean plate count within males at 24 hours after diesel exhaust exposure relative to clean air. The significant sex difference in platelet count at 6 hours

indicates that females had a stronger systemic inflammatory response to diesel exhaust exposure than males relative to clean air.

Appendix B

Table B.1. Systemic Inflammation Markers.

Blood Cell Values	
Variables	**Times Measured**
Platelet WBC RBC MPV	1. Baseline 2. 6hrs Post exposure 3. 24hrs Post Exposure
Variables	**Times Measured**
Neutrophils Lymphocytes Monocytes Eosinophils Basophils	1. Baseline 2. 6hrs Post exposure 3. 24hrs Post Exposure
Variables	**Times Measured**
Neutrophils Lymphocytes Monocytes Eosinophils Basophils	1. Baseline 2. 6hrs Post exposure 3. 24hrs Post Exposure
Variables	**Times Measured**
CRP Il-6	1. Baseline 2. 6hrs Post exposure 3. 24hrs Post Exposure
Variables	**Times Measured**
Fibrinogen	1. Baseline 2. 24hrs Post Exposure

WBC = White Blood Cells and RBC = Red Blood Cells.
Units of RBC = 10e6/µL;
Units of WBC and Cell Blood Count absolute variables $10e^3$/µL;
Units of percent variables = %;
Unit of CRP = mg/l; Unit of IL-6 = pg/ml and Unit of Fibrinogen = mg/dl.

Table B.2. CRP Mean Values.

Sex	Exposure	Time	N	Min	Max	Mean	SD
Males	DE	1	55	0.02	7.50	1.32	1.44
		6	49	0.02	6.69	1.27	1.47
		24	55	0.00	6.26	1.27	1.28
	CA	1	56	0.01	8.47	1.20	1.45
		6	48	0.00	9.99	1.27	1.69
		24	57	0.01	5.30	1.14	1.11
Females	DE	1	32	0.01	7.04	1.65	1.93
		6	30	0.01	6.77	1.61	1.86
		24	32	0.01	8.20	1.75	2.10
	CA	1	31	0.01	6.16	1.62	1.48
		6	30	0.01	5.43	1.48	1.35
		24	31	0.01	7.96	1.70	1.85

Table B.3. IL-6 Mean Values.

SEX	Exposure	Time	N	Min	Max	Mean	SD
Males	DE	1	49	0.17	527.77	52.67	116.44
		6	48	0.17	509.99	54.23	122.65
		24	26	1.02	530.43	86.75	147.82
	CA	1	48	0.19	527.15	59.61	124.43
		6	45	0.65	522.20	50.64	110.27
		24	27	0.67	528.07	89.36	149.58
Females	DE	1	21	0.80	43.37	8.29	9.16
		6	21	0.33	14.99	6.00	4.67
		24	13	1.20	77.58	10.05	20.55
	CA	1	20	0.33	99.64	11.66	21.59
		6	20	0.17	12.33	4.75	3.85
		24	14	0.59	10.94	4.02	3.27

Table B.4. WBC Mean Values.

SEX	Exposure	Time		N	Min	Max	Mean	SD
Males	DE	1	WBC	60	3.70	10.00	5.77	1.26
		6	WBC	60	3.80	9.90	6.61	1.54
		24	WBC	60	2.90	9.80	5.63	1.53
	CA	1	WBC	58	3.10	9.40	5.81	1.32
		6	WBC	55	4.40	10.40	6.63	1.49
		24	WBC	60	3.20	8.30	5.50	1.18
Females	DE	1	WBC	32	2.60	9.30	5.53	1.54
		6	WBC	33	3.60	11.50	6.41	1.79
		24	WBC	34	2.60	8.10	5.14	1.20
	CA	1	WBC	35	2.50	10.40	5.78	1.62
		6	WBC	34	3.50	10.90	6.52	1.70
		24	WBC	33	2.80	9.60	5.31	1.35

Table B.5. Cell Blood Counts Means (Absolute Values).

SEX	Exposure	Time		N	Min	Max	Mean	SD
Males	DE	1	Neutrophils	60	1410	6230	3328.48	948.11
		6	Neutrophils	60	1733	7108	3903.00	1211.08
		24	Neutrophils	60	1505	7614	3243.40	1164.24
	CA	1	Neutrophils	59	1081	6420	3336.27	1059.80
		6	Neutrophils	56	1989	6753	3903.50	1219.43
		24	Neutrophils	60	1284	5312	3140.38	876.44
Females	DE	1	Neutrophils	32	1427	6408	3196.72	1123.04
		6	Neutrophils	33	1698	7613	3716.00	1443.11
		24	Neutrophils	34	1161	5190	2932.18	957.93
	CA	1	Neutrophils	35	1263	7363	3456.26	1375.62
		6	Neutrophils	35	1425	7281	3677.51	1446.16
		24	Neutrophils	33	1627	6739	3018.61	1115.83
Males	DE	1	Lymphocytes	60	761	2643	1809.62	404.84
		6	Lymphocytes	60	975	3258	2002.80	509.66
		24	Lymphocytes	60	765	3420	1777.48	480.15
	CA	1	Lymphocytes	59	1240	2647	1781.81	332.98
		6	Lymphocytes	56	1334	3115	1969.38	429.31
		24	Lymphocytes	60	838	2770	1765.58	416.64
Females	DE	1	Lymphocytes	32	892	3054	1809.38	568.17
		6	Lymphocytes	33	1001	3396	2081.42	608.55
		24	Lymphocytes	34	924	2643	1716.59	498.95
	CA	1	Lymphocytes	35	973	3191	1777.40	512.95
		6	Lymphocytes	35	1139	3394	2142.23	548.78
		24	Lymphocytes	33	885	2950	1782.33	518.78
Males	DE	1	Monocytes	60	176	860	442.50	136.36
		6	Monocytes	60	279	833	516.40	133.79
		24	Monocytes	60	184	892	432.07	141.41
	CA	1	Monocytes	59	232	979	462.15	149.40
		6	Monocytes	56	285	878	514.39	120.93
		24	Monocytes	60	188	730	417.03	128.34
Females	DE	1	Monocytes	32	165	559	367.66	94.371
		6	Monocytes	33	234	733	443.88	128.79
		24	Monocytes	34	188	558	347.56	97.83
	CA	1	Monocytes	35	170	771	390.26	112.31
		6	Monocytes	35	233	796	466.20	133.48
		24	Monocytes	33	190	608	362.73	95.64
Males	DE	1	Eosinophils	60	19	618	165.92	117.11
		6	Eosinophils	60	14	583	159.88	115.97
		24	Eosinophils	60	11	644	151.08	114.00
	CA	1	Eosinophils	59	31	630	180.03	131.76
		6	Eosinophils	56	8	624	169.23	131.29
		24	Eosinophils	60	17	592	156.22	120.86
Females	DE	1	Eosinophils	32	14	524	133.41	109.77
		6	Eosinophils	33	14	542	134.55	124.07
		24	Eosinophils	34	16	400	120.21	92.87
	CA	1	Eosinophils	35	17	464	134.03	101.16
		6	Eosinophils	35	17	661	139.03	137.58
		24	Eosinophils	33	17	363	117.30	86.858
Males	DE	1	Basophils	60	5	61	23.80	10.72
		6	Basophils	60	9	72	29.58	14.21
		24	Basophils	60	0	70	22.82	11.68
	CA	1	Basophils	59	6	59	22.90	10.79
		6	Basophils	56	13	64	31.25	12.26
		24	Basophils	60	8	89	24.53	12.79
Females	DE	1	Basophils	32	5	47	24.16	10.88
		6	Basophils	33	12	55	30.52	10.18
		24	Basophils	34	0	53	21.82	11.14
	CA	1	Basophils	35	6	64	25.11	13.08
		6	Basophils	35	0	90	35.37	17.02
		24	Basophils	33	8	108	28.09	18.66

Table B.6. Cell Blood Counts (Percent Values).

SEX	Exposure	Time		N	Min	Max	Mean	SD
Males	DE	1	Neutrophils	60	38.10	73.10	57.07	7.29
		6	Neutrophils	60	45.50	72.90	58.36	7.35
		24	Neutrophils	60	41.80	81.00	56.83	7.93
	CA	1	Neutrophils	58	31.80	71.20	56.69	7.50
		6	Neutrophils	55	42.70	73.40	58.52	7.47
		24	Neutrophils	60	34.70	75.70	56.65	7.55
Females	DE	1	Neutrophils	32	43.80	73.80	57.04	8.19
		6	Neutrophils	33	37.90	79.50	56.82	9.91
		24	Neutrophils	34	35.00	73.00	56.32	9.35
	CA	1	Neutrophils	35	40.50	75.70	58.29	9.82
		6	Neutrophils	34	40.20	76.10	55.64	9.74
		24	Neutrophils	33	39.10	73.60	55.95	8.91
Males	DE	1	Lymphocytes	60	14.10	55.00	31.98	7.03
		6	Lymphocytes	60	16.50	47.50	30.87	6.86
		24	Lymphocytes	60	15.00	52.40	32.38	7.53
	CA	1	Lymphocytes	58	15.80	51.50	31.76	6.45
		6	Lymphocytes	55	17.60	46.40	30.54	6.39
		24	Lymphocytes	60	17.10	53.50	32.56	6.77
Females	DE	1	Lymphocytes	32	18.30	48.70	33.31	7.98
		6	Lymphocytes	33	14.50	54.60	33.46	8.70
		24	Lymphocytes	34	20.20	55.30	34.02	8.75
	CA	1	Lymphocytes	35	16.70	50.70	31.94	8.82
		6	Lymphocytes	34	16.90	50.50	34.15	8.60
		24	Lymphocytes	33	20.10	52.50	34.29	8.59
Males	DE	1	Monocytes	60	3.80	11.60	7.71	1.85
		6	Monocytes	60	4.00	12.40	7.93	1.57
		24	Monocytes	60	3.50	12.40	7.75	1.66
	CA	1	Monocytes	58	3.90	15.30	8.10	2.21
		6	Monocytes	55	4.40	12.40	7.96	1.69
		24	Monocytes	60	4.70	12.70	7.58	1.64
Females	DE	1	Monocytes	32	4.50	9.00	6.81	1.37
		6	Monocytes	33	4.5	10.6	7.09	1.67
		24	Monocytes	34	3.6	9.1	6.90	1.69
	CA	1	Monocytes	35	3.5	16.4	6.99	2.11
		6	Monocytes	34	4.0	17.1	7.44	2.42
		24	Monocytes	33	4.6	15.6	7.04	2.02
Males	DE	1	Eosinophils	60	.3	9.5	2.83	1.76
		6	Eosinophils	60	.3	8.1	2.38	1.57
		24	Eosinophils	60	.3	9.9	2.63	1.74
	CA	1	Eosinophils	58	.8	10.7	3.04	2.11
		6	Eosinophils	55	.1	9.9	2.50	1.86
		24	Eosinophils	60	.4	10.1	2.76	1.91
Females	DE	1	Eosinophils	32	.3	8.4	2.39	1.79
		6	Eosinophils	33	.2	9.5	2.14	2.05
		24	Eosinophils	34	.3	7.5	2.32	1.66
	CA	1	Eosinophils	35	.3	8.3	2.35	1.76
		6	Eosinophils	34	.3	11.2	2.21	2.33
		24	Eosinophils	33	.4	7.4	2.19	1.54
Males	DE	1	Basophils	60	.1	.9	0.41	0.16
		6	Basophils	60	.1	1.3	0.46	0.21
		24	Basophils	60	.0	1.4	0.42	0.21
	CA	1	Basophils	58	.1	1.0	0.41	0.19
		6	Basophils	55	.2	1.0	0.48	0.17
		24	Basophils	60	.2	1.1	0.45	0.19
Females	DE	1	Basophils	32	.1	1.0	0.44	0.19
		6	Basophils	33	.2	1.0	0.50	0.19
		24	Basophils	34	.0	.9	0.44	0.19
	CA	1	Basophils	35	.1	.8	0.43	0.17
		6	Basophils	34	.0	1.7	0.56	0.31
		24	Basophils	33	.2	1.9	0.53	0.33

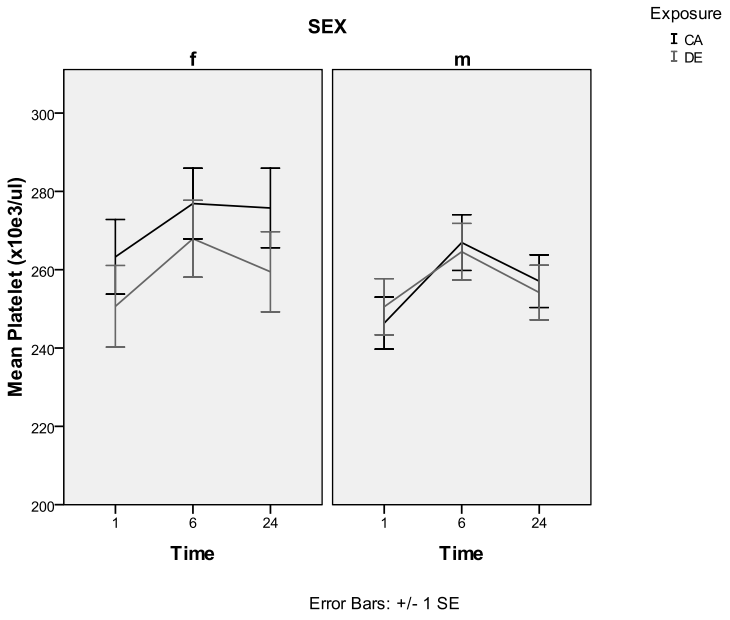
Table B.7. Fibrinogen Mean Values.

SEX	Exposure		Time	N	Min	Max	Mean	SD
Males	DE	1	Fibrinogen	57	148.60	428.40	248.41	58.42
		24	Fibrinogen	58	155.70	506.00	253.81	70.90
	CA	1	Fibrinogen	53	120.60	379.10	245.23	54.91
		24	Fibrinogen	54	134.50	450.20	245.55	55.49
Females	DE	1	Fibrinogen	24	182.50	432.50	280.10	56.66
		24	Fibrinogen	22	198.10	438.70	278.46	58.25
	CA	1	Fibrinogen	22	222.20	451.70	287.76	67.54
		24	Fibrinogen	27	154.50	444.70	282.65	63.21

Table B.8. Mean Platelet Counts (x10e^3/µl).

SEX	Exposure	Time	N	Min	Max	Mean	S.D.
Males	DE	1	60	151	399	250.53	55.610
		6	60	164	421	264.58	56.046
		24	60	163	376	254.17	54.061
	CA	1	59	171	362	246.36	51.001
		6	56	174	382	266.93	53.311
		24	60	174	387	257.07	52.106
Females	DE	1	32	152	358	250.66	58.975
		6	33	181	364	267.91	56.363
		24	34	152	370	259.47	59.703
	CA	1	35	167	381	263.29	56.414
		6	35	181	363	276.89	53.421
		24	33	169	373	275.76	58.314

DE = Diesel Exhaust; CA = Clean Air; S.D. = Standard deviation.



Graph B.1. Mean Platelet Count by Sex.

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Chapter 4

Analysis of Sex Differences in Self Reported Symptoms after Acute Exposure to Diesel Exhaust

4.1 Abstract

Background: Inhalation of diesel exhaust has been associated with range of health effects from eye irritation to cardiovascular effects. Sex differences have been observed in self reported somatic symptoms after exposure to an injurious substance.
Aims: To evaluate possible sex differences in self reported health symptoms after acute exposure to diesel exhaust in a controlled environmental facility.
Method: In a crossover design study, healthy subjects were exposed to 300µg/m^3 of diesel exhaust and clean air in random order for 1 hour each in a controlled environmental exposure chamber on two different days (≥1 week washout). Subjects completed eight symptom questionnaires during the study session which were analyzed using a generalized linear equation model.
Results: There were statistically significant diesel exhaust induced sex differences in somatic ($p = 0.0501$) and lower respiratory ($p < 0.0001$) symptoms with females primarily having higher mean symptom severity ratings than males.
Conclusion: In healthy volunteers, acute exposure to 300µg/m^3 of diesel exhaust for an hour in a controlled environmental study resulted in females having statistically significantly higher self reported somatic and lower respiratory symptom severity ratings than males relative to clean air exposure. This indicates that females may be more sensitive to the effects of diesel exhaust exposure than males.

4.2 Introduction

Diesel exhaust is produced from the combustion of diesel fuel. Due to the superior performance characteristics of the diesel engine, it has been widely used to power vehicles and other machineries (USEPA HAD 2002). Compared to gasoline engines, diesel exhaust engines emit more particulate matter per mile driven (Tox Probe). This along with its wide use has made diesel exhaust a significant contributor to ambient air pollution and the largest single source of airborne particulate matter (PM) in many urban areas (Riedl and Diaz-Sanchez 2005).

Due to the ubiquitous nature of diesel exhaust and the potential for the high exposure of certain populations like workers using diesel-powered machinery and people who live close to diesel traffic, diesel exhaust has been one of the most studied

environmental pollutants in an attempt to determine its toxicological effects in humans and rodents. Inhalation of diesel exhaust is the primary route of exposure therefore experimental studies have often been done to examine the association between diesel exhaust exposure and respiratory and cardiovascular effects. The measures that have been used to assess these effects include cell changes (in-vivo and in-vitro), biomarkers, physiological changes and self reported symptoms following diesel exhaust exposure.

4.2.1 Symptoms of Diesel Exhaust Exposure

Epidemiological and clinical studies that have been done to date have shown that there are a range of symptoms that can occur after exposure to diesel exhaust via inhalation. Depending on the concentration of the exposure, acute exposure to diesel exhaust has been shown to cause the following symptoms and effects: acute irritation (e.g., eye, nose, throat, bronchial), neurophysiological symptoms (e.g., lightheadedness, nausea), respiratory symptoms (cough, phlegm); there is also evidence for an immunologic effect (the exacerbation of allergenic responses to known allergens and asthma-like symptoms) and cardiovascular effects (USEPA HAD 2002; Donaldson et al. 2001).

Following inhalation of diesel exhaust, not everyone will report the same types, frequency and severity of symptoms. This is because there are many determinants that affect the type, frequency and severity of self reported symptoms following the inhalation of a harmful substance like diesel exhaust. A broad definition of a symptom can be gotten from Gijsber's model of physical symptom perception where a symptom is defined as "an aversely perceived internal state" (Gijsbers et al 1997). By adapting this model to self

reported symptoms following an inhalation exposure and incorporating Becklake and Kauffmans (1999) definition of determinants of airway behavior, within this modified model, a determinant of a self reported symptom can be seen as anything that causes or does not cause a change in the occurrence and perception of a symptom, and anything that may increase or decrease the risk of perceiving the symptom. This definition encompasses a "biopsychosocial" (Gijsbers et al 1997) explanation of the factors that contribute to the types, frequency and severity of self reported symptoms following the exposure to an inhaled air pollutant like diesel exhaust. A key assumption of this model is that a self reported symptom is "the outcome of a perceptual process" (Gijsbers et al 1997). Two individual factors where the biological, psychological and environmental aspects of self reported symptom perception intercept are sex and gender.

4.2.2 Sex, Gender and Symptoms

In public health terms, sex can be thought to determine genetically based sensitivity to health determinants and gender can be thought to express some social forces that can influence exposure and responses to health determinants (Messing and Stellman 2006). The effects of sex and gender on the adverse health effects following the exposure to a harmful substance or stimuli often complement each other. Gender often has a big influence on the context, types and levels of the substance that males and females get exposed to especially in the occupational setting and sex often has a big effect on how males and females absorb, distribute, metabolize and excrete the xenobiotic, once the exposure has taken place (Arbuckle 2006 and Dimich-Wald et al 2006). Thus it is easy to

see how these two factors (sex and gender) are involved in the proposed biopsychosocial model of self reported symptoms and where they intersect with each other (figure 1).

It follows therefore that determinants of self reported symptoms related to exposures can be broadly grouped into biological determinants implicit in the word sex and sociocultural determinants implicit in the word gender (Becklake and Kauffmann 1999).

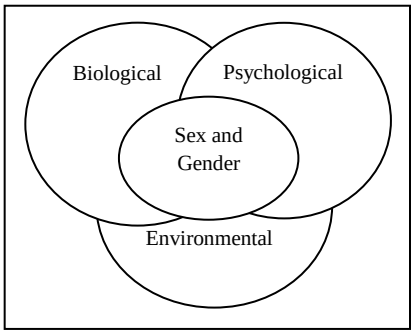


Figure 4.1. Intersetion of Sex and Gender and Self Reported Symptom Determinants.

This is especially so because sex differences with respect to symptoms are thought to involve differences in neuroanatomical, neurophysiological and neurobiological pathways that are activated upon exposure to an injurious substance and which produce differences in the perception, processing and modulation of the resulting symptoms (Barsky et al 2001). In contrast to that, gender related differences in symptom perception and sensitivity (Dimich et al 2006 and Barsky et al 2001) especially with regards to somatic symptoms are thought to involve innate differences in somatic and visceral perceptions, differences in the socializing process and differences in symptom labeling, description and reporting (Barsky et al 2001). Thus males and females often differ in the types, frequency and severity of self reported symptoms with females having been shown to report “more intense, more numerous and more frequent bodily symptoms than men” (Barsky et al 2001 and Kroenke et al 1990).

This observed gender difference with regards to somatic symptoms is one that has been well established and is independent of the symptom measure, response format, the time frame used and the population being studied in females (Gijsbers et al 1997). Looking at the proposed biological and social factors that are thought to contribute to both sex and gender differences (Table 1) in relation to reported somatic symptoms and presentations, it becomes obvious that it can be difficult to distinguish between the effects of sex and gender as they often work together or modulate one another in the perception and presentation of self reported symptoms.

Table 4.1. Proposed Determinants of Self Reported Symptoms.

Determinants	Examples
Biological / Physiological	1. Dimensional – there are differences in airway dimensions relative to lung size e.g. women exhibit on average a higher flow rate in relation to lung size than men. 2. Immunological – there are differences in total serum IgE levels. 3. Hormonal – Sex hormones influence many aspects of airway behavior e.g. female airways are responsive to their sex hormones and cyclical fluctuations. 4. Sensitivity – Laboratory and field studies have shown that women are more sensitive to external environmental cues (including stress) and men to internal physiological stimuli in noticing, defining and reacting to physical symptoms.
Psychosocial and Environmental	1. Some psychosocial factors which are more prevalent in women are strongly associated with symptom reporting, particularly depressive and anxiety disorders, as well as a history of sexual or physical abuse. 2. Other psychosocial antecedents that have been hypothesized include cultural factors permitting less stoicism and greater expressiveness among women; amplification of somatic symptoms; a lower threshold for seeking health care; and gender differences in social roles and responsibilities

Sources: Barsky et al 2001, Kroenke et al 1990 and Becklake and Kauffmann 1999

4.2.3 Sex and Respiratory Related Symptoms

Overall, there has been increasing evidence of the interaction of biological factors with socio-environmental factors in the perception, reporting, and interpretation of respiratory symptoms related to airway disease (Becklake and Kauffmann 1999). When looking at self reported symptoms in relation to an inhaled air pollutant like diesel exhaust, one is usually interested in assessing both primary (e.g. cough and irritation of the nose) and secondary (e.g., fatigue and body pain) respiratory related symptoms and morbidity. With regards to respiratory morbidity, comparatively little attention has been

given in the scientific literature to the potential effect of sex and there is a tendency to control for sex instead of examining sex based influences on health (Dimich-Wald et al 2006).

In 2006, Dimich-Ward et al looked at gender differences in respiratory morbidity based on surveys of food service workers, radiographers and respiratory therapists and they found that across occupations, women consistently had a greater respiratory morbidity for symptoms associated with shortness of breath while men usually had a higher prevalence of phlegm complaints.

Ernstgard et al in 2002 examined differences between men and women in acute health effects after a controlled chamber exposure to 2-propanol and m-xylene vapors. Within the self reported symptoms, they found that the rating for throat or airway discomfort increased more in women during exposure to 2-propanol or m-xylene and that the rating for fatigue was more increased in men after a one hour exposure to 2-propanol but more increased in women after a two hour exposure to 2-propanol (Ernstgard et al 2002).

This then raises the question of whether the observations of the studies above as well as the sex differences observed in relation to somatic symptom reporting could be present in self reported symptoms following diesel exhaust exposure and whether this trend extends beyond somatic symptoms to other respiratory symptoms. Therefore the purpose of this study is to investigate whether there are sex differences in the self reported symptoms of males and females after acute exposure to diesel exhaust.

4.3 Materials and Methods

4.3.1 Data Collection

The data that were used for this study are part of the datasets generated from a previous study of diesel exhaust effects. The study was funded by the Department of Defense (DOD) through a grant to the University of Medicine and Dentistry of New Jersey (UMDNJ) (Grant # DAMD17-03-1-0537; Principal Investigator: Dr. Nancy Fiedler).

4.3.2 Exposure Conditions and Experimental Design

A crossover design was used in which each subject underwent two exposure conditions on two separate days in random order; one was to a diluted diesel exhaust normalized to PM_{10} concentration of 300μg/m^3 and the other was to filtered clean ambient air of Busch campus in Piscataway, a suburban campus of Rutgers University. Both exposure conditions were 1 hour in duration and took place at the Environmental and Occupational Health Sciences Institutes' (EOHSI) Controlled Environmental Facility (CEF). Each exposure condition was presented with and without an acute psychological stressor to two groups of subjects high and low in self reported chemical intolerance (CI). Subjects were also asked to fast overnight the day before the study until after each exposure session and to also abstain from any exercise.

There were no significant differences in the exposure conditions for all subjects. Overall, the concentration of PM_{10} that the subjects were exposed to was 276±13μg/m^3 which was close to the target concentration of 300μg/m^3.

Table 4.2. Mean Air Characteristics and Composition.

	Diesel Exhaust	Clean Air
PM_{10} ($\mu g/m^3$)	276 ± 13	6 ± 5
NO_X (ppm)	3.63 ± 0.90	0.02 ± 0.02
CO (ppm)	3.79 ± 0.76	0.91 ± 0.21
1 Nitropyrene (ng/m^3)	2.68 ± 0.51	<0.06

4.3.3 Subject Selections and Demographic Data

110 healthy non-smoking men and women between the ages of 18 and 50 were recruited from the local community in Piscataway, New Jersey, based on the established study criteria; 100 subjects completed the study.

As mentioned previously, some subjects were simultaneously exposed to a psychological stressor and to diesel exhaust. Due to the fact that we were analyzing self reported symptoms which have the potential to be heavily influenced by psychological stress, therefore, all subjects who were also exposed to the psychological stressor were excluded from the analysis leaving 49 subjects.

Table 4.3. Demographic Information and Baseline Subject Characteristics.

	Females N = 22				Males N = 27			
	Min	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**
AGE (years)	19	43	24.09	5.67	18	34	23.11	3.57
Education (Years)*	12	22	15.64	2.35	12	20	15.91	2.26
Pulse [M]	50	94	70.18	10.81	56	86	69.15	7.86
Respiration [M]	14	20	16.36	2.01	10	20	15.38	2.10
Temperature ^oF [M]	96	99	98.20	0.65	96	99	98.09	0.79
Height (inches)	60	73	64.05	2.69	65	75	69.06	2.67
Weight(lb)	104	200	134.50	25.79	136	236	173.00	25.06
Race	**Frequency (%)**				**Frequency (%)**			
Asian / Pacific Islander	9	(40.91)			10	(37.04)		
Black not Hispanic	1	(4.55)			4	(14.81)		
Hispanic or Latino	4	(18.18)			6	(22.22)		
Other/Unknown	1	(4.55)			0	(0)		
White not Hispanic	7	(31.81)			7	(25.93)		

[M] = Some subjects had missing data; For Pulse and Respiration N = 26 for Males; For Temperature N = 19 for Females and N = 25 for Males. *****Education Calculated as High school (12 years) + Years of college or other.

4.3.4 Symptom Questionnaire

A symptom questionnaire that consisted of 37 symptom questions that could be grouped into the following symptom clusters: 1) somatic 2) eye irritation 3) anxiety 4) lower respiratory, 5) upper respiratory, 6) central nervous system (CNS) 7) acute phase reaction (APR) and 8) no load (general symptoms) was used (see table 3 for a list of individual symptoms).

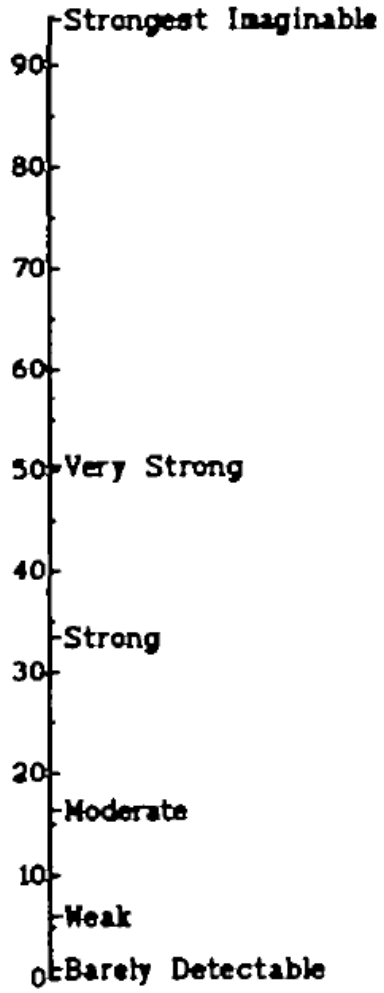


Figure 4.2. Labeled Magnitude Scale.
Source: Green et al 1996 (Reproduced with permission from Chemical Senses).

These clusters were chosen because they represent categories of symptoms that have been directly associated with either upstream responses to diesel exhaust exposure such as upper and lower respiratory symptoms or which are thought to be an indirect

representation of downstream systemic changes in response to diesel exhaust exposure such as acute phase reaction symptoms which reflect the presence and intensity of systemic inflammation (Gabay et al. 1999).

The symptoms were rated using a labeled magnitude scale that was developed by Green et al. (1996) and which ranged from 0 (barely detectable) to 100 (strongest imaginable) (see Figure 2).

4.3.5 Questionnaire Administration Protocol

The questionnaire was administered at eight times before, during and after the experimental exposure to diesel exhaust and clean air (see figure 3).

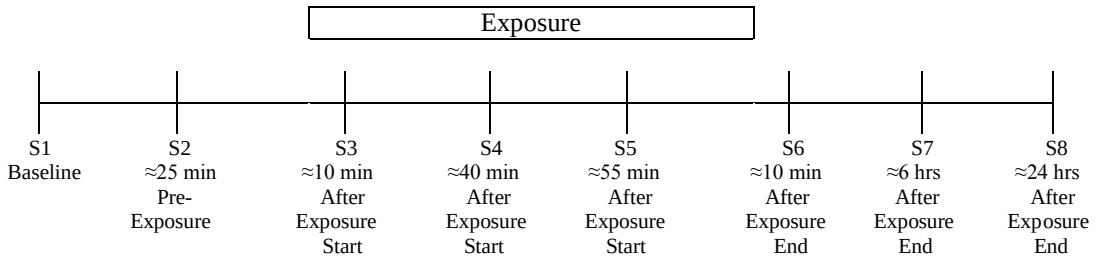


Figure 4.3. Questionnaire Study Protocol.
S = Symptom questionnaire.

4.4 Statistical Methods

Descriptive statistical analysis, histograms and box plots were used to evaluate the distribution of all the variables and to check for outliers. Based on the fact that the data were right skewed, a Poisson distribution was used in the final analysis.

4.4.1 Calculation of Response Measures

The individual severity ratings for the 37 symptoms were added together within the previously mentioned eight categories (see table 4) to give eight total summary symptom severity response measures.

Table 4.4. Description of Symptom Categories and The Creation of the Summary Severity Scores.

Symptom Categories		Individual Symptoms	Range of Total Summary Score
Somatic	=	Skin irritation + Ear ringing + Leg cramp + Perspiration + Leg tingling + Back pain	0 - 600
Eye Irritation	=	Eye Irritation + Running Eyes	0 – 300
Anxiety	=	Jittery + Nervous + Palpitation + Tense + Worried	0 – 500
Lower Respiratory	=	Shortness of breath + Chest pain + Wheezing + Chest tightness + Coughing + pain upon deep inspiration	0 – 800
Upper Respiratory	=	Nasal congestion + Sneezing + Choking + Throat irritation + Nose irritation	0 – 500
Central Nervous System	=	Disoriented + Dizzy + Headache + Light headed	0 – 400
Acute Phase Reaction	=	Fatigue + Drowsy + Concentration + Nausea + Body temperature + Body ache + Stomach ache	0 – 700
No Load	=	Bad taste + Facial temperature	0 – 200

To address the aim of the paper, a Poisson regression using a generalized linear model with repeated analysis and contrast was done to see if there was a significant three way interaction between exposure (diesel exhaust and clean air), sampling time (Time 1 to Time 8) and sex (males and females). The final model contained sex as a between subjects variable, exposure and time as within subjects variables with the eight symptom outcome variables as the dependent variables. The ‘exposure*time*sex’ three way interaction was examined to assess the hypothesis of interest.

Table 4.5a. Mean Total Symptom Severity Ratings by Category and the Time Reported.

			T1	T2	T3	T4	T5	T6	T7	T8
			Mean (S.D.) N	Mean S.D. N	Mean S.D. N	Mean S.D. N	Mean S.D. N	Mean S.D. N	Mean S.D. N	Mean S.D. N
Somatic	**Males**	**DE**	0.56 (1.60) 27	2.59 (8.01) 27	1.67 (4.16) 27	2.96 (7.63) 27	1.48 (4.96) 27	1.11 (4.06) 27	0.56 (2.12) 27	0.74 (2.67) 27
		CA	1.67 (6.05) 27	0.93 (2.42) 27	2.41 (5.26) 27	3.85 (6.97) 26	3.89 (6.70) 27	2.41 (5.78) 27	0.58 (2.16) 26	0.74 (2.67) 27
	Females	**DE**	0.91 (2.51) 22	1.82 (3.95) 22	2.95 (6.30) 22	4.55 (9.87) 22	7.73 (13.69) 22	2.73 (4.81) 22	1.82 (4.24) 22	2.27 (5.92) 22
		CA	3.64 (6.93) 22	1.59 (3.58) 22	3.64 (7.27) 22	2.50 (5.72) 22	2.05 (5.27) 22	1.14 (3.43) 22	2.50 (7.98) 22	3.18 (7.49) 22
Eye Irritation	**Males**	**DE**	2.04 (5.42) 27	2.41 (6.41) 27	6.67 (19.32) 27	4.63 (7.96) 27	4.44 (5.94) 27	1.30 (4.07) 27	0.19 (0.96) 27	0.00 (0.00) 26
		CA	2.78 (6.84) 27	2.41 (8.70) 27	4.81 (15.35) 27	2.12 (6.19) 26	1.48 (6.77) 27	0.19 (0.96) 27	0.19 (0.98) 26	1.48 (4.77) 27
	Females	**DE**	0.91 (2.51) 22	1.14 (2.64) 22	4.77 (9.57) 22	6.59 (11.06) 22	5.00 (10.80) 22	1.14 (3.06) 22	0.45 (1.47) 22	0.68 (2.34) 22
		CA	0.68 (1.76) 22	1.36 (5.39) 22	2.62 (9.83) 21	1.67 (6.58) 21	2.27 (9.60) 22	0.45 (1.47) 22	1.14 (3.43) 22	0.91 (3.32) 22
Anxiety	**Males**	**DE**	4.07 (8.78) 27	4.26 (12.91) 27	4.44 (6.84) 27	9.07 (33.69) 27	5.37 (13.86) 27	3.52 (12.77) 27	0.74 (3.01) 27	0.74 (3.01) 27
		CA	3.33 (6.50) 27	4.44 (9.84) 27	2.41 (4.47) 27	1.73 (4.23) 26	1.67 (3.67) 27	0.93 (3.93) 27	0.38 (1.96) 26	1.48 (7.70) 27
	Females	**DE**	2.27 (4.81) 22	3.64 (5.16) 22	6.14 (12.43) 22	7.50 (11.93) 22	8.86 (16.54) 22	1.82 (4.51) 22	0.00 (0.00) 22	0.00 (0.00) 22
		CA	7.73 (9.48) 22	5.23 (8.52) 22	5.24 (12.79) 21	4.76 (7.82) 21	2.73 (5.51) 22	0.68 (2.34) 22	1.59 (4.73) 22	1.59 (5.43) 22
Lower Respiratory	**Males**	**DE**	0.74 (3.01) 27	1.11 (4.24) 27	3.15 (7.86) 27	6.11 (11.88) 27	3.15 (9.42) 27	0.56 (2.12) 27	1.30 (3.28) 27	0.19 (0.98) 26
		CA	1.48 (3.62) 27	1.48 (3.04) 27	1.48 (4.34) 27	2.12 (4.04) 26	1.11 (2.53) 27	0.19 (0.96) 27	0.77 (3.06) 26	0.19 (0.96) 27
	Females	**DE**	0.23 (1.07) 22	1.14 (3.43) 22	3.64 (7.10) 22	4.55 (9.99) 22	7.05 (13.77) 22	0.68 (3.20) 22	0.91 (2.94) 22	0.91 (2.94) 22
		CA	2.27 (7.67) 22	2.77 (7.52) 22	0.68 (3.20) 22	1.59 (4.73) 22	2.05 (6.67) 22	0.23 (1.07) 22	3.86 (13.88) 22	1.36 (4.41) 22

T1 = Symptom severity reported at the start of the study session.
T2 = Symptom severity reported at ≈25 min after the start of the study session.
T3 = Symptom severity reported at ≈10 min after the start of the exposure.
T4 = Symptom severity reported at ≈40 min after the start of the exposure.
T5 = Symptom severity reported at ≈55 min after the start of the exposure.
T6 = Symptom severity reported at ≈10 min after the end of the exposure.
T7 = Symptom severity reported at ≈6 hrs after the end of the exposure.
T8 = Symptom severity reported at ≈24 hrs after the end of the exposure.
DE = Diesel Exhaust and CA = Clean Air.

Table 4.5b. Mean Total Symptom Severity Ratings by Category and the Time Reported (continued).

			T1	T2	T3	T4	T5	T6	T7	T8
			Mean (S.D.)	Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.
	Sex	Exp	N	N	N	N	N	N	N	N
Upper Respiratory	Males	DE	5.56 (12.35) 27	2.22 (6.25) 27	10.56 (28.90) 27	8.33 (10.65) 27	9.81 (14.31) 27	1.48 (3.88) 27	2.04 (4.65) 27	1.60 (4.50) 25
		CA	7.96 (11.79) 27	3.15 (7.09) 27	4.81 (10.79) 27	3.85 (9.41) 26	2.78 (7.51) 27	1.11 (3.49) 27	0.58 (1.63) 26	3.52 (6.17) 27
	Females	DE	2.95 (3.67) 22	3.18 (5.89) 22	7.95 (10.54) 22	7.95 (14.36) 22	8.86 (14.14) 22	1.59 (3.58) 22	2.27 (4.56) 22	1.36 (3.16) 22
		CA	6.82 (10.86) 22	5.00 (13.09) 22	4.00 (7.36) 20	4.29 (9.78) 21	3.18 (5.68) 22	1.14 (2.64) 22	2.05 (3.67) 22	4.55 (11.22) 22
Central Nervous System	Males	DE	0.19 (0.96) 27	1.15 (5.88) 26	6.67 (12.56) 27	7.96 (13.18) 27	8.70 (12.37) 27	2.41 (6.56) 27	0.74 (2.67) 27	0.37 (1.93) 27
		CA	1.11 (4.00) 27	0.56 (2.89) 27	1.48 (4.12) 27	0.58 (1.63) 26	1.30 (3.82) 27	0.74 (3.85) 27	0.00 (0.00) 26	0.74 (3.85) 27
	Females	DE	0.23 (1.07) 22	2.05 (4.54) 22	9.55 (11.74) 22	13.64 (23.56) 22	13.18 (25.15) 22	1.59 (3.23) 22	0.45 (1.47) 22	0.23 (1.07) 22
		CA	1.59 (4.19) 22	0.91 (3.32) 22	2.27 (4.29) 22	4.32 (12.08) 22	4.55 (13.44) 22	0.91 (3.32) 22	1.59 (4.19) 22	0.68 (2.34) 22
APR	Males	DE	8.81 (17.25) 27	8.41 (21.72) 27	10.37 (13.72) 27	17.04 (24.11) 27	16.11 (20.58) 27	7.78 (15.53) 27	3.52 (8.97) 27	4.81 (11.89) 27
		CA	5.67 (11.08) 27	9.07 (13.52) 27	13.33 (16.53) 27	11.54 (13.55 26	11.30 (14.58) 27	4.26 (9.38) 27	4.62 (9.99) 26	4.81 (13.55) 27
	Females	DE	8.86 (11.64) 22	18.64 (21.89) 22	23.41 (18.09) 21	24.77 (24.95) 21	18.86 (18.12) 22	6.14 (8.01) 22	4.77 (5.87) 22	3.18 (5.24) 22
		CA	15.00 (16.48) 22	20.45 (25.40) 22	32.14 (32.58) 22	19.52 (30.41) 22	14.77 (20.27) 22	8.18 (10.64) 22	9.77 (15.92) 22	6.36 (11.04) 22
No Load	Males	DE	1.30 (4.72) 27	0.74 (3.01) 27	2.41 (7.77) 27	4.26 (9.88) 26	3.15 (9.92) 27	0.37 (1.93) 27	0.19 (0.96) 26	0.37 (1.33) 27
		CA	2.04 (6.39) 27	1.30 (4.07) 27	1.11 (4.87) 27	0.96 (2.84) 27	0.93 (3.41) 27	0.56 (2.89) 27	0.00 (0.00) 27	0.00 (0.00) 27
	Females	DE	0.91 (3.32) 22	1.14 (3.43) 22	2.05 (4.80) 22	1.82 (3.63) 22	1.82 (3.95) 22	0.68 (2.34) 22	0.00 (0.00) 22	0.00 (4.72) 22
		CA	2.73 (7.52) 22	2.95 (5.49) 22	2.50 (4.82) 22	2.73 (6.31) 22	2.50 (5.51) 22	1.82 (4.51) 22	1.82 (4.51) 22	1.36 (4.41) 22

T1 = Symptom severity reported at the start of the study session.
T2 = Symptom severity reported at ≈25 min after the start of the study session.
T3 = Symptom severity reported at ≈10 min after the start of the exposure.
T4 = Symptom severity reported at ≈40 min after the start of the exposure.
T5 = Symptom severity reported at ≈55 min after the start of the exposure.
T6 = Symptom severity reported at ≈10 min after the end of the exposure.
T7 = Symptom severity reported at ≈6 hrs after the end of the exposure.
T8 = Symptom severity reported at ≈24 hrs after the end of the exposure.
DE = Diesel Exhaust and CA = Clean Air.

Tests of the three interactions were conducted using Type 3 score tests (Liang and Zeger, 1986) of the interaction between exposure, time and sex. Contrasts were used to test whether males and females differed with respect to the effect of exposure on changes in the symptom severity ratings from baseline to each subsequent time point. Contrasts were also used to test whether within males and within females, changes in the symptom severity ratings from baseline to each subsequent time point differed between exposures.

All statistical analysis for this paper were done using SAS software, Version 9.1 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA) and SPSS software, Version 17.0 (SPSS inc., Chicago IL). Also, some graphs were created using Microsoft Excel 2007. P-values less than or equal to 0.05 were considered to be significant.

4.5 Results

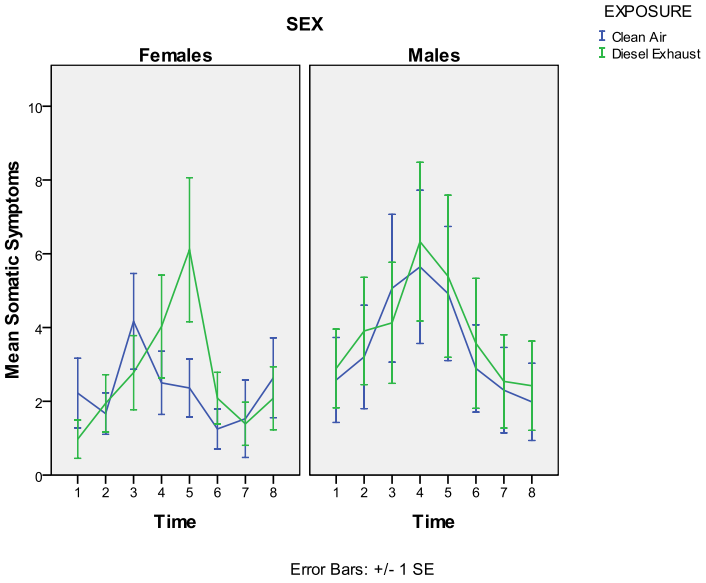
Interaction Effect of Exposure, Time and Sex on Symptoms:

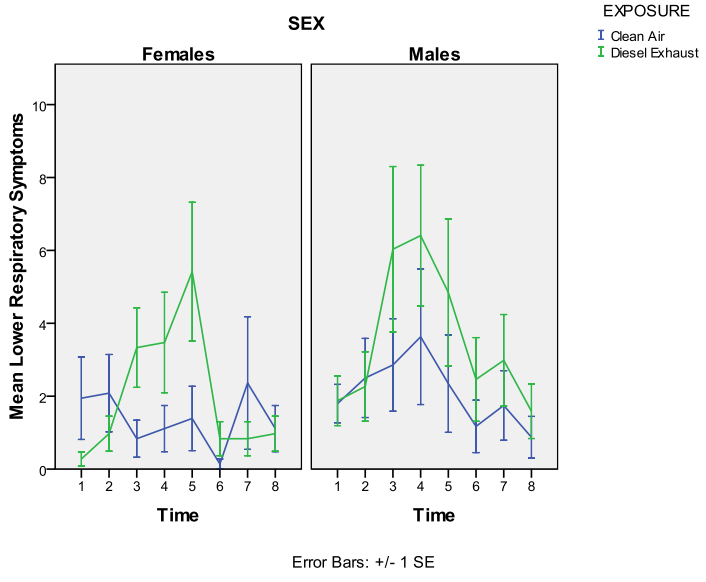
The results of the analysis showed that out of the eight symptom categories that were assessed, there was a statistically significant three way 'exposure*time*sex' interaction effect on somatic ($p = 0.0501$) and lower respiratory symptom severity ratings ($p < 0.0001$). Upper respiratory and acute phase reaction did not show a significant three way 'exposure*time*sex' interaction effect (see table 6).

Table 4.6. Three Way Interaction Results.

	Exposure*Time*Sex		
Variables	**DF**	**Chi Square**	**p Value**
Somatic	7	14.06	0.0501
Eye Irritation	–	–	–
Anxiety	–	–	–
Lower Respiratory	7	157215	<0.0001
Upper Respiratory	7	2.73	0.91
CNS	–	–	–
APR	7	9.10	0.25
No Load	–	–	–

- = No results due to lack of convergence.
E = Exposure (Diesel Exhaust and Clean Air); T = Time (1 – 8);
S = Sex (Females and Males). $p \leq 0.05$ is significant.





Graph 4.1a-b. Line Graph of Mean Self reported Somatic and Lower Respiratory Symptoms by Sex.

For the remaining four symptom clusters (eye irritation, anxiety, central nervous system and no load) the majority of subjects had a zero severity rating and therefore there was very little variability between the groups to analyze. Therefore the four clusters could not converge in the model and so did not produce results (see tables 7a – 7d).

Table 4.7a. Count of Reported Eye Irritation Symptoms (N/Y) by Sex, Exposure and Time.

		Females				Males			
		Clean Air		Diesel Exhaust		Clean Air		Diesel Exhaust	
Time	N/Y	Freq	%	Freq	%	Freq	%	Freq	%
T1	N	19	86.4	19	86.4	21	77.8	22	81.5
	Y	3	13.6	3	13.6	6	22.2	5	18.5
T2	N	20	90.9	18	81.8	27	100.0	27	100.0
	Y	2	9.1	4	18.2	22	81.5	22	81.5
T3	N	18	81.8	15	68.2	5	18.5	5	18.5
	Y	4	18.2	7	31.8	27	100.0	27	100.0
T4	N	19	86.4	12	54.5	23	85.2	17	63.0
	Y	3	13.6	10	45.5	4	14.8	10	37.0
T5	N	20	90.9	13	59.1	27	100.0	27	100.0
	Y	2	9.1	9	40.9	21	77.8	16	59.3
T6	N	20	90.9	19	86.4	6	22.2	11	40.7
	Y	2	9.1	3	13.6	27	100.0	27	100.0
T7	N	19	86.4	20	90.9	25	92.6	14	51.9
	Y	3	13.6	2	9.1	2	7.4	13	48.1
T8	N	20	90.9	20	90.9	27	100.0	27	100.0
	Y	2	9.1	2	9.1	26	96.3	23	85.2

N = Symptom severity rating of "0". Y = Symptom severity rating > "0". Freq = Frequency and % = Percent.

Table 4.7b. Count of Reported Anxiety Symptoms (N/Y) by Sex, Exposure and Time.

		Females				Males			
		Clean Air		Diesel Exhaust		Clean Air		Diesel Exhaust	
Time	N/Y	Freq	%	Freq	%	Freq	%	Freq	%
T1	N	9	40.9	17	77.3	19	70.4	20	74.1
	Y	13	59.1	5	22.7	8	29.6	7	25.9
T2	N	12	54.5	13	59.1	18	66.7	21	77.8
	Y	10	45.5	9	40.9	9	33.3	6	22.2
T3	N	14	63.6	13	59.1	20	74.1	16	59.3
	Y	8	36.4	9	40.9	7	25.9	11	40.7
T4	N	13	59.1	13	59.1	22	81.5	20	74.1
	Y	9	40.9	9	40.9	5	18.5	7	25.9
T5	N	17	77.3	14	63.6	22	81.5	21	77.8
	Y	5	22.7	8	36.4	5	18.5	6	22.2
T6	N	20	90.9	18	81.8	25	92.6	23	85.2
	Y	2	9.1	4	18.2	2	7.4	4	14.8
T7	N	19	86.4	22	100.0	25	92.6	25	92.6
	Y	3	13.6	0	0.0	2	7.4	2	7.4
T8	N	19	86.4	22	100.0	26	96.3	25	92.6
	Y	3	13.6	0	0.0	1	3.7	2	7.4

N = Symptom severity rating of "0". Y = Symptom severity rating > "0". Freq = Frequency and % = Percent.

Table 4.7c. Count of Reported Central Nervous System Symptoms (N/Y) by Sex, Exposure and Time.

		Females				Males			
		Clean Air		Diesel Exhaust		Clean Air		Diesel Exhaust	
Time	N/Y	Freq	%	Freq	%	Freq	%	Freq	%
T1	N	19	86.4	21	95.5	25	92.6	26	96.3
	Y	3	13.6	1	4.5	2	7.4	1	3.7
T2	N	20	90.9	16	72.7	26	96.3	25	92.6
	Y	2	9.1	6	27.3	1	3.7	2	7.4
T3	N	16	72.7	8	36.4	23	85.2	16	59.3
	Y	6	27.3	14	63.6	4	14.8	11	40.7
T4	N	17	77.3	10	45.5	23	85.2	18	66.7
	Y	5	22.7	12	54.5	4	14.8	9	33.3
T5	N	18	81.8	10	45.5	24	88.9	15	55.6
	Y	4	18.2	12	54.5	3	11.1	12	44.4
T6	N	20	90.9	17	77.3	26	96.3	22	81.5
	Y	2	9.1	5	22.7	1	3.7	5	18.5
T7	N	19	86.4	20	90.9	26	96.3	25	92.6
	Y	3	13.6	2	9.1	1	3.7	2	7.4
T8	N	20	90.9	21	95.5	26	96.3	26	96.3
	Y	2	9.1	1	4.5	1	3.7	1	3.7

N = Symptom severity rating of "0". Y = Symptom severity rating > "0". Freq = Frequency and % = Percent.

Table 4.7d. Count of Reported No Load (General) Symptoms (N/Y) by Sex, Exposure and Time.

		Females				Males			
		Clean Air		Diesel Exhaust		Clean Air		Diesel Exhaust	
Time	N/Y	Freq	%	Freq	%	Freq	%	Freq	%
T1	N	19	86.4	20	90.9	23	85.2	25	92.6
	Y	3	13.6	2	9.1	4	14.8	2	7.4
T2	N	16	72.7	19	86.4	23	85.2	25	92.6
	Y	6	27.3	3	13.6	4	14.8	2	7.4
T3	N	16	72.7	18	81.8	25	92.6	21	77.8
	Y	6	27.3	4	18.2	2	7.4	6	22.2
T4	N	17	77.3	17	77.3	23	85.2	19	70.4
	Y	5	22.7	5	22.7	4	14.8	8	29.6
T5	N	17	77.3	17	77.3	25	92.6	21	77.8
	Y	5	22.7	5	22.7	2	7.4	6	22.2
T6	N	18	81.8	20	90.9	26	96.3	26	96.3
	Y	4	18.2	2	9.1	1	3.7	1	3.7
T7	N	18	81.8	22	100.0	26	96.3	26	96.3
	Y	4	18.2	0	0.0	1	3.7	1	3.7
T8	N	20	90.9	22	100.0	27	100.0	25	92.6
	Y	2	9.1	0	0.0	0	0.0	2	7.4

N = Symptom severity rating of "0". Y = Symptom severity rating > "0". Freq = Frequency and % = Percent.

The results of the contrast analysis showed that between males and females with respect to the effect of diesel exhaust on somatic symptom severity ratings reported after baseline, there was a statistically significant change in symptoms reported at time T5 (55 minutes after the start of diesel exhaust exposure; $p = 0.0036$) and time T6 (10 minutes

after the end of the exposure to diesel exhaust; p = 0.0139) with females having higher mean severity ratings than males.

For lower respiratory symptom severity ratings, the contrast analysis showed that diesel exhaust had a borderline significant effect on severity ratings at time T3 (10 minutes after the start of the exposure; p =0.0925) and time T5 (55 minutes after the start of diesel exhaust exposure; p = 0.0762) between the sexes (see table 8) after controlling for baseline ratings with females having higher mean severity ratings than males.

Table 4.8. Contrast Between Sex (Males vs. Females) Test Results.

		Exposure by Sex Effect On T2Δ	Exposure by Sex Effect On T3Δ	Exposure by Sex Effect On T4Δ	Exposure by Sex Effect On T5Δ	Exposure by Sex Effect On T6Δ	Exposure by Sex Effect On T7Δ	Exposure by Sex Effect On T8Δ
		Chi Square (S.E.) p-Value	Chi Square (S.E.) p-Value	Chi Square (S.E.) p-Value	Chi Square (S.E.) p-Value	Chi Square (S.E.) p-Value	Chi Square (S.E.) p-Value	Chi Square (S.E.) p-Value
Variables	**Exp**							
Somatic	**DE vs CA**	0.35	0.16	2.00	8.46	6.06	0.00	0.00
		(1.02)	(1.12)	(0.81)	(0.89)	(0.79)	(1.43)	(0.89)
		0.55	0.69	0.16	0.0036*	0.0139*	1.00	0.96
Lower Respiratory	**DE vs CA**	1.32	2.83	1.63	3.15	0.71	0.11	0.48
		(1.05)	(1.50)	(1.24)	(1.02)	(1.92)	(1.21)	(1.52)
		0.25	0.0925**	0.20	0.0762**	0.40	0.74	0.49
Upper respiratory	**DE vs CA**	0.20	0.34	0.16	0.09	0.28	0.53	0.00
		(0.84)	(0.60)	(0.85)	(0.79)	(1.00)	(1.01)	(0.83)
		0.66	0.56	0.69	0.76	0.60	0.46	0.99
APR	**DE vs CA**	3.66	3.01	3.00	2.71	0.02	0.74	0.12
		(0.50)	(0.54)	(0.46)	(0.52	(0.56)	(0.64)	(0.78)
		0.0556**	0.0830**	0.0831**	0.10	0.89	0.39	0.72

ΔT2 = Change from baseline (T1) in symptom severity reported at t ≈25 min after the start of the study session.
ΔT3 = Change from baseline (T1) in symptom severity reported at ≈10 min after the start of the exposure.
ΔT4 = Change from baseline (T1) in symptom severity reported at ≈40 min after the start of the exposure.
ΔT5 = Change from baseline (T1) in symptom severity reported at ≈55 min after the start of the exposure.
ΔT6 = Change from baseline (T1) in symptom severity reported at ≈10 min after the end of the exposure.
ΔT7 = Change from baseline (T1) in symptom severity reported ≈6 hrs after the end of the exposure.
ΔT8 = Change from baseline (T1) in symptom severity reported at ≈24 hrs after the end of the exposure.
APR = Acute Phase Reaction. DE = Diesel Exhaust. CA = Clean Air.
Signt = Significant.
Effect of Sex (Males vs. Females) = the effect of sex on the diesel exhaust induced changes in symptom severity reported from baseline. * = statistically significant at p = 0.05 ** = borderline statistically significant at p = 0.05

Within females, the contrast analysis showed that there was a significant increase in the somatic and lower respiratory symptom severity ratings from baseline for all time points except T7 *(*6 hrs after the end of the exposure) and T8 (24 hrs after the end of the exposure) (Table 7) due to the main effect of diesel exhaust.

Within males, the contrast analysis showed that there was a significant increase in somatic severity ratings from baseline to T2 (25 minutes after the start of the study session) and there was a significant increase in lower respiratory severity ratings from baseline to T4 (40 minutes after the start of diesel exhaust exposure) through to T6 (10 minutes after the end of the exposure to diesel exhaust) due to the main effect of diesel exhaust.

Table 4.9. Contrast Within Sex Test Results.

		Exposure Main Effect On On T2 Δ	Exposure Main Effect On T3 Δ	Exposure Main Effect On T4 Δ	Exposure Main Effect On T5 Δ	Exposure Main Effect On T6 Δ	Exposure Main Effect On T7 Δ	Exposure Main Effect On T8 Δ
Variables	**Sex**	**Chi Square (S.E.) p-Value**	**Chi Square (S.E.) p-Value**	**Chi Square (S.E.) p-Value**	**Chi Square (S.E.) p-Value**	**Chi Square (S.E.) p-Value**	**Chi Square (S.E.) p-Value**	**Chi Square (S.E.) p-Value**
Somatic	**Males**	5.78	0.61	1.96	0.03	0.27	0.93	3.37
		(0.89)	(0.93)	(0.60)	(0.74)	(0.63)	(0.11)	(0.60)
		0.016	0.43	0.16	0.86	0.60	0.34	0.066
	Females	8.78	3.68	13.24	30.14	22.45	1.39	2.53
		(0.51)	(0.61)	(0.54)	(0.49)	(0.48)	(0.90)	(0.66)
		0.0003	0.055	0.0003	<0.0001	<0.0001	0.24	0.11
Lower Respiratory	**Males**	0.20	2.43	3.89	6.33	3.45	1.78	0.83
		(0.91)	(0.93)	(0.90)	(0.69)	(0.96)	(0.94)	(0.93)
		0.66	0.12	0.0487	0.0119	0.063	0.18	0.36
	Females	9.96	11.29	15.72	22.37	4.22	1.26	2.50
		(0.51)	(1.18)	(0.85)	(0.75)	(1.66)	(0.76)	(1.20)
		0.0016	0.0008	<0.0001	<0.0001	0.0399	0.26	0.11
Upper respiratory	**Males**	0.00	7.06	4.82	7.47	0.80	4.87	0.38
		(0.53)	(0.43)	(0.52)	(0.59)	(0.72)	(0.76)	(0.58)
		0.98	0.0079	0.0281	0.0063	0.37	0.027	0.54
	Females	0.35	13.08	5.03	12.57	2.93	1.97	0.38
		(0.65)	(0.41)	(0.67)	(0.52)	(0.68)	(0.67)	(0.60)
		0.56	0.0003	0.0249	0.0004	0.0867	0.16	0.54
APR	**Males**	2.21	2.25	0.02	0.04	0.12	2.17	0.69
		(0.35)	(0.46)	(0.35)	(0.45)	(0.47)	(0.50)	(0.53)
		0.14	0.13	0.90	0.85	0.73	0.14	0.41
	Females	1.49	0.75	6.31	8.65	0.60	0.23	0.09
		(0.35)	(0.27)	(0.30)	(0.26)	(0.31)	(0.39)	(0.57)
		0.22	0.39	0.012	0.0003	0.44	0.63	0.77

ΔT2 = Change from baseline (T1) in symptom severity reported at t ≈25 min after the start of the study session.
ΔT3 = Change from baseline (T1) in symptom severity reported at ≈10 min after the start of the exposure.
ΔT4 = Change from baseline (T1) in symptom severity reported at ≈40 min after the start of the exposure.
ΔT5 = Change from baseline (T1) in symptom severity reported at ≈55 min after the start of the exposure.
ΔT6 = Change from baseline (T1) in symptom severity reported at ≈10 min after the end of the exposure.
ΔT7 = Change from baseline (T1) in symptom severity reported ≈6 hrs after the end of the exposure.
ΔT8 = Change from baseline (T1) in symptom severity reported at ≈24 hrs after the end of the exposure.
Signt = Significant.
* = statistically significant at p = 0.05 ** = borderline statistically significant at p = 0.05

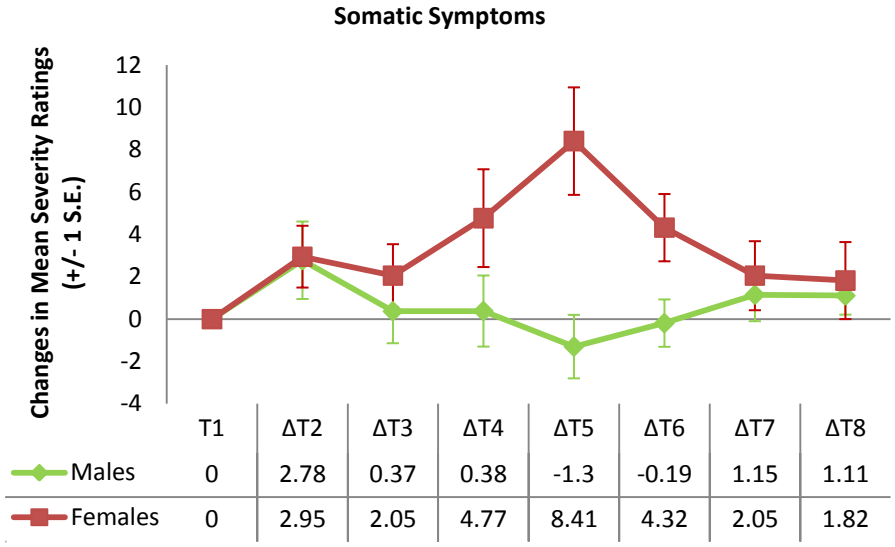
In general, looking at the mean change in the difference in somatic symptom severity ratings after both exposures (diesel exhaust – clean air ratings) relative to baseline for the remaining seven time points it was observed that females showed a

higher increase in somatic symptom severity ratings. While males showed a mixed response with some increased severity ratings and some decreased severity ratings during this time (see table 10a).

Table 4.10a. Mean Change (DE-CA) from Baseline in Symptom Severity Ratings for Males and Females.

		T1	ΔT2	ΔT3	ΔT4	ΔT5	ΔT6	ΔT7	ΔT8
Symptoms	**Sex**	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**
Somatic	**Males**	-1.11 (6.10) 27	2.78 (9.54) 27	0.37 (7.84) 27	0.38 (8.59) 26	-1.30 (7.79) 27	-0.19 (5.80) 27	1.15 (6.37) 26	1.11 (4.67) 27
	Females	-2.73 (6.12) 22	2.95 (6.84) 22	2.05 (7.01) 22	4.77 (10.85) 22	8.41 (11.89) 22	4.32 (7.45) 22	2.05 (7.66) 22	1.82 (8.53) 22
Eye Irritation	**Males**	-0.74 (8.85) 27	0.74 (7.93) 27	2.59 (13.75) 27	3.46 (9.36) 26	3.70 (7.92) 27	1.85 (7.61) 27	0.77 (8.80) 26	0.77 (7.83) 26
	Females	0.23 (2.43) 22	-0.45 (5.10) 22	2.14 (13.93) 21	4.76 (11.34) 21	2.50 (4.01) 22	0.45 (2.63) 22	-0.91 (5.49) 22	-0.45 (4.61) 22
Anxiety	**Males**	0.74 (11.99) 27	-0.93 (10.19) 27	1.30 (8.73) 27	6.73 (29.53) 26	2.96 (9.33) 27	1.85 (11.11) 27	-0.38 (11.74) 26	-1.48 (13.00) 27
	Females	-5.45 (9.50) 22	3.86 (7.70) 22	6.67 (14.69) 21	7.62 (12.31) 21	11.59 (18.15) 22	6.59 (11.89) 22	3.86 (9.99) 22	3.86 (12.34) 22
Lower Respiratory	**Males**	-0.74 (4.94) 27	0.37 (5.18) 27	2.41 (8.81) 27	5.00 (12.00) 26	2.78 (6.98) 27	1.11 (3.76) 27	1.35 (5.21) 26	0.77 (4.17) 26
	Females	-2.05 (6.67) 22	0.91 (2.94) 22	5.00 (10.12) 22	5.00 (12.63) 22	7.05 (14.69) 22	2.50 (6.32) 22	-0.91 (6.48) 22	1.59 (5.85) 22

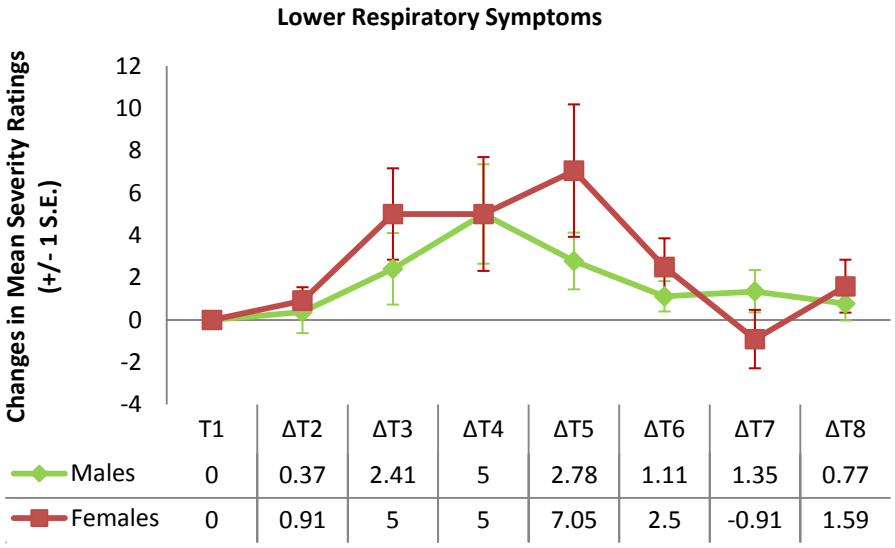
T1 = Symptom severity reported at the start of the study session (Baseline).
ΔT2 = Change from baseline (T1) in symptom severity reported at t ≈25 min after session start to DE relative to CA.
ΔT3 = Change from baseline (T1) in symptom severity reported at ≈10 min after exposure to DE relative to CA.
ΔT4 = Change from baseline (T1) in symptom severity reported at ≈40 min after exposure to DE relative to CA.
ΔT5 = Change from baseline (T1) in symptom severity reported at ≈55 min after exposure to DE relative to CA.
ΔT6 = Change from baseline (T1) in symptom severity reported at ≈10 min after exposure to DE relative to CA.
ΔT7 = Change from baseline (T1) in symptom severity reported ≈6 hrs after exposure to DE relative to CA.
ΔT8 = Change from baseline (T1) in symptom severity reported at ≈24 hrs after exposure to DE relative to CA.
DE = Diesel exhaust; CA = Clean air.



	T1	ΔT2	ΔT3	ΔT4	ΔT5	ΔT6	ΔT7	ΔT8
Males	0	2.78	0.37	0.38	-1.3	-0.19	1.15	1.11
Females	0	2.95	2.05	4.77	8.41	4.32	2.05	1.82

Graph 4.2. Changes in Mean Somatic Symptoms Severity Ratings.

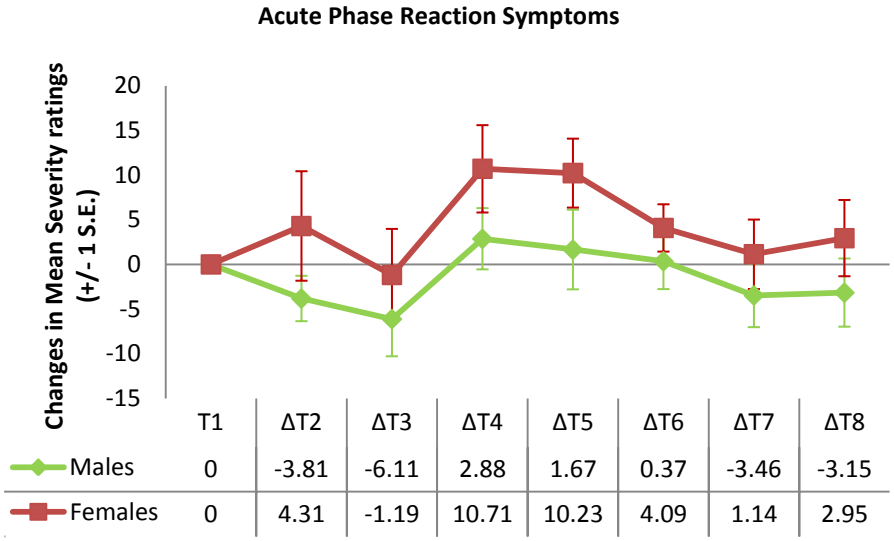
For lower respiratory symptoms, within males and females, there was an increase in the mean change in the difference in severity ratings after both exposures relative to baseline with females tending to have higher severity ratings than males relative to baseline.



	T1	ΔT2	ΔT3	ΔT4	ΔT5	ΔT6	ΔT7	ΔT8
Males	0	0.37	2.41	5	2.78	1.11	1.35	0.77
Females	0	0.91	5	5	7.05	2.5	-0.91	1.59

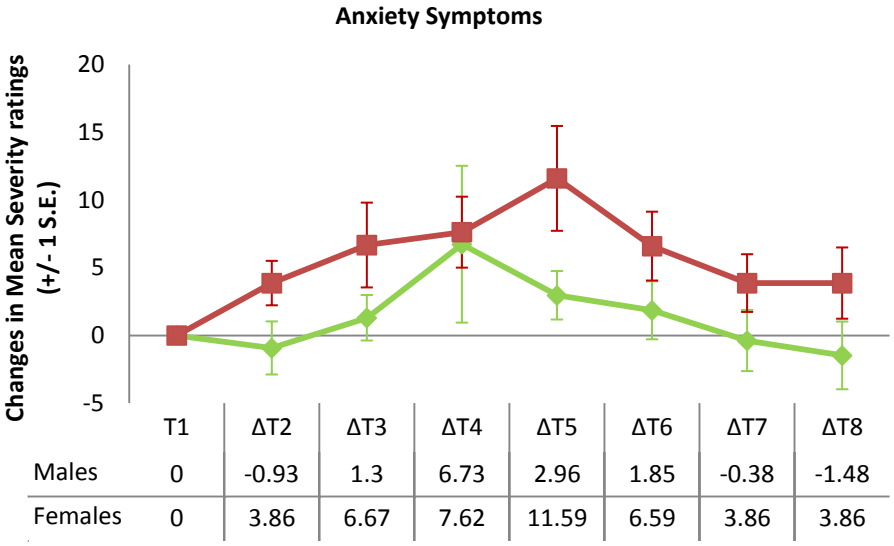
Graph 4.3. Changes in Mean Lower Respiratory Symptoms Severity Ratings.

Similar observations were made for acute phase reaction and anxiety symptom severity ratings but none of these observations were statistically significant.



	T1	ΔT2	ΔT3	ΔT4	ΔT5	ΔT6	ΔT7	ΔT8
Males	0	-3.81	-6.11	2.88	1.67	0.37	-3.46	-3.15
Females	0	4.31	-1.19	10.71	10.23	4.09	1.14	2.95

Graph 4.4 Changes in Mean Acute Phase Reaction Symptoms Severity Ratings.



	T1	ΔT2	ΔT3	ΔT4	ΔT5	ΔT6	ΔT7	ΔT8
Males	0	-0.93	1.3	6.73	2.96	1.85	-0.38	-1.48
Females	0	3.86	6.67	7.62	11.59	6.59	3.86	3.86

Graph 4.5. Changes in Mean Anxiety Symptoms Severity Ratings.

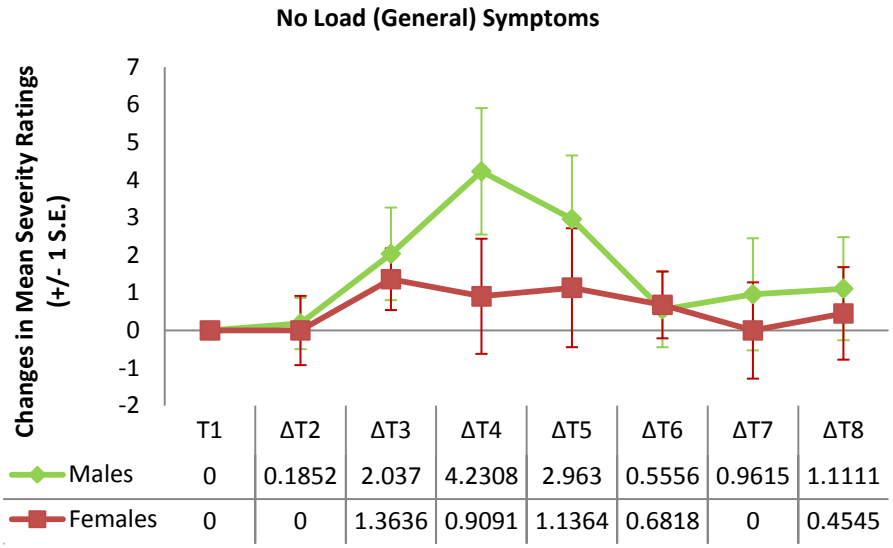
Although the no load (general) symptom severity ratings did not show a significant 'exposure*time*sex' three way interaction effect, they did show an opposite sex difference in response to diesel exhaust compared to clean air than was observed in somatic symptoms. It was observed that males primarily tended to show a mean higher increase in general symptom severity ratings than females after diesel exhaust exposure

compared to clean air and relative to baseline (see table 10b) although none of these observations were statistically significant.

Table 4.10b. Mean Change (DE-CA) from Baseline in Symptom Severity Ratings for Males and Females (continued).

		T1	ΔT2	ΔT3	ΔT4	ΔT5	ΔT6	ΔT7	ΔT8
		Mean (S.D.) N	Mean (S.D.) N	Mean (S.D.) N	Mean (S.D.) N	Mean (S.D.) N	Mean (S.D.) N	Mean (S.D.) N	Mean (S.D.) N
Upper Respiratory	**Males**	-2.41 (10.13) 27	1.48 (8.86) 27	8.15 (25.31) 27	6.35 (13.75) 26	9.44 (16.13) 27	2.78 (9.94) 27	4.04 (10.10) 26	1.60 (9.43) 25
	Females	-3.86 (11.33) 22	2.05 (13.77) 22	7.50 (11.750 20	8.10 (19.90) 21	9.55 (17.52) 22	4.32 (11.78) 22	4.09 (13.42) 22	0.68 (11.16) 22
Central Nervous System	**Males**	-0.93 (4.17) 27	1.54 (7.04) 26	6.11 (12.12) 27	8.65 (14.25) 26	8.33 (13.94) 27	2.59 (6.10) 27	1.73 (5.65) 26	0.56 (3.76) 27
	Females	-1.36 (4.41) 22	2.50 (7.20) 22	8.64 (11.46) 22	10.68 (18.34) 22	10.00 (20.93) 22	2.05 (5.04) 22	0.23 (5.23) 22	0.91 (5.26) 22
Acute Phase Reaction	**Males**	3.15 (16.93) 27	-3.81 (13.19) 27	-6.11 (21.69) 27	2.88 (17.51) 26	1.67 (23.19) 27	0.37 (16.28) 27	-3.46 (18.14) 26	-3.15 (19.85) 27
	Females	-6.14 (18.38) 22	4.31 (28.76) 22	-1.19 (24.23) 21	10.71 (22.93) 21	10.23 (17.83) 22	4.09 (12.41) 22	1.14 (18.25) 22	2.95 (20.04) 22
No Load	**Males**	-0.74 (7.81) 27	0.19 (3.53) 27	2.04 (6.39) 27	4.23 (8.57) 26	2.96 (8.80) 27	0.56 (5.25) 27	0.96 (7.62) 26	1.11 (7.12) 27
	Females	-1.82 (7.16) 22	0.00 (5.12) 22	1.36 (3.84) 22	0.91 (7.18) 22	1.14 (7.39) 22	0.68 (4.17) 22	0.00 (5.98) 22	0.45 (5.75) 22

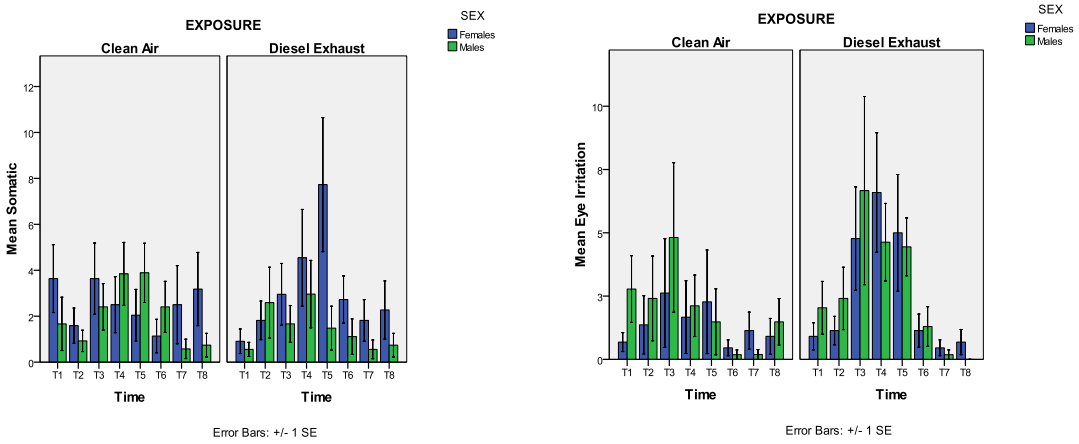
T1 = Symptom severity reported at the start of the study session.
ΔT2 = Change from baseline (T1) in symptom severity reported at t ≈25 min after session start to DE relative to CA.
ΔT3 = Change from baseline (T1) in symptom severity reported at ≈10 min after exposure to DE relative to CA.
ΔT4 = Change from baseline (T1) in symptom severity reported at ≈40 min after exposure to DE relative to CA.
ΔT5 = Change from baseline (T1) in symptom severity reported at ≈55 min after exposure to DE relative to CA.
ΔT6 = Change from baseline (T1) in symptom severity reported at ≈10 min after exposure end to DE relative to CA.
ΔT7 = Change from baseline (T1) in symptom severity reported ≈6 hrs after exposure to DE relative to CA.
ΔT8 = Change from baseline (T1) in symptom severity reported at ≈24 hrs after exposure to DE relative to CA.
DE = Diesel exhaust; CA = Clean air.

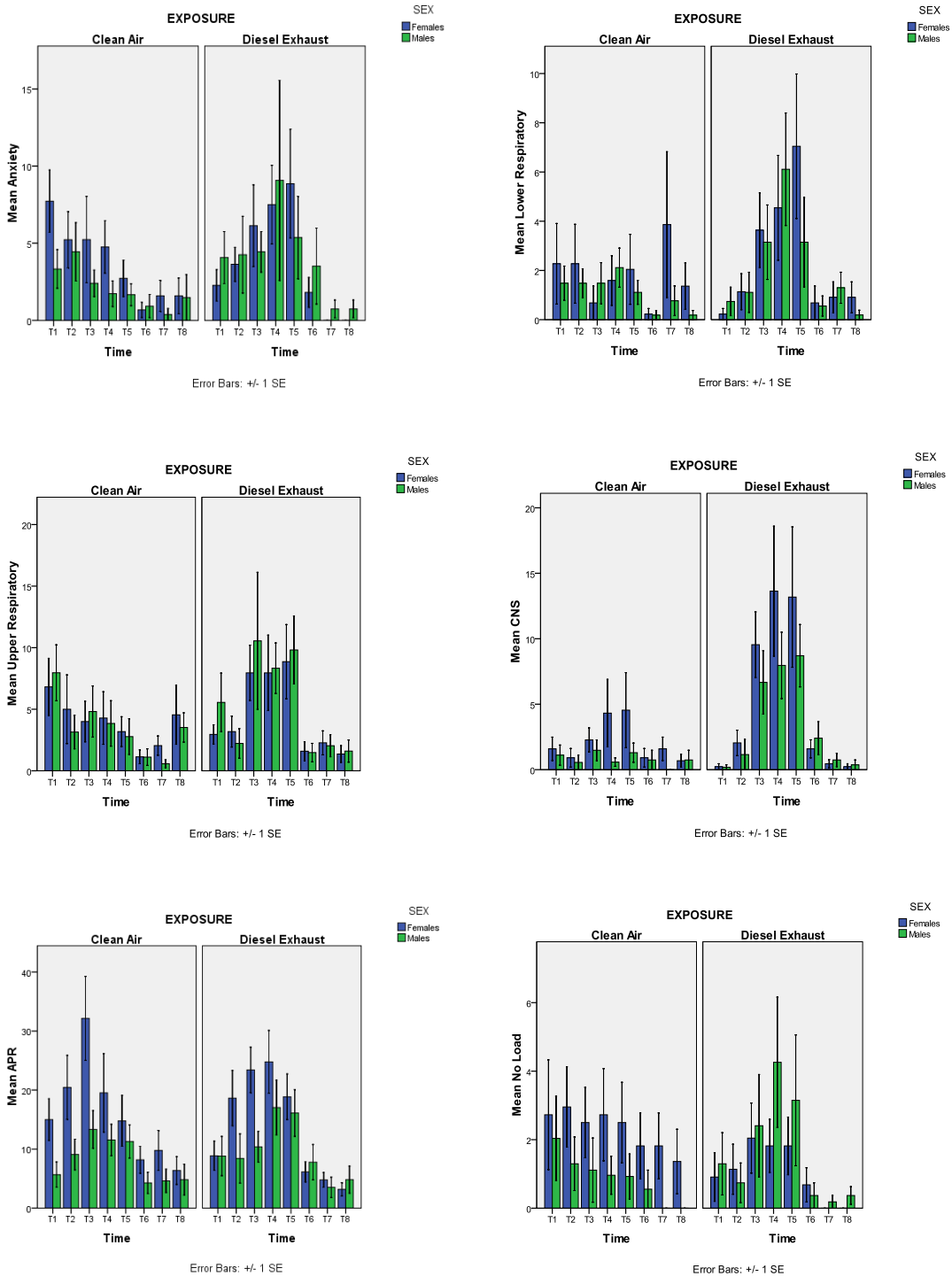


	T1	ΔT2	ΔT3	ΔT4	ΔT5	ΔT6	ΔT7	ΔT8
Males	0	0.1852	2.037	4.2308	2.963	0.5556	0.9615	1.1111
Females	0	0	1.3636	0.9091	1.1364	0.6818	0	0.4545

Graph 4.6. Changes in Mean No Load (General) Symptoms Severity Ratings.

For the rest of the symptom categories, there was an observed tendency of an increase in symptom severity ratings starting at about 25 minutes after the start of the study session to 10 minutes after the end of the exposure with negligible differences by sex that were not statistically significant.





Graph 4.7a-h. Distribution of Mean Symptom Severity ratings by Exposure, Time and Sex.

4.6 Discussion

The results showed that there was a significant three way (exposure*time*sex) interaction effect on the somatic and lower respiratory symptoms that were reported by males and females after a 1hour controlled human environmental exposure to a known concentration of diesel exhaust ($300\mu g/m^3$).

The results of this study are similar to what has been observed in other studies that have found a difference in the reporting of somatic symptoms between males and females (Kroenke and Spitzer 1998 and Barsky et al 2001). In this study we found that during the 1 hour exposure to diesel exhaust and immediately after the 1 hour exposure to diesel exhaust (time T2 to T6), females had statistically significant higher somatic severity ratings when compared to clean air exposure ($p \leq 0.00$).

After controlling for baseline lower respiratory symptom severity ratings, it was observed that the difference in lower respiratory symptom severity ratings by exposure (diesel exhaust ratings – clean air ratings) tended to be higher in females than in males. Previously in a review of gender differences in airway behavior done by Becklake and Kauffmann, they observed that shortness of breath (one of the lower respiratory symptoms) was more frequently reported by females than males in population based studies (1999). This observation can be attributed to gender related differences in socialization which leads to a variation in the perception and reporting of symptoms i.e. women feel more comfortable complaining about shortness of breath (Becklake and Kauffmann 1999 and Barsky et al 2001). On the other hand Becklake and Kauffmann also observed that in the population studies that they reviewed, for a given deficit in forced expiratory volume in 1 second (FEV_1), the rates of reporting of shortness of breath

was consistently higher in females as opposed to males. Thus proving evidence of a possible biological (sex) difference in the reporting of shortness of breath and therefore by inference a possible sex difference in the perception of lower respiratory symptoms in general.

This significant increase in self reported lower respiratory symptoms in females correlates with the observed higher concentration of cytokines in the fluid of the lungs of a subset of study females compared to a subset of study males after exposure to diesel exhaust relative to clean air. Although these observations in the fluid of the lungs were not statistically significant, they do suggest a biological basis for the significant sex difference in self reported lower symptom severity ratings that was observed.

In this study, although not statistically significant, females also tended to have higher severity ratings for acute phase reaction and anxiety symptoms. This could point to a difference by gender especially since "self reported generalized psychological distress has been found to be greater in women" (Barsky et al 2001). Despite this, acute phase reaction and anxiety symptoms do share a non-gender related biological (sex) link in that psychological stress can modulate or initiate acute phase reactions in people through the interaction of glucocorticoids with sex hormones on the immune system (Sapolsky et al 2000 and Rohleder et al 2001).

An interesting observation was that although the no load (general symptoms) did not show a statistically significant sex interaction effect (exposure*time*sex), they did show an opposite trend to what was observed with the somatic and lower respiratory symptoms. A trend of higher symptom severity ratings was observed in males after exposure to diesel exhaust when compared to clean air and relative to baseline values.

This trend may indicate a difference by sex in the types of self reported symptoms that males and females will report after exposure to diesel exhaust similar to what has been previously proposed by other studies that have looked at other symptom causing agents but which still needs to be investigated further in relation to diesel exhaust exposure.

4.6.1 Study Limitations

For the ascertainment of symptoms, the main study relied on self administered questionnaires and the data provided from them is based on subjective instead of objective measures of health effects (Dimich-Wald et al 2006). There is also the concern that by grouping the 37 individual symptoms into 8 categories, important information about the severity ratings of the individual symptoms by sex could be lost.

Lastly, since subjects were able to differentiate between clean air and diesel exhaust exposure this might have affected the perception, representation and reporting of symptoms.

4.7 Conclusions

In healthy volunteers, acute exposure to 300µg/m^3 of diesel exhaust for an hour in a controlled environmental study resulted in statistically significant changes in self reported somatic and lower respiratory symptoms by sex relative to clean air exposure with females having a tendency of higher mean somatic and lower respiratory symptom severity ratings than males. This suggests that females may be more sensitive than males to the effects of diesel exhaust exposure.

Finally, although not significant, there was also an observed trend of males having higher mean no load (general) symptom severity ratings than females after the diesel exhaust exposure compared to clean air exposure, suggesting that there may also be a variation by sex in the types of symptoms that are reported following diesel exhaust exposure relative to clean air.

Appendix C

Table C.1a. Mean Difference (DE-CA) in Symptom Severity Ratings for Males and Female.

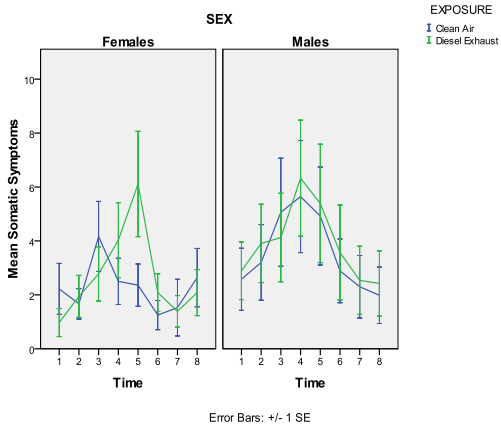
		T1	T2	T3	T4	T5	T6	T7	T8
Variables	**Sex**	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**
Somatic	**Males**	-1.11 (6.10) 27	1.67 (8.44) 27	-0.74 (5.83) 27	-0.77 (10.36) 26	-2.41 (8.48) 27	-1.30 (7.42) 27	0.00 (3.16) 26	0.00 (3.92) 27
	Females	-2.73 (6.12) 22	0.23 (2.43) 22	-0.68 (8.21) 22	2.05 (8.12) 22	5.68 (10.15) 22	1.59 (3.90) 22	-0.68 (8.35) 22	-0.91 (9.71) 22
Eye Irritation	**Males**	-0.74 (8.85) 27	0.00 (10.47) 27	1.85 (10.48) 27	2.69 (9.41) 26	2.96 (8.00) 27	1.11 (4.24) 27	0.00 (1.41) 26	-0.77 (3.06) 26
	Females	0.23 (2.43) 22	0.23 (3.93) 22	2.38 (13.10) 21	5.24 (10.89) 21	2.73 (3.69) 22	0.68 (1.76) 22	-0.68 (3.87) 22	-0.23 (3.26) 22
Anxiety	**Males**	0.74 (11.99) 27	-0.19 (17.12) 27	2.04 (8.69) 27	7.69 (35.05) 26	3.70 (14.05) 27	2.59 (13.61) 27	0.19 (0.98) 26	-0.74 (4.94) 27
	Females	-5.45 (9.50) 22	-1.59 (8.92) 22	1.19 (12.74) 21	3.10 (7.82) 21	6.14 (15.11) 22	1.14 (5.33) 22	-1.59 (4.73) 22	-1.59 (5.43) 22
Lower Respiratory	**Males**	-0.74 (4.94) 27	-0.37 (5.53) 27	1.67 (9.20) 27	4.23 (12.78) 26	2.04 (8.80) 27	0.37 (2.37) 27	0.58 (2.58) 26	0.00 (1.41) 26
	Females	-2.05 (6.67) 22	-1.14 (4.61) 22	2.95 (6.11) 22	2.95 (8.68) 22	5.00 (9.88) 22	0.45 (3.42) 22	-2.95 (11.92) 22	-0.45 (5.54) 22

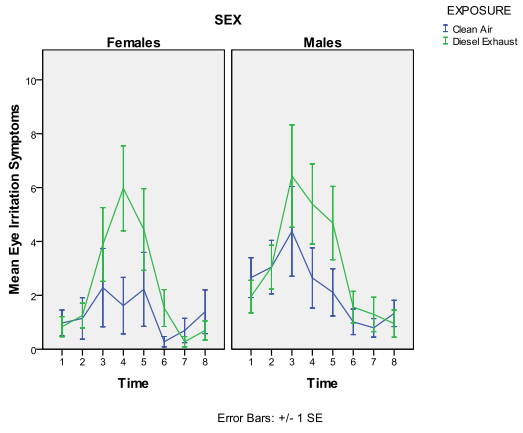
T1 = Symptom severity reported at the start of the study session.
T2 = Symptom severity reported at ≈25 min after the start of the study session.
T3 = Symptom severity reported at ≈10 min after the start of the exposure.
T4 = Symptom severity reported at ≈40 min after the start of the exposure.
T5 = Symptom severity reported at ≈55 min after the start of the exposure.
T6 = Symptom severity reported at ≈10 min after the end of the exposure.
T7 = Symptom severity reported at ≈6 hrs after the end of the exposure.
T8 = Symptom severity reported at ≈24 hrs after the end of the exposure.

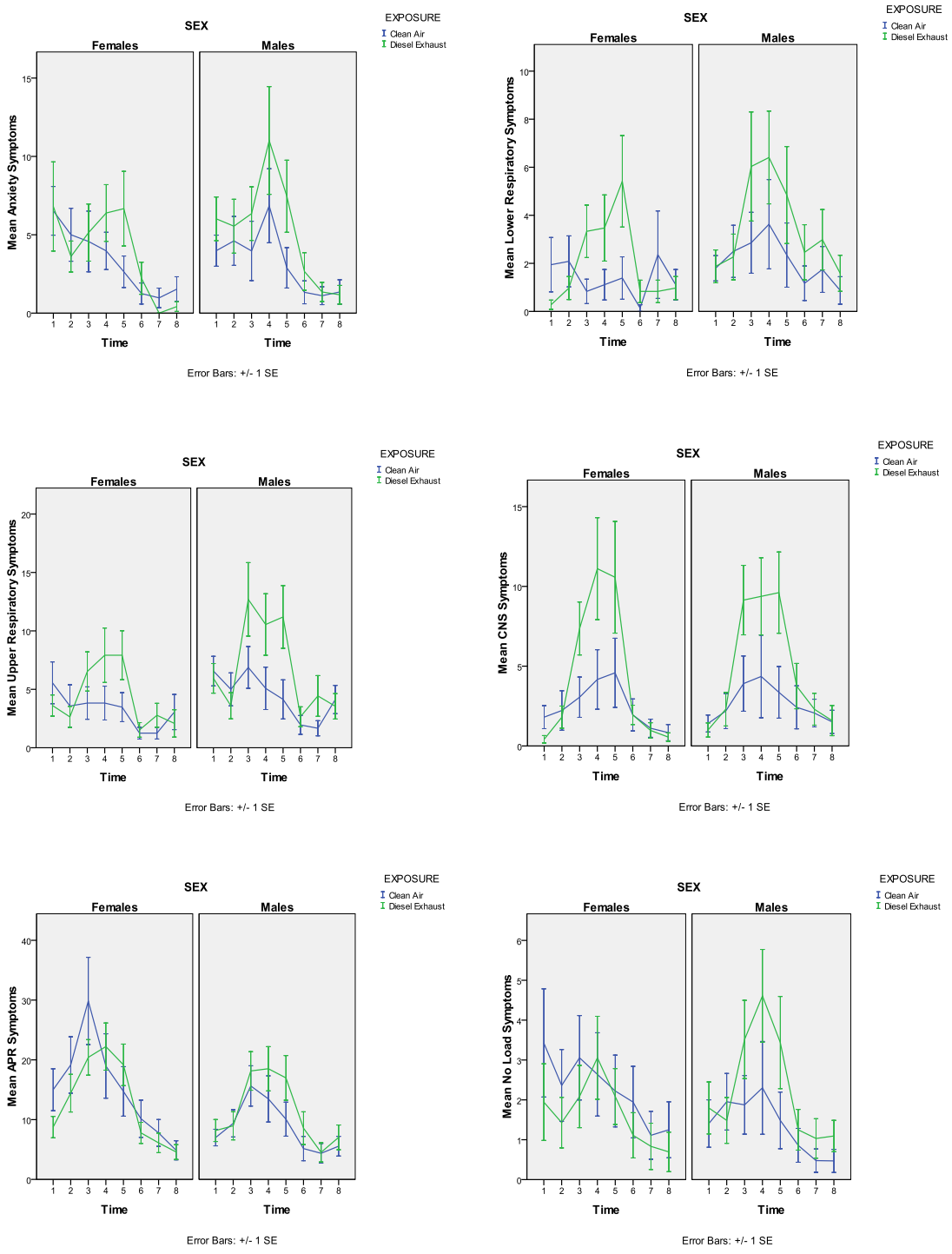
Table C.1b. Mean Difference (DE-CA) in Symptom Severity Ratings for Males and Females (continued).

		T1	T2	T3	T4	T5	T6	T7	T8
		Mean (S.D.) N	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**	**Mean (S.D.) N**
Upper Respiratory	**Males**	-2.40 (10.13) 27	-0.93 (8.55) 27	5.74 (22.09) 27	4.23 (14.19) 26	7.04 (15.83) 27	0.37 (4.79) 27	1.54 (5.05) 26	-1.60 (6.88) 25
	Females	-3.86 (11.33) 22	-1.82 (14.44) 22	3.75 (8.72) 20	4.05 (12.61) 21	5.68 (12.94) 22	0.45 (4.06) 22	0.23 (4.75) 22	-3.18 (11.19) 22
Central Nervous System	**Males**	-0.93 (4.17) 27	0.58 (6.68) 26	5.19 (10.96) 27	7.69 (13.80) 26	7.41 (12.66) 27	1.67 (3.92) 27	0.77 (2.72) 26	-0.37 (4.37) 27
	Females	-1.36 (4.41) 22	1.14 (5.76) 22	7.27 (10.20) 22	9.32 (16.35) 22	8.64 (17.74) 22	0.68 (3.20) 22	-1.14 (4.06) 22	-0.45 (2.63) 22
Acute Phase Reaction	**Males**	3.15 (16.93) 27	-0.67 (15.75) 27	-2.96 (16.89) 27	6.15 (20.36) 27	4.81 (21.95) 27	3.52 (14.33) 27	-0.96 (4.48) 26	0.00 (7.84) 27
	Females	-6.14 (18.38) 22	-1.82 (34.11) 22	-7.62 (32.39) 21	5.71 (21.75) 21	4.09 (15.40) 22	-2.05 (13.24) 22	-5.00 (15.51) 22	-3.18 (12.20) 22
No Load	**Males**	-0.74 (7.81) 27	-0.56 (5.25) 27	1.30 (9.47) 27	3.46 (10.75) 26	2.22 (10.59) 27	-0.19 (3.53) 27	0.19 (0.98) 26	0.37 (1.33) 27
	Females	-1.82 (7.16) 22	-1.82 (4.24) 22	-0.45 (5.32) 22	-0.91 (4.53) 22	-0.68 (6.23) 22	-1.14 (5.10) 22	-1.82 (4.51) 22	-1.36 (4.41) 22

T1 = Symptom severity reported at the start of the study session.
T2 = Symptom severity reported at ≈25 min after the start of the study session.
T3 = Symptom severity reported at ≈10 min after the start of the exposure.
T4 = Symptom severity reported at ≈40 min after the start of the exposure.
T5 = Symptom severity reported at ≈55 min after the start of the exposure.
T6 = Symptom severity reported at ≈10 min after the end of the exposure.
T7 = Symptom severity reported at ≈6 hrs after the end of the exposure.
T8 = Symptom severity reported at ≈24 hrs after the end of the exposure.







Graph C1a–h. Line Graph of Mean Symptom Severity Ratings by Sex.

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Chapter 5

Analysis of Sex Differences in the Urinary Concentration of 1-Aminopyrene after Acute Exposure to Diesel Exhaust

References

5.1 Abstract

Background: 1-Nitropyrene is one of the primary nitro-polycyclic aromatic hydrocarbons found in diesel exhaust. Therefore, it along with its metabolites such as 1-aminopyrene are thought of as potentially good specific biomarkers for diesel exhaust exposure. Physiological differences due to sex may affect the metabolism and excretion of 1-nitropyrene.

Aims: To investigate whether there are sex differences in metabolism of 1-nitropyrene as indicated by potential variations in the urinary levels of 1-aminopyrene excreted as a product of the nitro-reduction of 1-nitropyrene after a controlled environmental exposure to diesel exhaust.

Method: In a randomized crossover study, healthy human subjects were exposed to 300µg/m^3 of diesel exhaust and to clean for 1 hour each in a controlled environmental exposure chamber on two different days (≥1 week washout). Subjects were asked to collect 24 hour spot urine samples which were then analyzed for 1-aminopyrene using an HPLC-fluorescence. Primary statistical analysis was done using an analysis of variance.

Results: There was no statistically significant difference in the concentration of urinary 1-aminopyrene post exposure to diesel exhaust relative to clean air between males and females although females did show about a 12% mean increase in the time weighted average concentration of 1-aminopyrene compared to about a 0.004% increase that was observed in males after exposure to diesel exhaust relative to clean air exposure.

Conclusion: Although not statistically significant, we observed a difference by sex in the levels of urinary 1-aminopyrene 24 hours after acute exposure to 300µg/m^3 of diesel exhaust relative to clean air exposure. Females tended to secrete a higher concentration of diesel exhaust attributable 1- aminopyrene in urine than males, indicating that females may metabolize 1-nitropyrene via nitro-reduction more actively than males.

5.2 Introduction

Diesel exhaust is made up of a complex mixture of many chemicals including polycyclic aromatic hydrocarbons (PAHs) and nitro-PAHs in either gaseous or particulate form (CECBP 2008, USEPA HAD 2002). The particles are collectively called diesel exhaust particles (DEPs) and they are the largest single source of airborne particulate matter (PM) in many urban areas (Riedl and Diaz-Sanchez 2005). This means that for the general population, non-occupational exposure to diesel exhaust occurs frequently but

varies in concentration and length of exposure. Relatively high ambient exposures to diesel exhaust occur in ports, freeway intersections, and on school buses (CECBP 2008).

5.2.1 1-Nitropyrene

1-Nitropyrene (1-NP) is a nitro-PAH that is formed as a by-product of combustion and is the predominant nitrated polycyclic aromatic hydrocarbon emitted in diesel engine exhaust. 1-NP is also formed as a result of gas phase radical initiated atmospheric formation (USEPA HAD 2002) and is found in coal combustion, cigarette smoke, cooked meat products, wood burning, gas burner and kerosene heater emissions and airplane exhaust (W.H.O. 2003). Despite these other sources, the major route of exposure to 1-NP is through the inhalation of complex mixtures (e.g. diesel exhaust) and polluted urban air (USEPA HAD 2002). In many studies, the absorption and metabolism of 1-NP has been shown to occur rapidly with 1-NP mainly being metabolized by cytochrome P450 mediated ring oxidation and nitro reduction, followed by the conjugation and excretion of metabolites in urine and feces (Silvers et al 1994, W.H.O. 2003)

Since 1-NP is one of the primary nitro-PAHs found in diesel exhaust and also due to its strong association with diesel exhaust, it along with its metabolites such as 1-aminopyrene (1-APY) are thought of as potentially good specific biomarkers for DEPs exposure (Toriba et al 2007). This is important because 1-NP has been shown to have mutagenic properties and it is thought to be the main contributor of the direct acting mutagenicity of DEPs. It has been suggested that 1-NP in the body is mainly detoxified to 1-APY by nitro reduction (NTP 1996) but studies that have been done to date have shown mixed results about the urinary levels of 1-APY in relation to 1-NP exposure

(Seidel et al 2002 and Toriba et al 2007). Still there remains the possibility that 1-APY could be a potentially good biomarker for 1-NP exposure and thus diesel exhaust exposure.

5.2.1.1 Metabolism of 1-Nitropyrene

The metabolism of 1-NP can occur via nitro-reduction or cytochrome P450 mediated ring oxidation (Silvers et al 1997 and Chae et al 1999). The human Cytochrome P450 family of enzymes, in particular the P450 3A3 and 3A4 isomers are the predominant enzymes involved in the oxidative metabolism of 1-NP (Silvers et al 1997 and Chae et al 1999). In the mammalian system, 1-NP is metabolized by xanthine oxidase, DT-diaphorase or aldehyde oxidase (NTP 1996).

5.2.2 Sex Differences in Physiology

Many studies to date have shown that there is a large inter-individual variation in the excretion of nitro-PAHs and their metabolites and that this may be due to differences in the uptake and metabolism of these compounds and also the potential influence of confounders like age, environmental tobacco smoke, intake of charcoal broiled meat and residential area (USEPA HAD 2002, Nielsen et al 1996).

Males and females differ substantially with respect to physiology and these differences may have significant effects on the toxicity of any xenobiotic like nitro-PAHs and thus may affect how vulnerable males and females are to the effects of a xenobiotic and other environmental stressors (Gochfeld 2007). Specifically, sex may influence the uptake and also the type and function of metabolizing enzymes that either become

activated or deactivated upon exposure to nitro-PAHs. This may translate into differences in the rate of metabolism of nitro-PAHs, the types and severity of toxicity and the amount of nitro-PAHs metabolites that are excreted after exposure to diesel exhaust. In drug studies, female sex has been shown to be a risk factor for clinically relevant adverse drug reactions due to sex differences in pharmacokinetics, in pharmacodynamics, immunology, hormonal factors and differences in the dose (mg/kg) that was administered (Anderson 2008, Rademaker 2001).

Sex differences in the activity of the cytochrome P450 (CYP) and uridine diphosphate glucuronosyltransferase (UGT) enzymes and renal excretion also result in differences in clearance (Anderson 2008). Also, frequent and sometimes clinically relevant sex differences can possibly be identified for drug elimination processes and have predominantly been linked to the sex-specific expression of the metabolic enzyme systems, e.g. CYP3A4 and CYP1A2 (Beierle et al 1999).

The CYP3A gene subfamily is the most abundant P450 enzyme in human liver microsomes, the level of which can vary enormously (>10 fold) among individuals (Casarett and Doulls 2001). They (in particular CYP3A3 and CYP3A4) seem to be the enzymes involved in the human oxidative metabolism of 1-NP (W.H.O. 2003). There is evidence of females having lower activity of CYP1A2, CYP2E1, and UGT; higher activity of CYP3A4, CYP2A6, and CYP2B6; and no differences in CPY2C9 and CYP2D6 activity (Anderson 2008). As mentioned before, in the mammalian system, 1-NP is metabolized by xanthine oxidase, DT-diaphorase or aldehyde oxidase (NTP 1996) via nitro-reduction and in 1992 Relling et al showed that females had a higher activity of xanthine oxidase than males after exposure to a low dose of caffeine. Therefore, there is

the possibility that there may be a variation by sex in the concentration of 1-NP metabolites that are produced regardless of the metabolic pathway that is used

To summarize, with regards to physiology, women generally have a lower lean body mass, reduced hepatic clearance, differences in activity of cytochrome P450 (CYP) enzymes (40% increase in CYP3A4, varied decrease in CYP2D6, CYP2C19 and CYP1A2), metabolize drugs at different rates compared with men (Rademaker 2001) and have been shown to have a higher xanthine oxidase activity (Relling et al 1992). Other important factors include conjugation, absorption, protein binding and renal elimination, which may all have some sex-based differences (Rademaker 2001).

Sex differences have also been observed in baseline characteristics as well as in drug response, which might both, at least in part, be the consequence of modulation by sex hormones (Beierle et al 1999). All of these potential factors may affect the way that males and females react physiologically to a xenobiotic like diesel exhaust thereby influencing the rates and types of metabolism and excretion of the xenobiotic in males and females making one group either slow, intermediate or fast metabolizers (W.H.O. 2003) of the xenobiotic, thus influencing the levels and types of metabolites excreted.

The aim of the present study is to investigate whether there are sex differences in the metabolism of diesel exhaust as indicated by the potential variation in the amount of urinary 1-APY excreted as a product of the nitro reduction of 1-NP following diesel exhaust exposure. If there are variations in the amount of urinary 1-APY excreted following diesel exhaust exposure between males and females, a second aim of the paper is to determine if sex is a significant predictor of the amount of 1-APY that is excreted in urine.

5.3 Materials and Methods

5.3.1 Data Collection

The data used for this dissertation research are part of the datasets generated from a study of diesel exhaust effects and a subsequent add-on study of diesel exhaust exposure biomarkers. The parent study was funded by the Department of Defense (DOD) through a grant to the University of Medicine and Dentistry of New jersey (UMDNJ) (Grant # DAMD17-03-1-0537; Principal Investigator: Dr. Nancy Fiedler) and the add-on project was funded by the Environmental Protection Agency (EPA) through a STAR grant to UMDNJ (Grant # R832097; Principal Investigator: Dr. Junfeng Zhang).

5.3.2 Subjects

110 healthy non-smoking men and women between the ages of 18 and 50 were recruited from the local community in Piscataway, New Jersey, based on the established study criteria; 100 subjects completed the study. Out of these subjects, 55 subjects consented to provide urine samples as part of the add-on study (see table 1).

Table 5.1. Subject Characteristics by Sex.

	Females					Males				
	N	**Min**	**Max**	**Mean**	**SD**	**N**	**Min**	**Max**	**Mean**	**SD**
AGE (years)	21	19	44	25.19	7.24	29	19	43	24.97	6.70
Height (inches)	21	59.50	67.50	63.25	1.89	29	59.5	75	68.80	3.12
Weight(lb)	21	106	200	141.67	30.17	29	137	236	171.19	25.05
BMI* (kg/m^2)	21	19.08	37.17	24.94	5.52	29	21.28	34.35	25.41	3.20
Respiration	20 [M]	14	20	16.10	1.65	29	10	20	15.93	2.10
Temperature ^{o}F	19 [M]	97.30	99.40	98.34	0.41	28 [M]	97.5	99.1	98.30	0.43
Pulse	20 [M]	54	94	68.50	8.68	29	52	86	68.62	9.23
Log TWA 1-APY	21	-0.56	8.21	2.36	1.99	29	-3.30	5.83	1.91	2.17
Race	**Frequency (%)**					**Frequency (%)**				
Asian	10	(47.62)				12	(41.38)			
Black	2	(9.52)				1	(3.45)			
Hispanic	3	(14.29)				6	(20.69)			
Other	0	(0)				1	(3.45)			
White	6	(28.57)				9	(31.03)			

*BMI = (weight/height2) *703. [M] = some subjects had missing data.

5.3.3 Exposure Conditions and Experimental Design

A randomized crossover design was used in which each subject underwent two exposure conditions; one was to diluted diesel exhaust normalized to PM_{10} concentration of 300µg/m^3 and the other was to filtered clean ambient air of Busch campus in Piscataway, a suburban campus of Rutgers University. Both exposure conditions were 1 hour in duration and took place at the Environmental and Occupational Health Sciences Institutes' (EOHSI) Controlled Environmental Facility (CEF). Each exposure condition was presented with and without an acute psychological stressor to two groups of subjects high and low in self reported chemical intolerance (CI). Subjects were also asked to fast overnight the day before the study until after each exposure session and to also abstain from any exercise.

There were no significant differences in the exposure conditions for all subjects. Overall, the concentration of PM_{10} that the subjects were exposed to was 276±13µg/m^3 which was close to the target concentration of 300µg/m^3.

Table 5.2. Mean Air Characteristics and Composition.

	Diesel Exhaust	**Clean Air**
PM_{10} (µg/m^3)	276 ± 13	6 ± 5
NO_X (ppm)	3.63 ± 0.90	0.02 ± 0.02
CO (ppm)	3.79 ± 0.76	0.91 ± 0.21
1 Nitropyrene (ng/m^3)	2.68 ± 0.51	<0.06

5.3.4 Urine Sample Collection

Subjects were asked to collect 24 hour spot urine samples beginning on the morning of the day of exposure through to the following morning after exposure. They were provided with a cooler containing a dozen 50-ml bottles and they were instructed to use an empty and clean bottle to collect each urine sample. Each bottle had a label with the subject's identification number and the subjects were asked to write the sample

collection time on the labels. Along with the labels, the subjects were also given a simple time/activity diary with which to document potential sources of nitro-PAHs within the sampling period. Upon return to the laboratory, all the urine samples were stored at -40°C and protected from light before sample analysis.

5.3.5 Urine Sample Analysis

The urine samples were analyzed for 1-APY using an HPLC-fluorescence technique that was developed and described previously by Laumbach et al 2009. Briefly, the acetylated urinary 1-APY conjugate was hydrolyzed with concentrated HCl at 80ºC in a shaking bath for 1 hour before extraction. During the extraction, the pH of the mixture was first adjusted to 7 – 8 by adding about 1.1 ml of 10 M NaOH and a small quantity of concentrated acetic acid. The resulting solution was then centrifuged at 2500 g for 10 min with 5 ml of dichloromethane and extracted twice. The two dichloromethane extracts were then combined in a glass tube and evaporated to near dryness using nitrogen at room temperature. The residue was rinsed from the glass tube wall and made into a final solution of 1 ml with acetonitrile. Using a 0.2 µm PVDF liquid filter, the final solution was filtered through into a 1.8 ml amber vial that was sealed with a Teflon septum for HPLC analysis.

The HPLC system used for the 1-APY analysis included a 600E system controller, a 717 auto sampler and a 2474 fluorescence detector (Waters Co., Milford, MA). An Ascentis RP-Amide column (4.6mm x 250mm) (Supelco Co., Bellefonte, PA) was used to separate analytes. The mobile phase involved the use of two solutions; Solution A = 50% acetonitrile in water, and Solution B = 100% acetonitrile, with a linear gradient from 100% A to 100% B in 30 min at a flow rate of 1.0 ml/min. The injection

volume was 20µL. The fluorescence detector was set at an excitation wavelength of 254 nm and an emission wavelength of 425 nm. An external standard of 1-APY (97% purity, Aldrich Co., St. Louis MO) was used to generate calibration curves for quantifying 1-APY concentrations. All calibration curves had a near zero intercept and a $R^2 > 0.99$. This method had a detection limit of 0.02 ng/ml, expressed as 3 times the standard deviation of a low concentration calibration standard (N = 8). The coefficient spiked into real urine sample (with concentration in the original urine subtracted), the method recovery was 88.3%.

5.3.6 Creatinine Measurement

Creatinine concentrations were measured spectrophotometrically and were used to normalize 1-APY concentrations. Since subjects were asked to collect spot urine samples, the whole urine output per void was not collected and so the mass of 1-APY excreted in urine voids could not be determined. Therefore creatinine adjusted concentrations were used in an attempt to account for differences in the dilution of the spot urine samples.

5.3.7 Exclusion of Invalid Data and Calculation of Time Weighted Average Concentrations

The total number of subjects that provided samples for the study was 55; the total number of spot urine samples collected following diesel exhaust (DE) exposure was 373 and the total number of spot urine samples collected following clean air (CA) exposure was 359.

After excluding all invalid samples (those samples for which the creatinine levels were out of the valid range i.e. <30 and > 300 mg/dl (Barr et al 2005, ACGIH 199)) and

any urine samples that did not have a corresponding creatinine measurement, the total number of subjects left with valid samples was 50 (21 Females and 29 Males) leaving 249 Clean Air exposure sample measurements and 249 Diesel exhaust exposure sample measurements.

After the creatinine correction, the time weighted average (TWA) of 1-APY concentration per subject was calculated using the formula below.

$$C_{i.Exp} = \frac{\sum_{j=1}^{J_{iExp}} t_{ijExp} C_{ijExp}}{\sum_{j=1}^{J_{iExp}} t_{ijExp}}$$

Source: Laumbach et al 2009

$C_{i.\,Exp}$ = Time weighted average concentration after exposure j ;
T_{ijExp} = time that the concentration was measured for an exposure j in minutes;
C_{ij} = Concentration measured at time i for exposure j.

Table 5.3. TWA 1-APY Concentrations for All Subjects and by Sex.

	All		Females		Males	
	DE	**CA**	**DE**	**CA**	**DE**	**CA**
Mean	35.73	21.08	37.87	2.99	34.19	34.19
SD	51.46	85.08	55.41	3.27	49.34	110.62
Max	237.46	503.85	215.40	12.13	237.46	503.85
Median	17.02	2.34	12.88	2.09	18.86	2.96
Min	1.65	0.03	1.65	0.06	1.73	0.03
Total	50	50	21	21	29	29

1-APY = 1 – Aminopyrene. Concentrations as presented above are in ng/ml.

5.3.8 Exclusion of Baseline Values

There was a difference between the mean baseline 1-APY concentrations for all subjects, with subjects having a higher mean baseline 1-APY concentration prior to diesel exhaust exposure than prior to clean air exposure (7.10 ng/ml vs. 2.82 ng/ml). This trend continued when looked at by sex with males having a higher mean baseline 1-APY

concentration than females both prior to diesel exhaust exposure (7.51 ng/ml vs. 6.49 ng/ml) and prior to clean air exposure (3.09 ng/ml vs. 2.49 ng/ml).

Table 5.4. Baseline 1-APY Concentrations.

	All Subjects		Females		Male	
	DE	**CA**	**DE**	**CA**	**DE**	**CA**
Mean	7.10	2.82	6.49	2.49	7.51	3.09
SD	14.47	4.30	12.18	4.48	16.03	4.22
Max	74.57	15.40	47.83	15.40	74.57	15.20
Median	0.60	0.65	0.37	0.32	1.34	1.07
Min	0.02	0.01	0.02	0.01	0.02	0.03
Total	47*	43*	19*	19*	28*	24*

*Some subjects were missing baseline measurements. Concentrations as presented above are in ng/ml
1-APY = 1 – Aminopyrene. DE = Diesel Exhaust; CA = Clean Air.

Since every subject did not have a baseline 1-APY sample (concentration of 1-APY measured in urine that was collected on the morning of exposure prior to the start of exposure), all baseline 1-APY samples were excluded from the dataset. Prior to their exclusion, a sensitivity analysis was done using a 2 by 2 between group analysis of covariance (ANCOVA) to assess whether mean TWA 1-APY concentrations for diesel exhaust and clean air were significantly different for males and females after the baseline 1-APY values were controlled for. This was done to determine if the baseline 1-APY concentrations had an effect on the amount of TWA 1-APY that was secreted by exposure and by sex. The TWA 1-APY and the baseline 1-APY values were log transformed prior to the ANCOVA analysis due to the fact that their distributions were right skewed.

In the ANCOVA analysis, the independent variables were exposure (DE, CA) and sex (males, females). The dependent variable was the log transformed 24 hour TWA 1-APY concentrations measured following exposure to diesel exhaust and clean air. The log transformed baseline values were used as a covariate in the analysis

Table 5.5a. Results of the ANCOVA Sensitivity Analysis.

Source	Type III SS	Mean Square	F Value	p Value
Exposure type	101.18	101.18	38.74	<.0001
SEX	3.97	3.97	1.52	0.22
Log Baseline	6.88	6.88	2.64	0.11
Exposure type x SEX	1.08	1.08	0.41	0.52

Concentrations as presented above are in ng/ml. SS = Sum of squares.
1-APY = 1 – Aminopyrene. TWA = Time Weighted Average.

Table 5.5b. Mean and Adjusted Mean Concentrations of Baseline TWA 1-APY by Sex.

Log TWA 1-APY	N	Mean	Adjusted Mean*
Females			
Clean Air	19	0.24	0.30
Diesel Exhaust	19	2.68	2.68
Males			
Clean Air	24	0.98	0.95
Diesel Exhaust	28	2.90	2.88

*Means adjusted for by log baseline values. Concentrations as presented above are in ng/ml.
1-APY = 1 – Aminopyrene. TWA = Time Weighted Average.

Since the significance level of the main effect of the log baseline covariate and the exposure type by sex interaction were both greater than 0.05 ($p = 0.11$ and $p = 0.52$ respectively), it indicated that there was no significant relationship between the log baseline 1-APY concentrations (covariate) and the log TWA 1-APY concentrations (dependent variable) by exposure and by sex and therefore it was appropriate to exclude the baseline 1-APY concentrations from the main analysis.

5.3.9 Calculation of Response Measure

Urinary creatinine concentration and specific gravity are two of the accepted methods for adjusting for dilution of a spot urine sample (Barr et al 2005). With regards to sex, it has been shown that sex plays a role in the concentration-dilution of spot urine samples thereby influencing both the urinary concentration of creatinine and specific gravity that are used to correct these spot samples (Carrieri et al 2001).

This means that there is the potential that the use of creatinine concentrations to control for the effect of hydration in this study might skew the results because creatinine concentration is influenced by muscle mass which in turn differs by sex with women

generally having lower lean body mass (Ernstgard et al 2003, Rademaker 2001). Therefore in an attempt to remove the potential sex based bias of creatinine correction on the concentrations of 1-APY secreted, the ratio of the creatinine corrected TWA 1-APY concentrations secreted after exposure to diesel exhaust (DE) and the creatinine corrected TWA 1-APY concentrations secreted after exposure to clean air (CA) was calculated and used as the study response measure in an attempt to cancel out the influence of the effect of creatinine variation by sex.

TWA 1-APY$_{DE}$/TWA 1-APY$_{CA}$ = TWA 1-APY $_{DE/CA}$ = **Relative Δ** (Response measure)

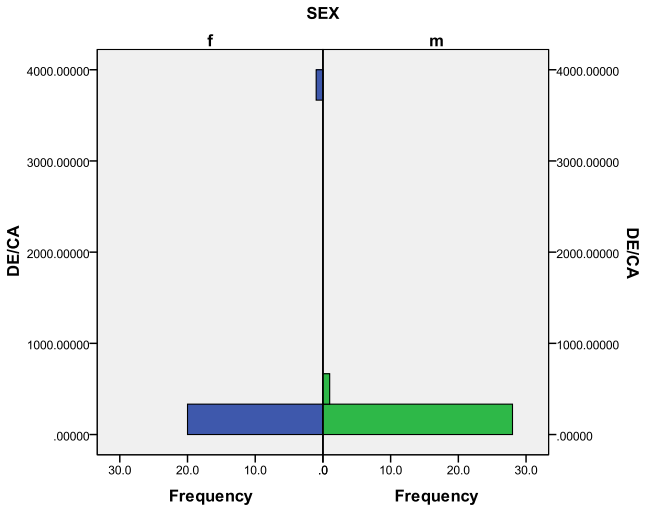


Figure 5.1. Distribution of the TWA 1-APY $_{DE/CA}$ by Sex.
TWA = Time Weighted Average.
DE/CA = ratio of the TWA concentration of 1-Aminopyrene measured after DE exposure to the concentration of 1-Aminopyrene measures after CA exposure.

Table 5.6. Mean TWA 1-APY $_{DE/CA}$ for All Subjects and by Sex.

1-APY	All	Females	Males
Mean	101.28	197.17	31.84
SD	518.79	797.78	67.10
Max	3673.75	3673.75	341.50
Median	8.26	8.59	8.23
Min	0.04	0.57	0.04
Total	50	21	29

1-APY = 1 – Aminopyrene. TWA = Time Weighted Average.
DE/CA = ratio of the TWA concentration of 1-APY measured after DE exposure to the concentration of 1-APY measures after CA exposure.

5.3.10 Body Mass Index

Along the same lines, another factor which also had the potential to skew the results of the analysis was body mass index (BMI). Although it is not clear whether sex has a causal association with BMI, meaning that BMI is one mechanism by which sex modifies health outcomes or whether BMI functions independent of sex, BMI is still known to vary by sex. Therefore BMI was calculated using height and weight and was then stratified by sex in order to evaluate the mean BMI by sex. The mean BMI for females (Mean = 24.94 and SD = 5.52) and the mean BMI for males (Mean = 25.41 and SD = 3.20) were about the same for the study population so there was no need to control for BMI in this analysis.

5.4 Statistical Methods

Descriptive statistical analysis, histograms and box plots were used to evaluate the distribution of the variables. The distribution of the response measure TWA 1-APY $_{DE/CA}$ was right skewed and so the final variable on which all subsequent statistical analysis was performed was log transformed (log TWA 1-APY $_{DE/CA}$).

Table 5.7. Mean Log TWA 1-APY $_{DE/CA}$.

1-APY $_{DE/CA}$	All	Females	Males
Mean	2.10	2.36	1.91
SD	2.09	1.99	2.17
Max	8.21	8.21	5.83
Median	2.11	2.15	2.10
Min	-3.30	-0.56	-3.30
Total	50	21	29

1-APY = 1 – Aminopyrene. TWA = Time Weighted Average.
DE/CA = log of the ratio of the TWA concentration of 1-APY measured after DE exposure to the concentration of 1-APY measures after CA exposure.

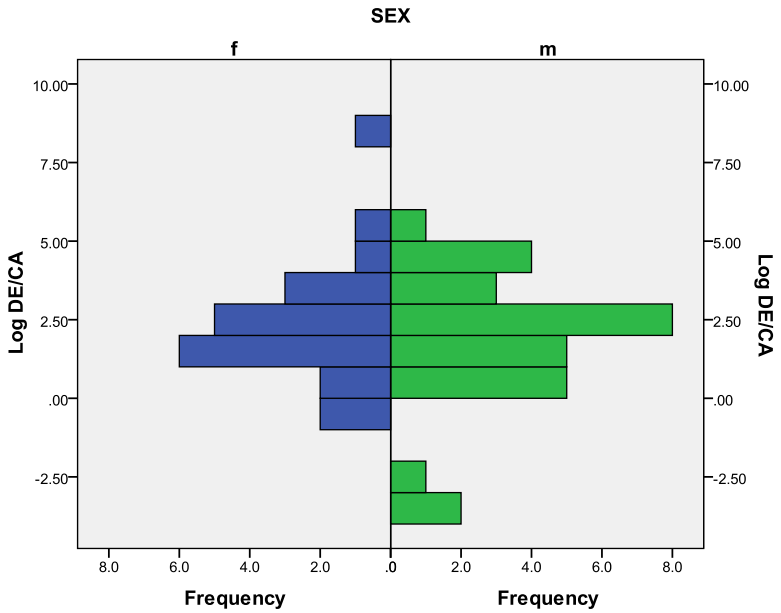


Figure 5.2a. Distribution of the Concentration of Log $TWA_{DE/CA}$ by Sex.
TWA = Time Weighted Average.
Log DE/CA = log of the ratio of the TWA concentration of 1-Aminopyrene measured after DE exposure to the concentration of 1-Aminopyrene measures after CA exposure.

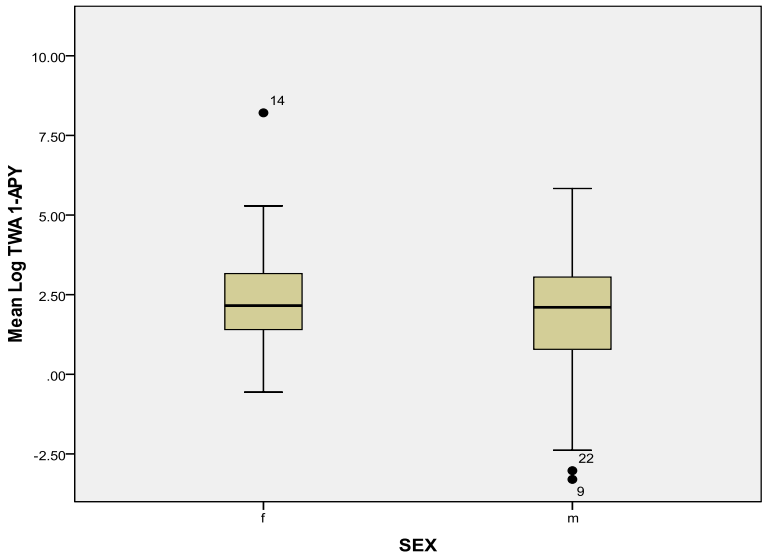


Figure 5.2b. Box Plot of the Mean Concentration of Log TWA 1-APY $_{DE/CA}$ by Sex.
1-APY = 1 – Aminopyrene. TWA = Time Weighted Average.
Log DE/CA = ratio of the TWA concentration of 1-Aminopyrene measured after DE exposure to the concentration of 1-Aminopyrene measures after CA exposure.

Three one sample t-tests were done to confirm the main effect of diesel exhaust exposure on the concentration of log TWA 1-APY $_{DE/CA}$ for all subjects (t-value = 7.10;

df = 49; $p < 0.0001$), within females (t-value = 5.44; df = 20; $p < 0.0001$) and also within males (t-value = 4.73; df = 28; $p < 0.0001$) relative to clean air exposure.

To address the first aim of the paper, a analysis of variance (ANOVA) was performed to determine if there was a significant difference in log TWA 1-APY $_{DE/CA}$ concentrations between sex groups (male, females). As mentioned before, since some subjects were also exposed to stress (a potential confounder), stress (stress, no stress) was initially included in the model to analyze the 'sex*stress' interaction. The interaction was not significant, so stress was excluded and the final model that was used contained sex as the independent classification variable and log TWA 1-APY $_{DE/CA}$ as the dependent variable.

To address the second aim of the paper, a backward regression analysis was done to determine if sex was the greatest predictor of log TWA 1-APY $_{DE/CA}$ excreted in urine. Based on similar studies that have been done so far, age, body mass index (BMI), pulse, respiration, temperature and race were included as potential independent predictors along with sex into the initial regression model with log TWA 1-APY $_{DE/CA}$ as the dependent variable. At each stage of the subsequent analysis, the independent predictors that showed the least contribution to the model were excluded from the model. Leaving a final model that contained only those independent predictors that were significant at the 0.10 level.

For all tests, where appropriate the variables were log transformed prior to analysis in an attempt to give them a more normal distribution.

All statistical analysis for this paper were done using SAS software, Version 9.1 of the SAS System for Windows (*SAS* Institute Inc., Cary, NC, USA) and SPSS software,

Version 17.0 (SPSS inc., Chicago IL). Also, some graphs were created using Microsoft Excel 2007. P-values less than or equal to 0.05 were considered to be significant.

5.5 Results

5.5.1 Aim 1: Are there sex differences in urinary levels of 1-aminopyrene (Log TWA 1-APY $_{DE/CA}$) post exposure to diesel exhaust?

The ANOVA analysis showed no statistically significant difference (F value = 0.58; df = 1, 48; p = 0.452) between the mean log TWA 1-APY $_{DE/CA}$ concentrations for females (2.36) and the mean log TWA 1-APY $_{DE/CA}$ concentrations for males (1.91).

Table 5.8. ANOVA Results.

Source	Type III Sum of Squares	Df	Mean Square	F	p-Value	Partial Eta Squared
Corrected Model	2.54[a]	1	2.54	0.58	0.45	0.01
Intercept	222.38	1	222.38	50.44	0.00	0.51
SEX	2.54	1	2.54	0.58	0.452	0.01
Error	211.62	48	4.41			
Total	434.64	50				
Corrected Total	214.16	49				

[a] R Squared = .012 (Adjusted R Squared = -.009)

Although not significant, there was a sex difference in the mean TWA 1-APY concentrations that were observed after exposure to diesel exhaust when compared to the mean TWA 1-APY concentrations that were observed after exposure to clean air. With females showing about a 12% overall mean increase in TWA 1-APY excretion following diesel exhaust exposure relative to clean air, while males showed a very slight overall mean increase in TWA 1-APY excretion of about 0.004% following diesel exhaust exposure relative to clean air.

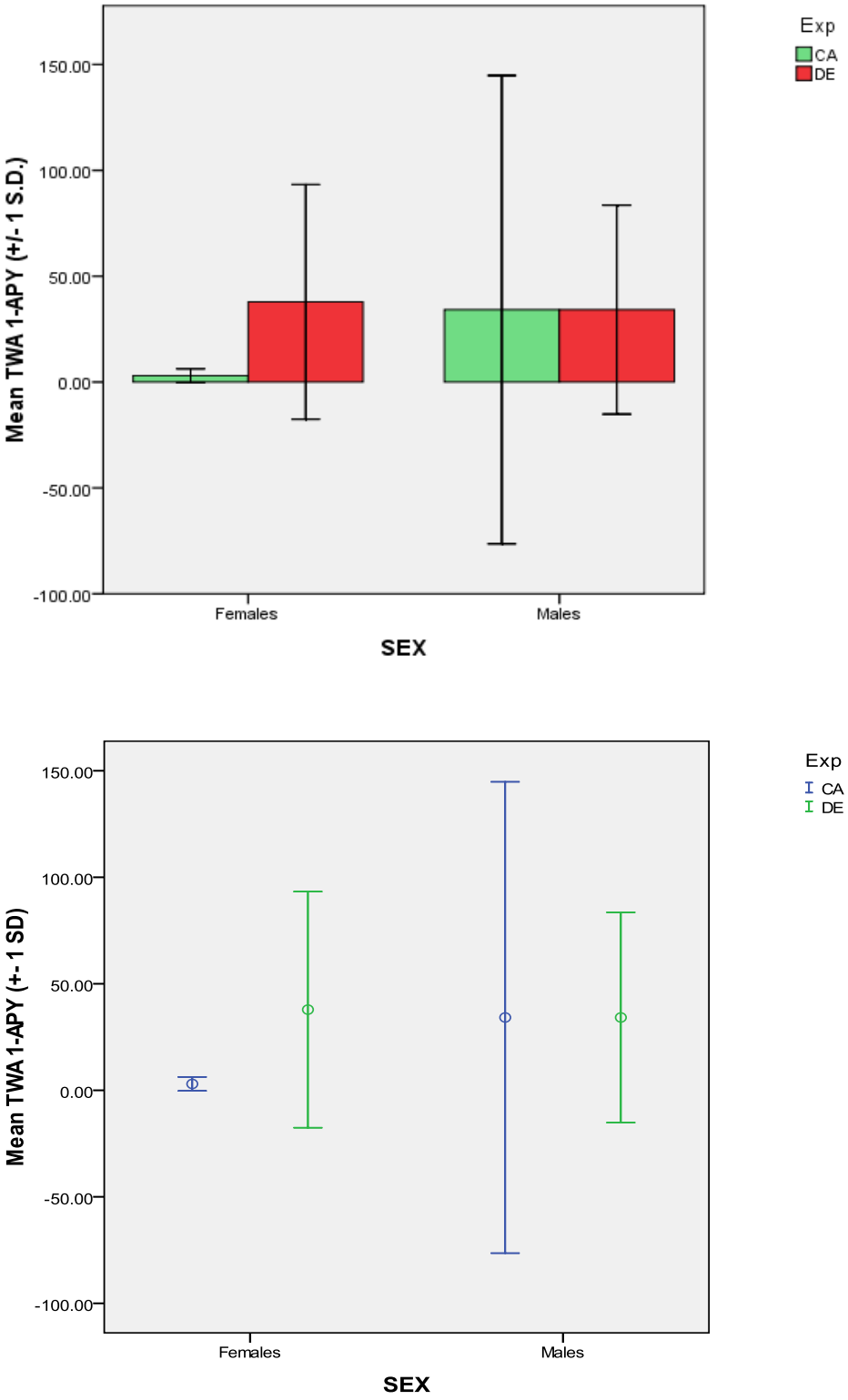


Figure 5.3. Mean TWA 1-APY Concentrations Secreted by Exposure and by Sex.

5.5.2 Aim 2: Can sex predict urinary levels of 1-aminopyrene (Log TWA 1-APY $_{DE/CA}$) post exposure to diesel exhaust?

At the 0.10 significance level, none of the proposed predictor variables were retained in the final regression model, indicating that none of them (including sex) were significant independent predictors of log TWA 1-APY $_{DE/CA}$.

Table 5.9. Summary of Backward Elimination Regression of TWA 1-APY $_{DE/CA}$.

Step	Variable Removed	# of Variables in Model	Partial R-Square	Model R-Square	C(p)	F value	p Value
1	Age	6	0.00	0.15	6.14	0.14	0.71
2	Pulse	5	0.01	0.15	4.48	0.35	0.56
3	Sex	4	0.02	0.13	3.33	0.88	0.35
4	Respiration	3	0.02	0.11	2.33	1.05	0.31
5	Temperature	2	0.04	0.07	2.08	1.82	0.18
6	BMI	1	0.03	0.03	1.67	1.62	0.21
7	Race	0	0.03	0.00	1.24	1.58	0.22

5.6 Discussion

The most striking result of this study was the sex difference in the observed concentrations of TWA 1-APY measured in urine after exposure to diesel exhaust relative to clean air even though these observed differences were not statistically significant. This lack of significance between the sex groups may be due to the small sample size of the two groups. Most studies that have been done so far looking at urinary levels of 1-APY in humans have been occupational exposure studies and these studies did not look at sex differences as we did in this study so there is nothing to compare our current findings to.

Previously Ernstgard et al in 2003 looked at sex differences in the toxicokinetics of m-Xylene under controlled environmental conditions and they found that overall males had a significantly higher cumulative excretion of m-methyhippuric acid in urine ($p = 0.005$) and that this was consistent with their higher net uptake of m-xylene. They concluded that the results indicated slight differences between women and men which

were consistent with anatomical sex differences and thus body size and body composition (physical constitution) seemed to be more important determinants than sex in the toxicokinetics of m-xylene.

In this study, we found opposite results with females showing about a 12% overall mean increase in TWA 1-APY excretion following diesel exhaust exposure, while males showed a very slight overall mean increase of about 0.004% (see Figure 3). Although none of these observations were statistically significant they do highlight a trend of a sex difference in the excretion of TWA 1-APY and indicate the possibility of an underlying mechanism that may influence the metabolism of 1-nitropyrene by sex and not anatomical differences. If this trend that was observed by sex was due to anatomical differences, and males did indeed inhale a higher amount of DEPs than females, then based on the previous study by Ernstgard et al, we would expect to see the opposite of what we found with males excreting a larger amount of TWA 1-APY in urine than females after exposure to diesel exhaust, which was not the case in this study.

The metabolism of 1-NP can occur via nitro-reduction or cytochrome P450 mediated ring oxidation (Silvers et al 1997 and Chae et al 1999). 1-APY is one of the major nitro-reduction metabolites of 1-NP so the observed differences in the urinary concentrations of 1-APY after diesel exhaust exposure between males and females could indicate that females may metabolize 1-NP via nitro-reduction more actively than in males where the oxidative metabolic pathways may be more active. Since the urinary concentration of the oxidative metabolites of 1-NP were not measured in this study it is hard to definitively hypothesize if this is the case.

5.6.1 Study Limitations

This possible trend needs to be studied with larger balanced populations especially since the sample size used for this study was small and unbalanced. A power analysis using the observed mean and standard deviations of this study indicates that with the study sample size (21 for females and 29 for males) the likelihood to detect a 5% sex difference in urinary 1-aminopyrene is 0.12. This means that at the observed effect size (0.22) a total sample size of 514 subjects will be needed to achieve an acceptable power of 0.70 to detect sex differences in urinary 1-aminopyrene excretion.

Also, since creatinine excretion is influenced by muscle mass (Masi et al 2004) a different method for correcting for dilution of the urine samples should be used instead of creatinine when looking at sex differences because muscle mass is also influenced by sex and thus the use of creatinine correction in such a study could potentially lead to the skewing of the data.

In addition, a potential source of error that was not investigated was the potential additional exposure of subjects to DEPs or 1-nitropyrene outside of the exposure chamber during their diverse daily activities. This might be a potential source of error as indicated by both the baseline pre-exposure levels of 1-APY and also the within subject variability in the 1-AP secreted after exposure to diesel exhaust and clean air.

Lastly, there is the possibility that the length and intensity of the study exposure was not adequate enough to produce statistically significant changes.

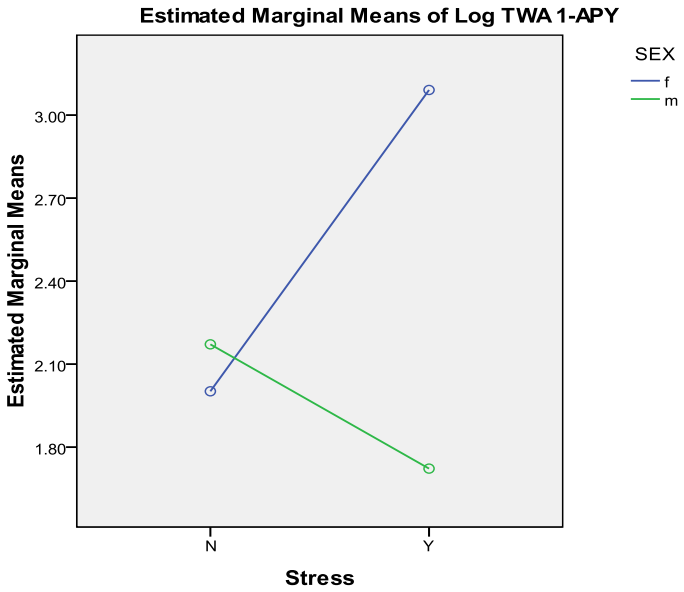
5.7 Conclusions

Although not statistically significant, the results of this study showed a trend of a difference by sex in the metabolism of 1-NP following acute exposure to diesel exhaust relative to clean air as indicated by differences in the urinary concentrations of TWA 1-APY between males and females after exposure to diesel exhaust relative to clean air. Females tended to secrete a higher concentration of TWA 1-APY after diesel exhaust exposure relative to clean air exposure than males. This observation was not statistically significant however, the results suggest that females may metabolize 1-nitropyrene via nitro-reduction more actively than males.

Appendix D

Table D.1. One Sample t-test results of Log TWA 1-APY by Stress for All Subjects and also by Sex.

Log TWA 1-APY $_{DE/CA}$	Mean	S.E.	t Value	p-Value
All	-0.04	0.60	-0.07	0.95
F	-1.09	0.91	-1.19	0.24
M	0.45	0.83	0.54	0.59



Sex	Stress	Mean	S.E
F	N	2.00	0.56
	Y	3.09	0.80
M	N	2.17	0.61
	Y	1.72	0.51

Figure D.1. Profile Plot of Log TWA 1-APY by Stress for All Subjects and also by Sex.

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Chapter 6

Summary, Implications and Future Research Directions

6.1 Summary of Results

The findings from this study on sex differences in response to acute exposure to 300μg/m^3 of diesel exhaust in a controlled environment can be summarized as follows:

6.1.1 Local Airway Immune and Inflammatory Responses

- In nasal lavage, significant sex differences were observed in tumor necrosis factor – α concentrations with males having a mean higher concentration than females twenty four hours after diesel exhaust exposure relative to clean air. Although not significant, males also tended to have higher mean concentrations of other upper airway inflammatory markers than females twenty four hours after diesel exhaust exposure relative to clean air.
- In sputum, it was observed that females tended to have mean higher concentrations of lower airway inflammatory markers e.g. tumor necrosis factor – α and interleukin 6 than males six hours after exposure to diesel exhaust relative to clean air. None of these observations were statistically significant.
- The observed differences in the sites of the suggested inflammatory response between males and females could indicate that males are more sensitive to the effects of the fine particles of diesel exhaust which tend to deposit in the upper airways while females are more sensitive to the effects of the ultra fine particles of diesel exhaust which tend to deposit in the lungs.

6.1.2 Systemic and Acute Phase Reactions

- There was a statistically significant effect of sex on platelet count. Six hours after exposure to diesel exhaust there was a statistically significant difference in

platelet count levels between males and females, with males showing a mean decrease in platelet count following exposure to diesel exhaust relative to clean air, after subtracting baseline counts compared to an observed mean increase in platelet count in females.

- Within males, twenty four hours after exposure to diesel exhaust there was a statistically significant mean decrease in platelet count relative to clean air,
- The rest of the systemic and acute phase response markers showed a mixed response trend with no clear cut significant sex difference.

6.1.3 Self reported Symptoms

- There was a statistically significant effect of sex on self reported somatic and lower respiratory symptoms severity ratings with females tending to have higher mean severity ratings than males after exposure to diesel exhaust relative to clean air, after subtracting baseline severity ratings.
- Although not statistically significant, mean acute phase reaction and anxiety symptoms severity ratings tended to be higher in females than in males following exposure to diesel exhaust relative to clean air, after subtracting baseline severity ratings.
- General symptoms were observed to show an opposite trend with males tending to have higher mean symptom severity ratings than females following exposure to diesel exhaust relative to clean air, after subtracting baseline severity ratings. This observation was not statistically significant.

6.1.4 Metabolism of 1-Nitropyrene

- Sex differences were observed in the time weighted average urinary concentration of 1-aminopyrene following exposure to diesel exhaust relative to clean air. Females had a higher mean increase in the time weighted average urinary concentration of 1-aminopyrene than males. However, this observed difference was not statistically significant.
- Despite the above observation, using a backward regression analysis model, sex did not appear to be a significant predictor of the time weighted average urinary concentration of 1-aminopyrene.

6.1.5 Integration of Study Results

Overall, the observations of this study were in the direction of the study hypothesis with females appearing to be more sensitive to the effect of inhaled diesel exhaust (ultra fine) particles than males as hypothesized. Upon exposure to diesel exhaust females had a stronger inflammatory response in the lower airway (as suggested by the observed cytokine concentrations in sputum which were not statistically significant). This was accompanied by a stronger systemic inflammatory response in females (as indicated by the significant sex difference in platelet counts at 6 hours) and a reporting of higher symptom severity ratings by females (as indicated by the significant sex difference in somatic and lower respiratory symptom severity ratings).

Also, as hypothesized there was an observed difference by sex in the concentration of 1-aminopyrene in urine with females tending to secrete more 1-aminopyrene in urine than males. Although this observation was not statistically

significant it does suggest that the nitro-reduction metabolic pathway is may be more active in females than in males.

Contrary to the study hypothesis, males had a stronger inflammatory response in the upper airway (as indicated by the significant increase in TNF-α concentrations in the nasal lavage of males compared to a decrease in females). This could highlight the fact that males may be more sensitive to the effects of inhaled diesel exhaust (fine) particles in the upper airway.

6.2 Implications

Although not all the sex differences observed in this study were statistically significant, they do have important research and clinical implications.

The study observations suggest that there may be a sex difference in the toxicokinetics of diesel exhaust. This means that for a similar exposure event, the difference in the internal physiology of females and males due to sex could cause a difference in the toxicological profile and effect of diesel exhaust, ultimately affecting which symptoms appear or fail to appear.

This potential sex driven difference in the toxicokinetics of diesel exhaust was highlighted by the following significant study findings:

- The fact that TNF-α concentrations in nasal lavage and platelet counts in blood were significantly different between males and females.
- The fact that somatic and lower respiratory symptoms were significantly different between males and females.

The research implications of this potential sex driven difference in the toxicokinetics of diesel exhaust include the following areas:

- Subject Selection – Depending on the subject composition by sex in an experimental study looking at diesel exhaust inducible physiological effects, the results have the potential to be underestimated or overestimated if this sex driven difference in the toxicokinetics diesel exhaust holds true.
- Diesel Exhaust Composition – Variations in the particle size composition of diesel exhaust used in studies may lead to differences in the observed effects due to the suggested sensitivity of males to fine particles and females to ultra fine particles of diesel exhaust.
- Biomarkers – If there is a difference by sex in the metabolic pathways used to metabolize 1-nitropyrene then this could lead to variations in the urinary concentration of 1-nitropyrene metabolites and so this has to be taken into consideration when trying to select an appropriate biomarker of exposure to diesel exhaust based on 1-nitropyrene metabolites.
- Health Assessment Tools – With respect to self reported health symptoms, since there may be a sex difference in the types and severity of symptoms reported after diesel exhaust exposure which may be linked to the proposed sex driven difference in the toxicokinetics of diesel exhaust, it is important to consider sex when planning to use self reported symptom questionnaires as a data collection tool in the analysis of diesel exhaust attributable health effects.

Overall, regarding research, the observations from this study provides further support for not just controlling for sex as has been historically done in environmental exposure studies but actually including sex as part of the analysis.

The clinical implication of the observed tendency of females in this study to generally report higher symptom severity ratings than males after a controlled exposure to the same external concentrations of diesel exhaust could indicate that females may be more physiologically sensitive to diesel exhaust exposure and thus females may be more susceptible to diesel exhaust exposure.

Along those lines, the public health implications of the females potentially being more physiologically sensitive to diesel exhaust exposure include the following areas:

- Risk Assessment – If this hypothesis holds true then female as a whole (not just pregnant women) may be another sensitive population that has to be considered when risk assessments of environmental pollutants like diesel exhaust are done especially with regards to occupational settings and exposure.
- Policy Measures – Also, the standard inter-individual uncertainty factor of 10 used to come up with acceptable exposure levels of environmental air pollutants like diesel exhaust may not be protective enough if females really are more sensitive than males. This means that an additional uncertainty factor may need to be used but more data is needed which would provide more evidence in support of this potential sex difference as well as provide more information about the potential magnitude of variation in the response of females compared to males. These data are needed for the proper evaluation of whether the current uncertainty factors that are used are adequate.

6.3 Future Research Directions

More studies should be done using larger and balanced sample sizes to try to further elucidate the possible sex differences in response to diesel exhaust exposure that was suggested by this study.

- Further research on the pro-inflammatory nature of diesel exhaust should take into account the possible sex difference in the sensitivity and toxicokinetics of diesel exhaust during study design.
- Also, other possible non-study related environmental exposures to diesel exhaust should be controlled for if feasible in order to prevent the potential confounding of results.
- Studies should be done to help clarify the potential mechanism involved in the diesel exhaust attributable reduction in platelet count that was observed in males in this study versus an observed increase in females.
- Studies should be done to further evaluate the sex difference in somatic and lower respiratory symptoms that were seen in this study to see how the individual symptoms that make up the two symptom categories (somatic and lower respiratory) vary between males and females.
- Studies should also be done to see if these self reported somatic and lower respiratory health symptom cans be correlated with other objective physiological measures of diesel exhaust induced health effects.
- Lastly, a study should be done to look at the concentrations of the major metabolites of oxidation and reduction of diesel exhaust in order to ascertain whether there are sex differences in the concentration of metabolites based on the

metabolic pathway used. This will help to better understand if there is a difference by sex in the metabolic pathways that are activated upon diesel exhaust exposure and if these are correlated with sex linked genetic polymorphisms. This could possibly have important ramifications for the selection of biomarkers of exposure to diesel exhaust.

6.4 Uncertainties

There are some uncertainties regarding the study design and the mechanism of diesel exhaust toxicity that has to be kept in mind when reviewing and interpreting the results and observations of this study. These include the following:

- Since the study subjects consisted of volunteers, there is the possibility that only those people who were not bothered by diesel exhaust participated in the study. If true, this would introduce a possible selection bias into the study and might account for the greater number of males in the study than females (64 versus 36 respectively) especially if females are more sensitive to effects of diesel exhaust and so are not as willing to volunteer for such studies. Therefore the results of the study may be different if conducted in a different population.
- The composition of diesel exhaust may not be similar to those used in other studies. Therefore this has to be considered when comparing the effect seen in this study to those seen in other studies.
- Despite all the studies that have been done so far, there is still a general lack of consensus about the underlying mechanism by which diesel exhaust causes the health effects that have been observed. Although this study is based on the hypothesis of diesel exhaust induced inflammation related health effects, there is

also the possibility that the observed health effects could be due to the direct systemic effect of translocated diesel exhaust particles which has also been hypothesized as a potential mechanism of diesel exhaust toxicity.

- The effects of diesel exhaust could be additive or synergistic depending on whether its exposure is simultaneous with other air pollutants (USEPA HAD 2002).
- The toxicity of diesel exhaust is not only dependent on individual physiology but also on the individual internal exposure which is a function of both the variable concentration of diesel exhaust in the environment and the individual breathing and particle retention patterns (USEPA HAD 2002).
- Lastly, since this is an exploratory study, we chose not to correct for multiple comparisons, but this is still a potential issue and so the results from this study should be interpreted with caution.

Curriculum Vitae

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EDUCATION

2005 – 2010	University of Medicine and Dentistry of New Jersey School of Public Health, Piscataway, NJ **Ph.D in Public Health**
2002 – 2004	University of Medicine and Dentistry of New Jersey School of Public Health, Piscataway, NJ **M.P.H in Public Health**
2000 – 2002	Rutgers University, Newark, NJ **M.S. in Biology**
1995 – 1999	Rutgers University, New Brunswick, NJ **B.A. Biology**

EXPERIENCE

2006 – 2009	University of Medicine and Dentistry of New Jersey, Piscataway, NJ **Research Teaching Assistant**
2005 – 2006	GlaxoSmithKline, Parsippany, NJ **Asset Manager Analyst**
2002 – 2005	GlaxoSmithKline, Parsippany, NJ **Data Management Coordinator**
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