EFFECT OF A CONTROLLED DIESEL EXHAUST EXPOSURE ON AIRWAY OXIDATIVE STRESS IN HUMANS: ANALYSIS OF EXHALED BREATH CONDENSATE

by

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ABSTRACT

Introduction:

The purpose of this study was to characterize the effects of a controlled diesel exhaust (DE) exposure on airway oxidative stress in humans by measuring two biomarkers of interest in exhaled breath condensate (EBC). As there is evidence that antioxidant supplementation plays a role in reducing respiratory health effects associated with DE, this study also assessed whether antioxidant supplementation helps mitigate DE-related oxidative stress in humans.

Methods:

EBC was taken from subjects participating in a randomized, three-way crossover study (i.e. 3 different exposures: fresh air + placebo [FAP], DE + placebo [DEP], and DE + antioxidant [DEN]) at baseline as well as 2, 6 and 30 hours after exposure. Analysis for 8-isoprostane was performed using liquid chromatography with tandem mass spectrometry; pH was analyzed using standard de-aeration protocol and pH meter reading. Linear mixed effects models in SPLUS 8.0 were used for statistical analysis.

Results:

A total of 27 participants had their EBC collected and analysed for biomarker content: 23 for 8-isoprostane and 17 for pH. 8-isoprostane was consistently higher after DEP relative to FAP and was consistently lower after DEN relative to DEP, but none of these trends were statistically significant. Effects of exposure on pH were less consistent. The effect of exposure on 6 hour pH was significantly modified by sex ($p=0.03$); males showed a significant acidification after DEP relative to FAP ($p=0.003$), females showed a significant acidification after DEP relative to DEN ($p=0.03$). Other covariates did not significantly modify the interaction between exposure and biomarker levels.

Conclusion:

Amongst all subjects, exposure had no significant effect on EBC oxidative stress biomarkers. According to a secondary analysis, DEP lowered EBC pH 6 hours after exposure in males. Short-term diesel exhaust at concentrations typical of occupational settings does not significantly alter EBC oxidative stress in a controlled study with modest sample size. However, trends

towards an effect on pH and apparent effect modification by gender warrant consideration of further study using a larger sample size.

PREFACE

This thesis was made possible by the supervision of Dr. Christopher Carlsten, Dr. Karen Bartlett and Dr. Michael Brauer, from the UBC Faculty of Medicine.

Method development for analysis of EBC 8-isoprostane, detailed in Appendix A, had significant contribution by Philippe Provencher, B.Sc Chemistry and Dr. Winnie Chu, Faculty of Medicine.

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Mike Brauer, thank you for your wonderful guidance towards helping me obtain a better understanding on how to show, analyze and interpret results. Without your help, I probably would have submitted less thorough analyses and less critical evaluation of my results.

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Dedication

I dedicate this thesis to my mom.

I wouldn’t ever have dreamed to come this far if it wasn’t for your love and encouragement.

Chapter 1: Introduction

1.1 Overview

Over the past few decades, diesel exhaust particles (DEPs) -- a primary component of air pollution-related particulate matter -- have gained significant attention as a public health problem (1-2). Indeed, studies have shown that modest increases in particulate matter (PM) may contribute to significant increases in respiratory morbidity and mortality (3-6). Diesel fuel combustion results in the production of diesel exhaust particles (DEPs), the majority of which are classified as fine (2.5-0.1 mm) or ultrafine (<0.1 mm) particles (UFPs) (2). Diesel engines produce less greenhouse gas emissions than gasoline engines; however they contribute a 100 times more particles per distance traveled (2, 7-8). This is problematic given that DEPs are disproportionately UFPs, which appears to be the most toxic fraction of PM (9-10). This becomes increasingly problematic since the use of diesel engines is on the rise, due to their increased operating efficiency, reliability and fuel economy as compared to gasoline engines (9). Consumption of diesel fuel has increased markedly since 1995 (9, 11): freight transport volumes have more than doubled since 1970, to about 3000 billion tonne-kilometres (9). The consumption of diesel fuel is projected to increase from 40% to 60% of the transportation fuel market by 2020 (9). In Canada, diesel fuel consumption has increased at an exponential rate from 1960 to 2010 while gasoline consumption has remained somewhat consistent and stable (12-13). Despite recent advances in diesel fuel and combustion technology that reduce PM emissions, such as lower sulfur content in fuels (9), after-treatment tools and filters (14-15), the overall global benefit will take decades to come, given the longevity of existing diesel engines and thus the slow penetration of new engines into the global fleet (16-17). Thus, exposed working and non-working populations will continue to be subjected to the particulate matter emissions from DE.

Particulate matter originating from road traffic has been shown to exacerbate asthma and asthma-like symptoms (18-20). *In vitro* and *in vivo* controlled exposure studies have revealed particulate matter to confer potent pro-inflammatory effects upon lung epithelial cells and alveolar macrophages (10, 21-23). Controlled exposure chamber studies provide a method by which to examine the acute mechanisms of individual air pollutants (10). In fact, diesel exhaust

controlled exposure studies on healthy human participants showed that diesel exhaust exposure was associated with increased airway resistance (24-25), bronchial inflammatory changes (26-29) and increased airway inflammation (23). However, it is still unclear if the aforementioned effects are greater in asthmatics versus non-asthmatics.

It has been suggested that diesel exhaust may play a role in airway oxidative stress (30-35). Particulate matter and diesel exhaust particles (DEPs) contain redox cycling organic chemicals such as quinones and aromatic hydrocarbons as well as transition metals which induce pro-oxidative and pro-inflammatory effects in the lung (31-32, 36). It has been shown in vitro that these oxidative responses are suppressed by N-acetylcysteine (NAC), a powerful scavenger of reactive oxygen species (37), which directly complexes to electrophilic DEP chemicals and exert additional antioxidant effects at the cellular level (31, 33, 36). Romieu *et al* showed that antioxidant supplements were associated with attenuation of pollution-associated decreases in lung function (38). However, the association between antioxidant supplementation and acute DE-related airway functional change has not been demonstrated in humans, asthmatic or healthy.

Exhaled breath condensate (EBC) has the potential to contribute to the understanding of the physiological and pathophysiological processes in the lungs when exposed to diesel exhaust in several ways (39). For instance, EBC pH has been shown to be a marker of airway inflammation and acidification (22, 40-44). Levels of 8-isoprostane have been shown to be a reliable marker for airway oxidative stress (45-48). Furthermore, EBC is non-invasive, easy to use, portable, reproducible and has a very safe collection method as compared to other invasive and semi invasive methods to assess the degree of airway inflammation such as bronchoscopies and sputum induction (22, 40, 44, 49). In an asthma study, the assessment of airway acidification using EBC pH was shown to be lower during exacerbations and to normalize with anti-inflammatory therapy (44). Also, 8-isoprostane formation increases dramatically *in vivo* post oxidant injury (50), levels have been shown to be modulated by antioxidant status (51-53), and unaffected by lipid content of the diet (54-55). Thus, EBC pH and 8-isoprostane levels may show the hypothesized beneficial effects of antioxidant treatment for airway oxidative response to diesel exhaust exposure.

1.2 Objectives

1. To investigate the effects of controlled diesel exhaust exposure on human airways' oxidative stress by analysis of pH and 8-isoprostane in exhaled breath condensate.
2. To characterize the effects of antioxidant treatment on potential DE-associated airway oxidative stress in terms of EBC pH and 8-isoprostane.

CHAPTER 2: Background

2.1 Diesel exhaust

2.1.1 Fuel, composition, and physical characteristics

Diesel exhaust (DE) is not one specific compound but a complex mixture of elements typically characterised by a carbon core in which certain polycyclic aromatic hydrocarbons (PAHs) find themselves adsorbed while others are volatilized; also in this mixture are various hydrocarbons of varying complexity and length, traces of metals, water vapor, carbon oxide gaseous species (CO/CO_2), nitrous oxide (NOx) gaseous species (NO/NO_2), as well as sulfur oxide compounds, depending on the type of diesel engine (56-58).

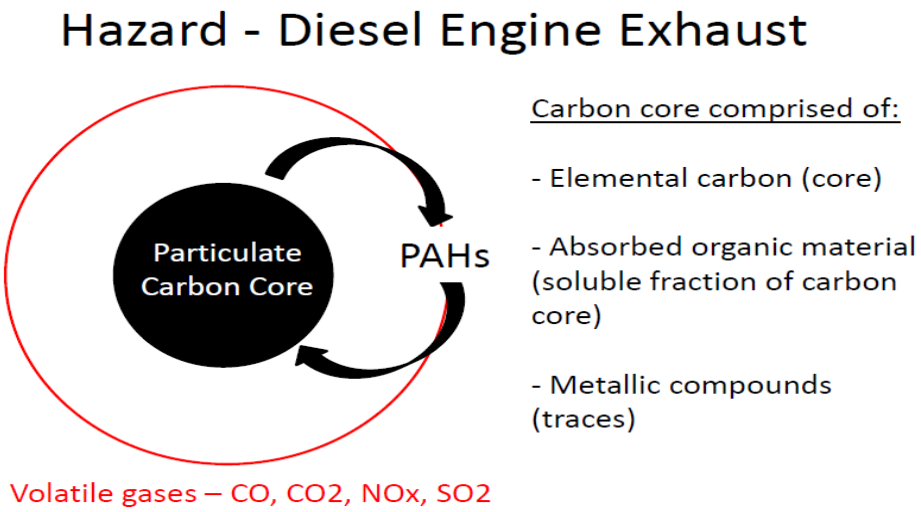

Figure 1. Diesel engine exhaust chemical structure substitute. Absorbed organic material refers to the PAHs which dissolve and get emitted by the carbon core depending on the ambient environment. Metallic compounds include lead, arsenic, etc, which are highly toxic materials. Depending on the type of diesel fuel, there may or may not be sulfur and sulfur oxides being emitted.

Diesel fuel combustion results in the production of diesel exhaust particles (DEPs), ranging from coarse (any particle with an aerodynamic diameter between 2.5 and 10µm) to ultrafine particulate size (any particle with an aerodynamic diameter of less than 0.1µm); indeed, the majority are classified as ultrafine particles (UFPs) which are highly respirable and capable of depositing in the lower airways (2). Diesel engines produce less greenhouse gas emissions than gasoline engines; however they produce 100 times more particles per distance traveled (7-8).

Ultrafine particulate matter, the predominant diesel-derived particle fraction, appears to be particularly toxic (9-10). This is problematic since the use of diesel engines is on the rise, due to their increased operating efficiency, reliability and fuel economy as compared to gasoline engines (9, 59). Consumption of diesel fuel has increased markedly since 1995 (9, 11) and freight transport volumes have more than doubled, since 1970, to about 3000 billion tonne-kilometres (9). The consumption of DE is projected to increase from 40% to 60% of the transportation fuel market by 2020 (9).

In recent years as well, the sulfur content of diesel fuels has substantially decreased due to better emission standards in both Europe and North America (9).These new standards have lead to the use of "clean diesel" which offers a significantly smaller amount of particulate matter emissions than regular fuel consumptions (9). According to a report by the WHO in 2005, particulate matter emissions from passenger cars have a projected decrease from 0.06g/km in 2000 to less than 0.01 in 2008; similarly for heavy duty engines, a projected decrease from 0.15g/KWh in 2000 to less than 0.05g/KWh in 2008 (9). The U.S. EPA published a report in 2010 identifying an overall 33% national decrease in PM_{10} emissions and 48% reduction in $PM_{2.5}$ emissions between 1990 to 2005 (60). Thus, ambient levels of particulate matter have decreased in recent decades in North America; however, PM including DEP is still an important exposure in occupational settings as described in section 2.4. Despite diesel fuel being "cleaner" in that combustion releases less particulate matter today than a decade ago, particulate matter emissions are still a current public health concern and have been linked to several different health effects such as described in section 2.2.

2.1.2 DE emission control technology

Diesel combustion is used in both stationary and mobile applications, particularly where power output is needed, such as trucks, railroad trains, agricultural equipment, marine vessels, etc (61). There has been a recent change in diesel engine technology of more recent diesel engines in an effort to decrease tailpipe emissions (61). Indeed, the innovation of high pressure, common rail, direct injection systems (62-63), the use of shape and multiple pulse fuel injection methods (64), and the development of PM and NOx species after treatment tools (14) have helped change diesel engines to match current emission standard limits for environmental air quality (61). The

most common tool used to reach emission standards is diesel emission after treatment methods such as diesel particulate filters (15).

Newer engines with emission control parameters will have significantly less NOx species emitted in light-duty diesel engine emissions, heavy-duty technology still being developed (65). Particulate matter control for both light- and heavy-duty engines have used filter materials and catalysts (17). Despite these advances in control technology, the implementation of such controls is slow and costly, particularly since the median lifetime of such engines is 18-19 yrs (16). As such, emissions from diesel engines will continue to be problematic, particularly in heavy diesel engine use industries since replacing of fixing these types of engines typically cost more than the average on road light duty vehicle (17). Lastly, there is evidence that newer engines may not decrease nano-particle emissions (16).

2.2 Health effects

2.2.1 General respiratory morbidity and mortality

Over the past few decades, diesel exhaust particles (DEPs) a primary component of air pollution-related particulate matter, have gained significant attention as a public health problem (1-2). Indeed, studies have shown that modest increases in particulate matter (PM) may contribute to increased respiratory morbidity and mortality (3-5, 66-70).

2.2.2 Acute respiratory effects

Particulate matter originating from road traffic has been shown to exacerbate asthma and asthma-like symptoms (18-20). *In vitro* and *in vivo* controlled exposure studies have revealed particulate matter to confer potent pro-inflammatory effects upon lung epithelial cells and alveolar macrophages (10, 21-24, 26-29, 71-76).

2.3 DE biochemistry, method of action

Furthermore, it has been suggested that diesel exhaust may play a role in airway oxidative stress (30-35). Diesel exhaust particles (DEPs) contain redox cycling organic chemicals such as quinones and aromatic hydrocarbons as well as transition metals which induce pro-oxidative and pro-inflammatory effects in the lung (31-32, 36). Indeed, *in vitro* and animal studies have shown

that DEPs can generate reactive oxygen species (ROS), which stimulate inflammatory cytokine production and induce apoptosis in lung cells and tissues (77-78).

DEP induces the release of granulocyte macrophage-colony stimulating factor in nasal and bronchial epithelial cells (79). This causes the phagocytosis of DEP and the activation of the phosphorylation pathway MAPK and transcription factors like NF-κB (79-81). It is thought that PAHs, part of DEPs, could be desorbed from DEP in the cell and be available to interact with cytosolic aryl hydrocarbon receptor and induce gene expression (82). The gene expression is thought to occur on CYP-450 1A1 genes which activates PAH metabolism thereby releasing electrophilic and reactive metabolites, including ROS (82). Quinones, which are also a part of DEPs in the lungs, are known to generate oxidative stress by being responsible for the production of $0_2^{-}\cdot$ and $OH^{-}\cdot$ radicals (83). Quinones are also directly involved with free radical production which has been linked to activating NADPH CYP 450 reductase that turns quinones into semi quinones, a reactive oxygen compound.

Bonvallot *et al.* 2002 made a diagram of the hypothesized pro-inflammatory pathway of diesel exhaust particles (Figure 2).

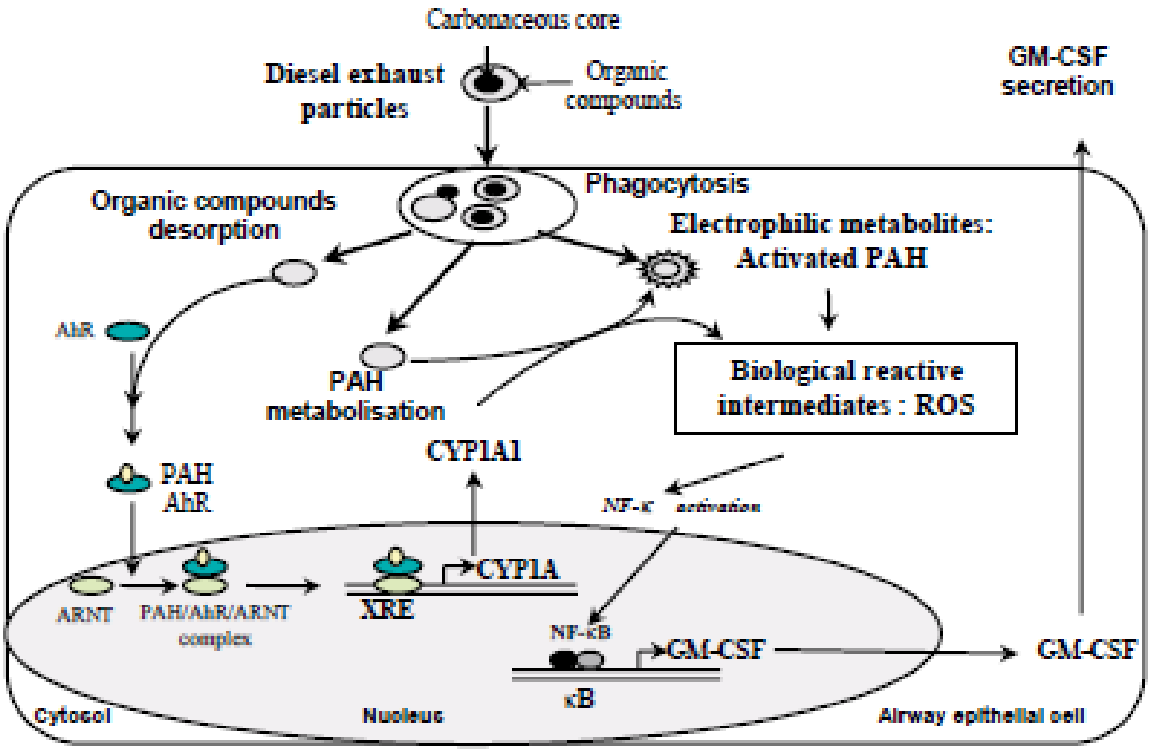

Figure 2. Hypothesized DEP pro-inflammatory response mechanism. Taken from Bonvallot et al. 2002 publication entitled "Diesel exhaust particles induce an inflammatory response in airway epithelial cells: involvement of reative oxygen species" which appeared in BioFactors (78).

2.4 Occupational exposures to DE in North America

Occupational exposure to DE particles is a significant health concern in North America. Scientific literature from the 1980s to present day has shown a multitude of occupations that involve substantial exposure to DE. Most occupational exposures to DE emissions come from off-road vehicles such as mining equipment, trains, ferries and trucks (84).

U.S. studies showed geometric mean (GM) exposures of respirable elemental carbon (EC) in local and long haul truck drivers of 6 µg/m^3 (Geometric Standard Deviation [GSD] 1.6) and 4-19 µg/m^3 (GSD 2.0-3.8, respectively (85). The same studies showed GM respirable particulate matter (PM) exposures ranging from 20 to 120µg/m^3 (GSD 1.5-2.5) (85). The truck mechanics were exposed to GM respirable PM concentrations of 152µg/m^3 (GSD 2.1) (85). U.S. mining studies showed respirable GM EC exposures in production and maintenance undergrounds crews of 111µg/m^3 (GSD 1.4-4.8) and 66µg/m^3 (GSD 1.7-4.6), respectively; as well as underground PM concentrations of 940 µg/m^3 (GSD 1.7-2.5) (86-88). Railroad industry workers in Canada

showed mean respirable GM EC concentration exposures of 3-6µg/m^3 (GSD 1.5-3.5). U.S. construction industry workers off heavy highway traffic sites and dockworkers were exposed to GM EC concentrations of 8µg/m^3 (GSD 2.7) and 2-7µg/m^3 (GSD 1.3-27.2), respectively; while tunnel workers were exposed to GM PM respirable concentrations of 254µg/m^3 (GSD 2.5-4.2) (89). Earlier U.S. studies showed 240 µg/m^3 exposures of respirable $PM_{2.5}$ in truck mechanical workers (90).

Pronk et al. reviewed studies which showed the highest respiratory exposures to submicron $PM_{2.5}$ occurred in the underground mining industry with geometric means ranging from 142-699µg/m^3, 364-3300µg/m^3 of respirable $PM_{2.5}$ as well as in underground construction with exposures of 1160-1700µg/m^3 of respirable $PM_{2.5}$ (84). High exposures of submicron respirable $PM_{2.5}$ of 106-1600µg/m^3 were observed for mechanics, emergency workers in fire stations, distribution workers at dock sites and workers loading and unloading inside a ferry (84).

These studies characterized worker exposures to DE, they also suggested that small exposures to DE should not be disregarded as they may have significance for long term chronic health effects (91). Interestingly, for some occupations such as dock workers and mechanics, exposures to DE emissions seemed to dramatically increase in cold temperatures; thus, DE exposure may be of increased concern in northern occupational populations (91).

2.5 Vulnerable populations

The health implications of DE exposure are of concern for many occupational groups but especially for vulnerable populations such as pregnant women, children, the elderly as well as those with respiratory diseases such as asthma (1, 92-96). Asthma is a complex chronic airway inflammatory syndrome which is characteristically associated with airflow obstruction and bronchial hyperresponsiveness (41). DE has been shown to exacerbate asthmatic phenomena such as airway hyperresponsiveness and wheeze (23, 97), and even induce occupational asthma in certain cases (98).

2.6 Asthma

Asthma is a complex chronic airway inflammatory syndrome that is characteristically associated with airflow obstruction and bronchial hyperresponsiveness (20, 99-101). Its phenotypic

plasticity makes for its challenging diagnosis through patient history, physical examinations, assessment of airway obstruction reversibility and elimination of other diseases and syndromes (41). Typically, this is done using lung function testing for airway hyperresponsiveness in conjunction with the assessment of clinical symptoms such as intermittent wheeze and cough (20, 41). There are several methods that clinicians use to assess airway inflammation and hyperresponsiveness which vary in invasiveness; these include invasive procedures such as bronchial lavages, bronchoscopies and bronchial biopsies; a semi-invasive technique such as sputum induction; and non-invasive methods such as fraction of exhaled nitric oxide (FeNO) and exhaled breath condensate (EBC) (42). Exhaled breath condensate is a non-invasive procedure from which a large number of biomarkers of lung disease can be sampled for (for example, pH and 8-isoprostane); therapeutic interventions may also be monitored using EBC (43).

Asthmatics exposed to DE have shown significant changes in airway inflammation and oxidative stress biomarkers. Indeed, asthmatics are thought to be at increased susceptibility to diesel exhaust exposure due to their inherent imbalance in antioxidant mechanisms (102-105).

2.7 Oxidative stress

2.7.1 Physiology

Oxidative stress is characterized by an imbalance between increased exposure to free radicals and antioxidant defences comprised of small molecular weight antioxidants such as glutathione and antioxidant enzymes such as superoxide dismutase (46).

During normal aerobic metabolism, the human body constantly produces reactive oxygen species (ROS) and reactive nitrogen species (RNS). Reactive oxygen species include superoxide anion, hydroxyl radical, hydrogen peroxide, and singlet oxygen; while RNS include nitric oxide (106).

Under normal circumstances, the human body is able to control the redox balance using a very sophisticated antioxidant defense system, which includes enzymes such as superoxide dismutase, catalase, and glutathione peroxidase as well as nutrients such as vitamins A, C, E, and glutathione, among others. However, in disease conditions such as chronic infection and inflammation excess, production of pro-oxidants such as ROS/RNS may result in depletion of

antioxidant stores resulting in oxidative stress (106-108). Excess production of ROS/RNS can cause oxidative damage to DNA as well as modify carbohydrates, proteins, and lipids to cause cellular injury (78, 81, 109-113).

Oxidative stress is a component of the inflammatory response (114-115), airways of asthmatics have increased oxidant injury and inflammatory burden (107-108, 116) and as such it is thought that DE causes increased oxidative stress (31, 35, 81, 117-118).

2.7.2 Antioxidant therapy

It has been shown *in vitro* that these oxidative responses are suppressed by N-acetylcysteine (NAC), a powerful scavenger of reactive oxygen species (37, 119), that directly complexes to electrophilic DEP chemicals and exerts additional antioxidant effects at the cellular level (31, 33, 36, 119). An animal study showed N-acetylcysteineamide to reduce oxidative stress and inflammation caused by diesel exhaust particles (120). Romieu et al. 2004 showed that antioxidant supplements were associated with an attenuation of pollution-associated decreases in lung function in asthmatic children of Mexico City (38, 121). However, the effect of antioxidant (NAC) supplementation on acute DE-related airway functional change has not been demonstrated in humans.

2.8 Exhaled Breath Condensate

2.8.1 EBC overview

Exhaled breath condensate (EBC) is obtained through the condensation of exhaled breath into its liquid phase. It is thought to originate from airway surface lining fluid (ASLF), which becomes aerosolized during exhalation (49, 122-124). The content of the condensate is thought to reflect the composition of the ASLF fluid (40, 125-127). EBC biomarkers vary in their amounts, composition, chemical characteristics and can be present as small ions, gaseous acids, low-molecular-weight compounds as well as large to small proteins (i.e. although large molecules may not aerosolize as well as small soluble molecules) (126, 128).

EBC can contribute to the understanding of the physiological and pathophysiological processes in the lungs when exposed to diesel exhaust in several ways (39). For instance, EBC pH has been

shown to be a marker of airway inflammation and acidification (22, 40-44). Levels of 8-isoprostane have been shown to be a reliable marker for airway oxidative stress (45-48).

2.8.2 Source and composition of EBC

EBC contains mostly fluid, about 99% of its contents being liquid, only a fraction of which is derived from non volatile molecules that may be expired into respiratory droplets and then condensed into the ASLF (43). EBC is thought to reflect the composition of the ASLF in the lower airways due to similarities between studies conducted on inflammatory mediators such as 8-isoprostane and hydrogen peroxide (45, 129-132). In addition, care must be taken prior to analysing of EBC results from patients with oral inflammatory diseases since they certain diseases may alter the levels of certain biomarkers (43).

Certain issues arise with characterizing EBC biomarkers due to the presence of this large variation in chemical characteristics in EBC biomarkers; EBC biomarker volatility affects their variability in the various analytical assays available to assess its composition (43, 128). For example, the issue of dilution for volatile components of EBC is one which has not been clearly identified or standardized yet since it varies among patients and over time (airway lining fluid dilution occurs during condensation of exhaled breath); however, dilution does not seem to affect non-volatile components of EBC such as pH, isoprostanes, aldehydes and nitrogen oxides (43, 128).

2.8.3 Methodology of collection

Typically, patients are asked to breathe tidally through a mouthpiece, which is connected to a collection device, previously cooled to 0°C (133). This procedure is done from 10 to 30minutes in order to obtain 1-3ml of condensate. The American/European Thoracic Society task force published a document in 2005 mentioning several key issues with regards to EBC collection for specific biomarkers of interest (43), the most important aspects mentioned below, specifically for 8-isoprostane and pH:

Sampling device: it is not clear whether or not several collection devices can be used and results compared. As such, it is important to keep only one method of collection for groups being

compared to one another (i.e. matched collection devices) throughout the study and compare results to other studies with caution (please see the following section, 2.8.4).

Temperature of collection: it is not clear whether or not the temperature of collection results in better or worse for certain mediators. As such, keep the temperature of collection constant throughout the study.

Duration of collection: sample volume, minute volume or duration of collection may affect EBC volatile mediators; as such, one parameter should be chosen and kept constant throughout the study. It has been shown that in order to assess EBC pH without confounding, collection should not take more than 20 minutes for accurate EBC pH results (40).

Nose clips: they are recommended to be worn during EBC collection to minimize exhalation through the nose and maximize exhaled breath collection. However, inhalation through either the nose or mouth has been shown to not affect EBC pH or other protein markers (134).

Contamination: NOx species are present on laboratory surfaces or emanate from laboratory materials. Because contaminants may affect EBC pH, the system of collection and storage should be assessed for this.

Storage: store at the coldest temperature available. Vaughan showed that long term storage of EBC samples had no effect on de-aerated EBC pH up to two years (40); this was reconfirmed by Do in 2007 with 20-24 month storage at -80°C (135).

Stability: Data should be presented on the marker stability in EBC or previous publications should be assessed for the marker referenced.

Food and drink: the current protocol is to restrict food or drink 1 hour prior the EBC collection to avoid gastro-intestinal reflux which would acidify EBC pH.

Salivary contamination: no need to report salivary contamination; however, efforts should be made to reduce it.

Other: factors which have not yet been researched as affecting EBC pH or 8-isoprostane levels, which need to be noted: circadian rhythm (i.e. time of day), age and sex, tobacco smoking, use of medications.

Specific to pH biomarker of interest: EBC pH is the most widely studied biomarker of interest. De-aeration with a CO_2-free gas is compulsory when results are to be compared to the current literature as failure to do so may bias samples to the ambient levels of CO_2. Furthermore, the pH of de-aerated samples is not affected by hyperventilation, duration of collection (≤20min), duration and manner of storage (up to two years), oral versus endo-tracheal collection, acute airway obstruction with methacholine or exclusion of ammonia (40, 43, 135-138).

2.8.4 Collection devices

EBC can be collected using commercially available devices such as the portable RTube® or non-portable EcoScreen®, or from home made devices (139-140).

Koczulla, *et al.* 2009 showed that pH from EBC collected by RTubes® and Ecoscreens® produced comparable results for healthy, asthmatics and patients with COPD (140). There were no significant differences between pH values for controls, asthmatics and COPD patients or between days with coefficient of reproducibility for RTube® and EcoScreen® of 0.47 and 0.42, respectively.

Rosias, *et al.* 2008 measured several biomarkers of interest, more specifically 8-isprostane, and EBC volume in EBC collected using four different condensers: glass, silicone, EcoScreen® and an optimized glass condenser (139). The glass condenser offered significantly more EBC volume (median 2.025µL); while the reproducibility of the new glass condenser was comparable to that of the EcoScreen® (19-20% coefficients of variation [CVs]), as well as significantly better than the silicone and older glass condensers (29-37% CVs). However, reproducibility of biomarker determination for all condensers was variable; while the new glass condenser yielded

significantly more 8-isoprostane concentrations. Overall, reproducibility was not influenced by condenser type but concentration of biomarkers was highest in the optimized glass condenser.

Another study by Rosias, *et al.* in 2006, showed that condenser coating affected levels of EBC biomarkers. Five coatings (silicone, glass, aluminum, polypropylene and Teflon) and the EcoScreen® condenser were tested to measure 8-isoprostane, as well as three coatings (silicone, glass, EcoScreen®) to measure albumin. Silicone and glass coating had higher albumin and 8-isoprotane levels compared to the other coatings (141).

Prieto, *et al.* 2007 showed that pH in 23 non smoking asthmatics, after dearation with argon, was significantly higher using the EcoScreen ® condenser compared to the RTube®; as well as, that storage of samples at -80°C had a significant influence on the pH of samples analyzed without de-aeration (142).

2.9 EBC biomarkers

As previously mentioned, EBC is comprised of 99% water and 1% other compounds like proteins, ions, large molecules, etc. There are many of these molecules that can be detected and that have been used to characterise respiratory health status of the lungs. EBC 8-isoprostane and pH were chosen as the two biomarkers for this study because of several factors which are described below. The choice of which EBC biomarker to use came from the biomarkers influence by the following factors:

- Dilution
- Long term freezer stability
- Biomarker stability
- Collection times
- Circadian rhythm
- Influence of collection environment

Furthermore, samples were already mostly collected prior to this thesis and as such, certain limitations arose when choosing which biomarker to identify.

Gessner *et. al*, 2001 showed that EBC volume was linearly related to the volume expired, as well as to the total protein and urea content found in EBC suggesting that these compounds accumulated in the collecting device by a similar mechanism as expired water vapour (143). This even distribution, however, is not necessarily true for all substances present in EBC and needs further study. EBC's largest component is water, dilution of the biomarkers must be taken into consideration. There have been some studies assessing EBC standardisation of dilution or by standardising EBC parameters such as exhaled volume (129), exhaled ions (144-146), urea(144, 147), protein concentration (143) or conductance of lyophilised samples (148) as 'internal standards, or by using external dilution markers. Biomarkers affected by dilution were mostly volatile components such as ammonia, nitrogen oxides, H_2O_2 and leukotrienes (149).

Collection times also affect biomarker levels (149). Although no direct comparison is available regarding other mediators, no difference can be found in the concentrations of H_2O_2, nitrite/nitrate, 8-isoprostane, pH, adenosine and MDA between studies using 10, 15 or 20 min for EBC sampling (149).

Circadian rhythm has been demonstrated for EBC H_2O_2 levels both in normal subjects and patients with COPD (149). No circadian rhythm was identified for pH in a study of 152 subjects (40). However, it is not clear if circadian rhythm affects EBC 8-isoprostane. Collections were performed as closely as possible to the same time every exposure day in an attempt to control for circadian rhythm. Furthermore, our study design assumes circadian rhythm will be controlled for.

Hydrogen peroxide (H_2O_2) was considered as a biomarker of interest; however, this method to produce reliable results requires samples to be frozen immediately at -70°C until analysis. Our study left samples at -20°C for a short amount of time before switching to a -80°C. Furthermore, some of our study samples were kept for 2 years in the -80°C which has been shown to be inappropriate for hydrogen peroxide biomarker stability (149-151).

Nitrite species were also considered; however, these samples are easily contaminated by environmental NOx species on lab surfaces (149). Furthermore, NOx species are not stable at

low pH or even at neutral pH (149). This effect was not considered when EBC samples were collected.

Other biomarkers which were not considered were adenosines, arachidonic acid metabolites, cytokines, leukotrienes, aldehydes and ammonia; however are plagued with methodological issues (such as standardized analysis methodology, influence of biological variation, etc) which cannot be addressed by this study (149).

EBC 8-isoprostane and pH were the two biomarkers which were the least affected by the above factors. Particularly for long-term stability, pH was stable at -80°C for up to two years with freeze-thaw cycles not affecting pH readings. 8-isoprostane was shown to have little sample loss up to 180 days at -80°C; however, was influenced by freeze-thaw cycles and will be discussed later on (149, 152). The subsequent sections will discuss these strengths and limitations.

2.10 EBC 8-isoprostane

2.10.1 Physiology

Isoprostanes are prostaglandin-like compounds formed by the free-radical lipid peroxidation of arachidonic acid and represent *in vivo* markers of oxidative stress (41, 153-154). A number of studies have shown these compounds to be extremely accurate markers of oxidative stress in exhaled breath condensate and have illuminated the role of oxidant injury in association with the production of nitrogen species (46-47). Indeed, isoprostanes have several favourable attributes that make them a reliable indicator for oxidative stress: they are specific compounds of lipid peroxidation (54), they are stable compounds (54), levels are present at detectable quantities in biological fluids (54), their formation increases dramatically *in vivo* post-oxidant injury (50) and is modulated by antioxidant status (51-53), and their levels are not affected by lipid content of the diet (54-55).

The most widely studied isoprostane group is the F2-isoprostanes since they are chemically stable compounds, are specific products of peroxidation and are detectable in all normal biological fluids and tissues (46, 155). Levels of 8-iso PGF2α (i.e. 8-isoprostane, hereby used interchangeably), the most commonly characterized isoprostane of the 64 other isomers, have

been characterized in exhaled breath condensate in respiratory diseases such as asthma (45), COPD (156), cystic fibrosis (157), interstitial lung disease (158) and acute lung injury/adult respiratory distress syndrome (132). The levels of 8-isoprostane are doubled in mild asthma and further increased in moderate and severe asthma (45). Recent data showed a good correlation between 8-isoprostane and small airways function, indicating that 8-isoprostane reflects small airway inflammation, thus suggesting it may be used complementary to spirometry in the monitoring of patients with asthma (159).

2.10.2 Normative levels of 8-isoprostane

There are many studies investigating levels of 8-isoprostane in EBC for humans. The following table summarises different levels in healthy and asthmatic patients.

Table 1: Normative EBC 8-isoprostane levels in various study populations

Patient Description	8-Isoprostane Levels (pg/ml)	Standard Deviation (pg/ml)	Analytical Technique Used	Reference
Healthy subjects	15.8	±1.6	EIA	Montuschi *et al.* 1999 (45)
Mild Asthmatics	33.7	±2.8	EIA	
Moderate Asthmatics	38.3	±3.7	EIA	
Severe Asthmatics	48.9	±5	EIA	
Healthy subjects	15.5	14.1-17.5	RIA	Montuschi *et al.* 2010 (160)
Atopic non asthmatics	15.8	13.9-20.1	RIA	
Steroid native, atopic asthmatics	29.8	26.0-34.3	RIA	
Steroid treated atopic asthmatics	33	28.5-35.8	RIA	
Smokers	0.30-8.3	N/A	EIA	Van Hoydonck *et al.* 2004(48)
Asthmatics	12	9.4-29.5	EIA	Baraldi *et al.* 2003 (161-162)
Healthy controls	2.6	2.1-3.0	EIA	
Healthy	34.2	±4.5	RIA	
Steroid-native asthmatics	56.4	±7.7	RIA	
Steroid-treated asthmatics	47.2	±2.3	RIA	
Asthma exacerbation	22.86	±11.83	EIA	Patel, MM 2007 (163)
Stable asthma	10.95	±9.98	EIA	

Patient Description	8-Isoprostane Levels (pg/ml)	Standard Deviation (pg/ml)	Analytical Technique Used	Reference
Normal subjects	1.5-21	0.2-7	EIA	Koutsokera 2008 (164)
Healthy subjects	3.6	2.9-7.6	ELISA	Battaglia *et al.* 2005 (159)
Healthy subjects	7.0	±4.0	GC-MS	Carpenter *et al.* (132)
Healthy subjects	16.4	±1.6	EIA	Samitas *et al.* (165)
Healthy subjects	47.0	±7.8	LC-MS/MS	Syslova *et al.* 2008 (152)
Healthy subjects	4.44	±2.01	SPE-LC-MS/MS	Wang *et al.* 2010 (166)

EIA=enzyme immune-assay, RIA=radio immune-assay, ELISA=enzyme-linked immuno separation assay, GC-MS=gas chromatography and mass spectrometry, LC-MS/MS= liquid chromatography and tandem mass spectrometry, SPE= solid phase extraction.

Furthermore, limits of detection for the various methods ranged from as low as 1pg/ml to 10pg/ml for the above methods. The reported limits of detection were of the analysis kit or instrument, they were not limits of quantification.

2.10.3 Methods of analyses

Gas chromatography and mass spectrometry (GC/MS) is the reference analytical method for isoprostane analysis in biological fluids; however, this method is time consuming and expensive (46, 167). The solid-phase extraction (SPE) component of the GC method requires a laborious sample preparation (167). This method may be biased to artefact generation. Morrow *et al.* 1995 demonstrated that 8-isoprostane results contained a mixture of four isomers rather than 8-isprostane alone (167-168). Analysis has also been performed using liquid chromatography with tandem mass spectrometry (LC-MS/MS), allowing a more specific measurement of 8-isoprostane when coupled with the GC SPE methodology and while having significantly less sample preparation (152). Finally, 8-isoprostane can also be measured by enzyme assays such as enzyme-linked immuno-sorbent assay (ELISA), enzyme immuno-assay (EIA) and radio immuno-assays (RIA) which although widely used, offer less reliability than the previously described methods (160, 167). Overall, LC-MS/MS has been shown to be a superior method for 8-isoprostane analysis in biological fluids as it gives more specificity compared to EIA methods and offers less intensive sample preparation than GC/MS (167).

2.10.3.1 *Enzyme immunoassays*

EIA kits have measured 8-isoprostane to a limit of detection as low as 3.9 pg/mL in EBC samples (45, 156, 158, 169-170). This method has been validated by GC/MS with high correlation between two methods; however, reproducibility for the EIA has had contradictory results reported by different groups (48, 171).

Enzyme-linked immune-sorbent assay (ELISA) methods have assay coefficients of variation (CVs) ranging from 21-32.8% for intra assay and 26.8-44.3% for inter assay, which are higher than most GC or LC methods (172).

Immunoassays for F2-Isoprostanes are associated with problems related to substances in biological fluids that interfere with the immunoassay (167, 173). In this way, less complex biological fluids will have better results than when trying to assess F2-Isoprostanes levels in a highly complex matrix, in which interference would occur more readily (167). More often than not, these complex biological samples must be purified to some extent before performing the assay, this may lead to sample loss along the way (167). In the case of 8-isoprostane, a methods inter- and intra-assay repeatability is crucial to relate concentrations in a longitudinal fashion (167).

2.10.3.2 *Gas Chromatography Mass Spectrometry (GC-MS)*

Gas chromatography with mass spectrometry (GC-MS) is a more sensitive and specific method for determination of 8-isoprostane in EBC (153). Many studies continue to use stable isotope dilution gas chromatography with negative ion chemical ionization mass spectrometry for measurement of F2-Isoprostanes, of which 8-isprostane is a member (167). Although mass spectrometric methodology is expensive and time-consuming with a large amount of sample preparation, it is highly specific and sensitive (174). The accuracy of this method is typically over 96% and the precision is 5% for most methods analysing biological fluids (174).

2.10.3.3 *Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS)*

Liquid chromatography (LC) has also been shown to be sensitive and specific with less required sample preparation than EIA or GC-MS methods (152, 166). A small number of studies have tried to utilize LC to identify 8-isprostane in EBC. Syslova *et al.* 2008 developed an LC method

identifying 8-iso PGF2α in EBC using an immunoaffinity separation step prior to LC analysis (152). A stable isotope dilution was used followed by an immune-separation prior to instrumental analysis by liquid chromatography-electrospray tandem mass spectrometry (LC/ESI-MS/MS) operating in a multiple reaction monitoring mode. This method, highly sensitive and selective, yielded a limit of detection of 1pg/ml with a limit of quantification of 5pg/ml. It also showed an imprecision lower than 8.8% with an inaccuracy of less than 9.6%. Wang *et al*., 2010 used an automated on-line solid phase extraction (SPE) LC-ESI-MS/MS method which, similar to our method (shown in Appendix A), utilized 8-iso-PGF2α-d4 internal standard for quantitative determination. Intra- and inter-day precisions showed coefficients of variance ranged from 6.5 to 8.0% and 5.2 to 6.3%, respectively as well as percent recoveries ranging from 98.9-98.8, (166).

The aforementioned analyses require the use of SPE cleanup methods, which are laborious, time consuming and presents a major source of error in analytical methods (42). SPE methods are more general in application, since a range of matrixes can be analyzed, from the cleanest, eg water, to the dirtiest, eg urine. EBC may be directly injected into a liquid chromatograph because the concentration of matrix interferents and other compounds is low enough that they may be separated by LC.

Thus, instrumentation is very important in 8-isoprostane analysis. Our lab, not having the same instrumentation set up as the Syslova and Wang methods, needed to develop its own method for 8-isoprostane analysis in EBC. Our instrumentation was closest to Saenger *et al*., 2007, a method analysing 8-isoprostane in urine (172). Using those chromatography directions, the method was then optimized in order to compare Syslova and Wang EBC analysis methods. During this process, we have, therefore, developed a faster, more sensitive and precise method of assessing 8-isoprostane levels in EBC (Appendix A for full method development details).

2.11 EBC pH

2.11.1 Physiology

2.11.1.1 *Mechanism of acidification*

On the apical surface of the airway epithelium lies a thin layer of liquid named the airway surface lining fluid (ASLF) (175). This ASLF is made of two layers, the first being mostly water situated between the airway cilia and the second of mostly mucous, covering the cilia (176). The composition of the ASL is critical for normal mucociliary clearance; it's composed of 96% water, 1% salt, 1% lipids, 1% proteins and 1% mucus (176). pH balance determines the viscosity of the mucus layer as well as pH-dependent ion changes across biological fluids and tissues (176). The volume, ionic composition, and pH of the ASLF are key physiological parameters that are related to airway hydration, reactivity, and antimicrobial activity (175). The determinants of ASLF volume and composition probably include the rate of evaporative water loss, the transporting properties of the airway epithelium, the composition of fluid secreted onto the airway surface by sub mucosal glands, and the convective transport of fluid from lower to upper airways (175). The buffer capacity of the ASLF is primarily controlled by the mucus layer and the HCO3-ion exchange pathway, mainly CFTR, the cAMP-activated CL- channel (176). Hydrogen ions are also exchanged between the ASL and epithelium through H+ channels, Na+-H+ ion exchange channels and H+-K+ ATPases (176).

Ricciardolo *et al*., 1999 showed that excessive acidity in the airways lead to bronchoconstriction (177), cough, mucus production, effects on leukocyte function and interaction with nitric oxide and superoxide metabolism in the airways (178), to impair the ciliary motility (176, 179), to increase the airway mucus viscosity (176, 180) and to induce damage to airway epithelium (181). It has been proposed that ASLF volume and composition are important factors in the pathophysiology of cystic fibrosis, asthma, and other diseases of the airways (44, 182-185).

Exhaled breath condensate pH is not a direct measure of ASLF pH but rather a marker of airway acidification or excessive acid production in one or more locations of the airways (178). Indeed, endogenous airway acidification, as assessed by pH in expired breath condensate, has been implicated in airway pathophysiology (49, 176, 178). Low EBC pH has been associated with

asthma exacerbations (49), COPD (138), stable/moderate asthma and bronchiestasis (44), cystic fibrosis (157) and acute respiratory distress syndrome (138, 186).

2.11.1.2 *Normative data for EBC pH*

There are an extensive amount of studies reporting EBC pH levels for healthy and diseased participants. Table 2 examines the various literature to identify normal pH values for difference study populations.

Table 2: Normative EBC pH levels in various study populations

Patient Description	EBC pH	Standard Deviation (pH units)	Healthy Subjects/ Sample No.	Reference
Healthy non-smokers	7.7	± 0.2‡	19/19	Hunt *et al.*2000 (49)
Adults	7.57	(7.51-7.64)	10/10	Kostikas *et al.* 2002(44)
Adults: gas standardized in a different manner	7.46	± 0.5‡	12/12	Gessner *et al.* 2003 (186)
Adults	7.7	± 0.5‡	100/773	Vaughan *et al.* 2003(40)
Adults; intubated	7.8	± 0.3‡	32/32	Vaughan *et al.* 2003 (40)
Adults; mean age 43	8.26		16/16	Niimi *et al.* 2004 (187)
Adults; mean age 26 yr old.	7.61	(7.52-7.70)	12/12	Borrill *et al.* 2005(188)
Adults: mean age 71.3 Adults: mean age 23.4	7.50 7.59	± 0.13‡ ± 0.09‡	48/48	Brooks *et al.* 2006(189)
Healthy non smoking	8.0	(7.8-8.1)	404/404	Paget-Brown *et al.* 2006 (190)
Asthmatic		7.95-8.0	0/60	McCreanor *et al.* 2007 (5)
Controls Severe Asthmatic	7.88 7.75	± 0.10‡ ± 0.37‡	32/42 10/42	Accordino *et al.* 2008 (191)
Adults	7.5-8.1		600	Hunt, J 2007 (192)
Healthy controls Asthmatic patients	8.27 8.20	± 0.19‡ ± 0.2‡	10/40 10/40	Koczulla *et al.* 2009 (140)
Healthy non smoking adults	7.9-8.1		10/40	Koczulla *et al.* 2010 (136)
Healthy Controls	6.03	± 0.03‡	36/94	Antus *et al.* 2010 (193)
Asthma Allergic Rhinitis Healthy controls	7.84 7.64 7.74	(7.65-8.00) (7.41-7.88) (7.51-7.96)	11/62 48/62 14/62	Prieto *et al.* 2011 (194)

Patient Description	EBC pH	Standard Deviation (pH units)	Healthy Subjects/Sample No.	Reference
Control	7.90	7.40-8.20	31/573	Liu *et al.* 2011 (195)
Mild/Moderate asthma	7.94	7.56-8.30	293/573	
Severe asthma	8.02	7.52-8.20	249/573	

Accordino *et al.* 2008 showed no relationship between EBC pH and gender (191). Accordino *et al.* showed high repeatability for both healthy and asthmatics participants over a 1 yr time frame. Greater differences were shown in asthmatics EBC pH over time than in healthy controls, Accordino suggested that airway pH fluctuated over time with the disease.

Hunt 2007 also reported that age and sex has no significant effect on EBC pH (192). Hunt's group has analysed over 6000 samples for EBC pH from approximately 600 subjects and has found the normal range to be between pH 7.5 and 8.1 (192). They also mention that nothing should be eating or drunk at least 30 minutes prior to EBC collection (192).

2.11.1.3 *Acidification and oxidative stress*

EBC pH has been characterized as an important component of airway pathophysiology in several airway diseases (39, 42, 44, 49, 183, 185, 196). This pathophysiology has been shown to be related to changes in ASLF homeostasis in humans and animals over the past 70 years (176).

This lowered airway pH is important physiologically since most biochemical processes are highly pH dependent (177). Of importance to this thesis are the issues regarding airway oxidative stress. Insults from oxidative species are more severe in an acidic airway (177). Many oxidant and antioxidant airway processes are markedly pH dependent, and the power of oxidizing and nitrating species are greater at lower pH (177). Experimental acidification of airway lining fluid has been shown to increases the local production of oxidant species in rats (197). Additionally, an acidic insult greatly augments oxidative injury caused by hyperoxia in the rat, in part because of a loss of antioxidant capacity (198). In fact, in the airways of patients with asthma, oxidative stress is abundant (115), nitration is prominent (199), and nitrate levels are increased (200).

2.11.2 Methodology

EBC pH has been shown to be a robust, reproducible assay of airway acidity (40, 135). pH is easily measurable, and is not affected by hyperventilation (or flow rate, thus tidal breathing is adequate) or temperature of collection (as long as it remains constant between patients and for the same patients (136)); it also remains unaffected by the duration of collection or storage and acute airway obstruction (40). Furthermore, EBC pH is not affected by circadian rhythms or the use of nose clips (40). However, EBC requires de-aeration with argon or standardization with CO_2 partial pressure in order to obtain adequate constant pH readings (43). De-aeration with argon creates a time dependence in the detection of pH in EBC samples (40). As previously mentioned dilution is not a problem for EBC pH analysis as it is for non-volatile components of EBC samples (43); furthermore, salivary contamination is not an issue as compared to other volatile or non-volatile components of EBC (49).

2.11.3 Disadvantages in methodology

Unfortunately, there are limitations of using EBC pH that will need to be addressed by this study project. It is unclear whether height, weight, age, sex may themselves affect EBC pH measurements (43); though not problematic in a crossover design, such concerns may confound interpretation of cross-sectional data. Furthermore, lack in standardization may affects protocols of de-aeration with argon or CO_2 partial pressure sample stabilization; both have been attempted to obtain EBC pH readings, it is not clear which method is better than the other (41, 43). The importance of sample stabilization of EBC samples lies in the effect of ambient CO_2 levels on pH measurements: without argon de-aeration or CO_2 partial pressure standardization, pH levels may be modulated as the ambient CO_2 dissolves into the EBC matrix thereby affecting acidity of the sample (201). Dissolved CO_2 can make the EBC pH more acidic by combining with water to produce H_2CO_2 and subsequently release H+ and HCO_2^- into the solution (49). Other disadvantages of EBC pH revolve around the collection device used to obtain the samples; different EBC collection devices yield pH results that are not comparable and as such the same device must be used on all patients of a study (137, 142). This may be problematic when comparing research results to other studies. Lastly, issues regarding medications taken by patients, food, drinks and even weather patterns have been suggested may affect EBC pH (22, 43).

2.12 Controlled DEE studies

Controlled exposure chamber studies provide a method by which to examine the acute mechanisms of individual air pollutants (10). Most studies previously mentioned utilized a diesel engine from the 1990s with exposures ranging from 1 to 2 hours of 100 to 300µg/m^3 of PM_{10}.

In fact, diesel exhaust controlled exposure studies on healthy human participants showed that diesel exhaust exposure was associated with:

- increased airway resistance (24-25, 29, 202),
- increased airway responsiveness (24),
- airway inflammatory changes (23, 26-29, 71-76, 202-204),

Some human exposure studies have suggested that particulate matter-induced lung injury is the result of oxidative stress and uncontrolled airway inflammation (205-208).

2.12.1 Controlled DEE studies and EBC

With regards to exhaled breath condensate (EBC), only one partially-controlled diesel exposure study in humans (Laumbach and Kipen, 2010) attempted to use EBC in its observed medical endpoints (209). Ambient exposure studies such as McCreanor *et al.* 2007 have used EBC biomarkers to investigate the effects of air pollution on healthy and asthmatic individuals (5).

Laumbach and Kipen, 2010 investigated the effects of real world exposure to traffic-related air pollution on EBC in healthy and asthmatic individuals (209):

- twelve healthy volunteers were exposed to two hours of highway rush hour car ride with and without a HEPA filter,
- twenty-six healthy individuals were exposed to freshly-generated diesel exhaust (DE) at 200ug/m^3 $PM_{2.5}$ as well as clean air in a controlled chamber,
- seventeen mild-moderate asthmatics were exposed to 2 hours of 300ug/m^3 of $PM_{2.5}$ in a controlled chamber.

EBC was collected pre and 5-6 hours after exposure. Ecoscreen® was used in healthy volunteers and RTube® for asthmatics. EBC nitrite was investigated (209).

Laumbach and Kipen, 2010 showed borderline-significant (p-value between 0.05 and 0.1) increases in nitrite concentrations after unfiltered road-way exposure compared to HEPA filter exposure. They showed a significant increase in nitrite concentration in participants after 2 hours of controlled DE exposure, as opposed to a decrease following fresh air exposure for the healthy participants. Lastly, they showed that asthmatics had a borderline significant increase in EBC nitrite after DE exposure. All increases in nitrite resolved by 6 hours after exposure to DE (209-210).

McCreanor *et al.* 2007 looked at the effects of traffic-related air pollution on humans using EBC pH. Participants were exposed to 2 hours on a path in Hyde Park and 2 hours along Oxford Street in London, where traffic was predominantly diesel transport. They showed a significant decrease in EBC pH in the exposed group after 3 and 6 hours in asthmatics, the trend being stronger in moderate asthmatics (see Figure 3).

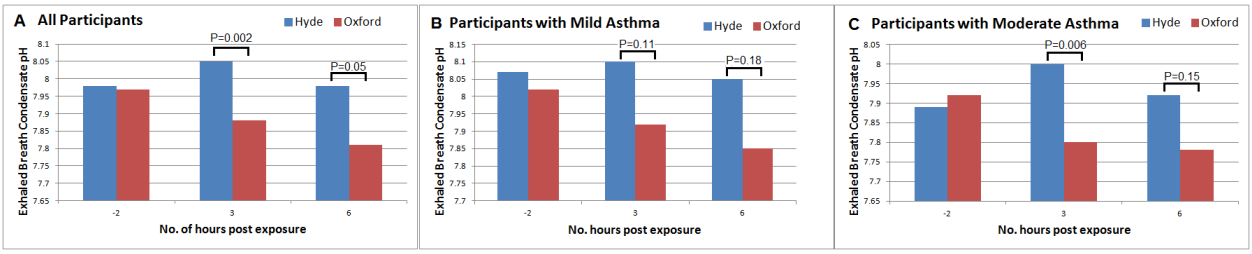

Figure 3. McCreanor et al. 2007 EBC pH. Taken from McCreanor et al. 2007 depicting EBC pH in A) all asthmatics together, B) only mild asthmatics and C) only moderate asthmatics at pre exposure, 3 and 6 hours after exposure to Hyde Park and Oxford Street.

The Zhang *et al.* 2009 Health Effects Institute report (describing the McCreanor study in more detail) further describes results found for EBC pH. In addition to the McCreanor *et al.* 2007 results, the mean % change was shown in both control and exposed groups. There was a significant decrease in % change for both 3 and 6 hours after exposure (211).

Overall, two controlled or semi controlled exposure studies use EBC biomarkers to investigate respiratory health effects, only one using pH and none for 8-isoprostane (to our knowledge).

2.12.2 Confounding and effect modification

Confounding is an important factor to consider for any biological study. Two variables may be correlated; however, one may not cause the other but is a result of a third variable common to both. As such, this third variable changes in tandem with the measured variables thereby giving the appearance of causality between the measured variables (212). These confounding variables are important to consider during observational studies, such as cross-sectional or retrospective cohorts. Experimental studies, such as randomized control studies, allow for the control of such confounding variables by the researcher.

The EBC samples collected for this thesis were from a randomized three-way crossover study design which inherently controls for biological variation as each participant acts as his or her own control (213). Typical confounding variables in epidemiological studies are smoking, age, sex, ethnicity, social economic status, body mass index (BMI), etc. With regards to oxidative stress, some of these factors may interact as effect modifiers.

Effect modification occurs when the measured variable in the presence of the exposure and a covariate (such as sex) is different than the results from the measured variable and the independent effects of the exposure and covariate (214). With respect to this thesis, an effect modification would occur if, for example, the effect observed between sex and treatment on 8-isoprostane levels was different than the effect observed for sex and treatment individually. If there is no difference between those two scenarios than there is no effect modification.

It is possible that certain biological factors about the participants in our study, although not confounding, may modify the relationship between our exposure and measured outcome variables. Some potential interaction terms to investigate would be sex, methacholine response status, atopic status, GSTP1 variant status, age, body mass index, ethnicity and diet. These are described below.

1. ***Sex***

Women and men may have biological differences with regards to asthma and oxidative stress. Ide *et al.* 2002 showed that men had higher plasma levels of 8-isoprostane when compared to pre-menopausal women and that those levels were decreased with supplementation with

antioxidant vitamins for 4 weeks (215). Sex was also found to be a significant determinant of 8-isoprostane levels in plasma in 298 adults where female exhibited higher levels of isoprostane than males (216).

EBC pH was determined not to be affected by sex through several different studies (190, 192). Thus, the sex of subjects will be evaluated for its potential effect modification on oxidative stress biomarkers.

2. Methacholine response status (MRS)

As methacholine responsiveness effects 8-isoprostane and pH, a lower dose of PC_{20} may be described as underlying oxidative stress at baseline. As such we may expect the 8-isoprostane levels to be generally higher for positive MRS individuals and pH to be lower for positive MRS subjects.

3. Atopic status

Allergic status is important to discuss, as a recent review paper synthesized the most recent evidence linking air pollution to sensitization (217). There has been increasing evidence on the role of air pollution with respect to allergic sensitization in children (218). The European birth cohort studies have suggested that exposure to air pollution prior to and during the first years of life may incur allergic sensitization (219-221). These studies have also suggested the link between air pollution and childhood asthma. Experimental studies have identified diesel exhaust particles (DEPs) to induce IgE responses (222). Furthermore, levels of 8-isoprostane were found significantly higher in individuals with atopic dermatitis when compared to controls (223).

4. Genetics GSTP1 variant

It is plausible that genetic variants involved in inflammation and protection against ROS may influence the response to air pollutants. Enzymatic defenses in the lungs mainly revolve around glutathione S transferases (GSTs) as well as super oxide dismutase (224). GSTP1 is one of the most strongly expressed GTSs in the lungs, among GTSM1 and GSTT1 (225-228). A study investigating the determinants of oxidative stress in children found that GSTP1 val/val phenotype was an important factor in the development of oxidant injury (224). Polymorphisms in oxidative stress gene P1 (GSTP1) have been associated with decreases in pulmonary function (121, 229-230), increased allergic responses (231-232), respiratory symptoms, and asthma (226,

233-237) in response to air pollutants including ozone and diesel exhaust particles (DEP). A recent study, using nitrogen oxides (NOx) as an indicator for local traffic air pollution, has reported interaction effects between GSTP1 polymorphisms and NOx levels on allergic sensitization to common allergens in children at 4 years of age (238). As such, the effect modification potential from GSTP1 variant will be investigated.

5. Age

2.10.1.2 of the literature review showed that age was not a significant factor for EBC pH between the ages of 11 and 60 years old (190). A positive correlation between levels of 8-isoprostane and increasing age bracket of ten years was shown in a study by Cruz *et al.* 2009 (239). Conversely, the younger age groups had a higher percentage of non-detects as compared to the oldest group perhaps biasing results (239). In contrast, a population based study investigating 8-isoprostane levels in plasma found age to have no association with lipid peroxidation (216). These varying results identify that age may be an effect modifier for 8-isoprostane.

6. Body mass index

In a cross sectional study, obese subjects exhibited higher levels of oxidative stress biomarkers than in control subject (240). Individuals with healthier lifestyles (i.e. better diet and exercise) showed lower levels of oxidant injury as compared to sedentary individuals who had a lower intake of antioxidants (241). Block *et al.* 2002 found BMI to be a significant predictor of 8-isoprostane levels in plasma, with highest levels being observed in the obese II category (216). Because oxidant injury is linked to inflammation, pH levels may also be affected by BMI. Higher BMI was associated with low EBC pH in a population based study on asthmatics (195). BMI thereby will be investigated for potential effect modification.

7. Ethnicity

There are no studies looking at ethnicity effect for EBC levels of pH and 8-isoprostane. Although race and ethnicity have been evaluated in epidemiologic studies with their relation to asthma, it was found that when controlling for socioeconomic status the effect disappeared (242-243) or remained strong (244-246). A population level study evaluated 8-isoprostane levels in plasma and found significantly higher levels of 8-isoprostane in white individuals, and lowest in blacks (216). However, this latter finding was inconsistent and unexpected due to inconsistencies in

oxidative stress biomarkers MDA and 8-isoprostane (216). Race was found to be associated with low pH in a population based study in asthmatics (195). Ethnicity may become an effect modifier to levels of oxidative stress. However, ethnicity will not be investigated due to study limitations such as availability of ethnicity data as well as reliability of self-reported ethnicity as opposed to race which was investigated in the aforementioned studies.

8. Diet

Several studies identified that isoprostanes were not confounded by lipid contents of the diet (247-249). Similarly to BMI, diet has a direct correlation with antioxidant content available for use in the human body. Block *et al.* 2002 found that plasma levels of ascorbic acid, a common dietary anti oxidant, significantly negatively correlated with plasma levels of 8-isoprostane (216). To minimize this potential confounding, diet was controlled during every exposure session: participants were asked to bring the same foods for every exposure day thereby minimizing the effects on oxidant concentration. Diet will not be evaluated as an effect modifier for this study.

2.13 Summary

Many controlled diesel exhaust exposure studies have attempted to characterise the respiratory health effects associated with DE in both healthy and asthmatics participants. However, not many of these published studies use exhaled breath condensate in order to assess lung health status in both healthy and asthmatic patients.

This study aimed at addressing the gaps in the literature concerning controlled DE exposures and its effects on EBC biomarker levels in both healthy and asthmatic individuals. In addition to characterising EBC pH and 8-isoprostane levels, this study attempted to investigate the potential suppression of DE-related health effects by supplementing the diet with antioxidants.

As such, this study may provide insight into the mechanism of action of DE as well as help link experimental results with broader public health epidemiological studies. EBC analysis of two biomarkers of airway oxidative stress may help regulatory agencies perform risks assessments and decision making with regards to exposure limits, in both occupational and environmental settings.

CHAPTER 3: DE Study Methods

3.1 Overview

A controlled diesel exhaust study was performed in order to assess the effect on airway oxidative stress by monitoring exhaled breath condensate levels of 8-isoprostane and pH. The following section outlines the methods by which health effects were assessed in this study.

3.2 Objectives

1. To investigate the effects of controlled diesel exhaust exposure on human airways' oxidative stress by analysis of pH and 8-isoprostane in exhaled breath condensate.
2. To characterize the effects of antioxidant treatment on potential DE-associated airway oxidative stress in terms of EBC pH and 8-isoprostane.

3.3 Participants

Non-smoking males and females aged 19-49 years of age were eligible to participate.
Asthmatics must have been physician-diagnosed with asthma for at least one year and have $PC_{20} \leq 8$mg/ml; healthy controls had to be never physician-diagnosed with asthma.
Exclusion criteria included: 1) pregnancy/ breastfeeding; 2) using inhaled corticosteroids, 3) using a bronchodilator medication more than 3 times per week, 4) taking any vitamins A, C, or E, 5) co-existing medical conditions, and 6) taking part in another study involving medications. Participants were told to have the same food on every exposure day in order to control for diet intake of antioxidants.

3.4 Methods

3.4.1 Exposure conditions

The experiment led by Dr. Christopher Carlsten was a crossover study with three exposure conditions:
1) DEP = DE (300µg $PM_{2.5}/m^3$) with placebo,
2) DEN = DE with antioxidant (600mg N-acetylcysteine [NAC] three times daily for 5 days prior to exposure),
3) FAP - filtered air with placebo.

Each exposure was 2hr in duration and included two 15-min periods of light bicycle exercise at an intensity that would elicit ventilation rates of 15L/min/m^2 in order to standardize breathing patterns and inhalation dose. Exposures were double-blinded, randomized and counter-balanced to order. Exposures were separated by a washout period of 14 days.

Exposures were performed at the Air Pollution Exposure Facility (A.P.E.L.) at the University of British Columbia (Vancouver General Hospital, Research Pavilion) which is a state-of-the-art facility that can generate DE accurately reflecting high-ambient and common industrial settings.

3.4.2 EBC collection

EBC was obtained from participants at baseline and 2, 6 and 30 hours after exposure. Seated participants were asked to breathe normally for 15 min into an R Tube™, a unique one-way valve into a polypropylene collection tube surrounded by an aluminum sleeve that was cooled to −20°C before sample collection (R Tube™ EBC collection system, Respiratory Research, Inc, Charlottesville, Virginia, USA), the circuit contained a saliva trap.

Temperatures and duration were noted for each experiment, nose clips were not worn as they were shown to not to affect EBC pH (40, 46-47).

This technique allowed collection of approximately 2 mL of EBC, 4x 500 µL aliquots. Aliquots were then temporarily stored at -20°C and subsequently transferred to a -80°C freezer until analysis for biomarker concentration.

3.4.3 EBC aliquot analysis allocation

EBC aliquots were separated into two in order to have enough for both EBC pH and 8-isoprostane analysis. Due to the variability in EBC volume collected (i.e 1 to 3ml) an allocation scheme was created in order to effectively separate EBC samples. Aliquots were favoured for 8-isoprostane analyses due to the increased variability around the analysis method (i.e. higher coefficients of variation) than for the EBC pH method. In this way, more aliquots were available for 8-isoprostane analyses than for EBC pH. When 1 or 3 aliquots was available, 8-isoprostane analysis was favoured. For 2 and 4 aliquots, aliquots were equally separated.

3.4.4 EBC 8-Isoprostane method development and sample analyses

Our lab had to develop its own method in order to analyse EBC levels of 8-isoprostane. The studies found analysing 8-isoprostane levels in EBC by liquid chromatography used a different instrumental set up and methodology which our lab could not reproduce without purchasing other equipment. As such, we utilised a urinary 8-isoprostane detection method which used similar instrumentation and methodology which our lab could reproduce and then optimized it for analysis of 8-isoprostane in EBC. Our chemist, Philippe Provencher, was solely responsible for developing this method; while our team provided feedback and guidance for this work.

Our study samples were then analysed using this method, for which the development and parameters are explained in detail in Appendix A.

3.4.5 EBC pH sample analyses

Samples were thawed prior to a 10 min de-aeration with argon using standard procedure (49, 250-251). Standardized de-aeration protocol provided by Hunt *et.al.*, 2000 was used on al EBC samples in order to remove the majority of CO2 from the sample (49). This methodology required de-aeration with argon for 10 minutes at 350 mL/min; Hunt *et al.*, 2000 as well as Ron Do, 2005 showed that pH readings stabilized after this point, suggesting that further CO_2 could not be removed (252). EBC pH was then measured using a calibrated PHE-2131 Omega ® KCl electrode with a Fisher Accumet Mini pH meter (Model 955, Waltham, MA). Calibration of the pH probe was done with two calibration points at pH 4.01 and 7.0.

3.4.5.1 Argon de-aeration procedure

Argon de-aeration was performed on the samples to remove dissolved CO_2 and stabilize pH readings in the EBC samples. The following steps were used in the procedure:

1. A plastic (PVC) tube was attached to the argon tank.
2. A long stem glass pipette was attached to the other end of the PVC tubing.
3. The glass pipette was held using a pipette holder attached to a stand.
4. Prior to and post de-aeration, the flow-rate was adjusted to 350 mL/min by a TSI^{TM} mass flow meter (4000 Series, Shoreview, MN).
5. The EBC sample was then placed on the pipette stand illustrated in Figure 4.

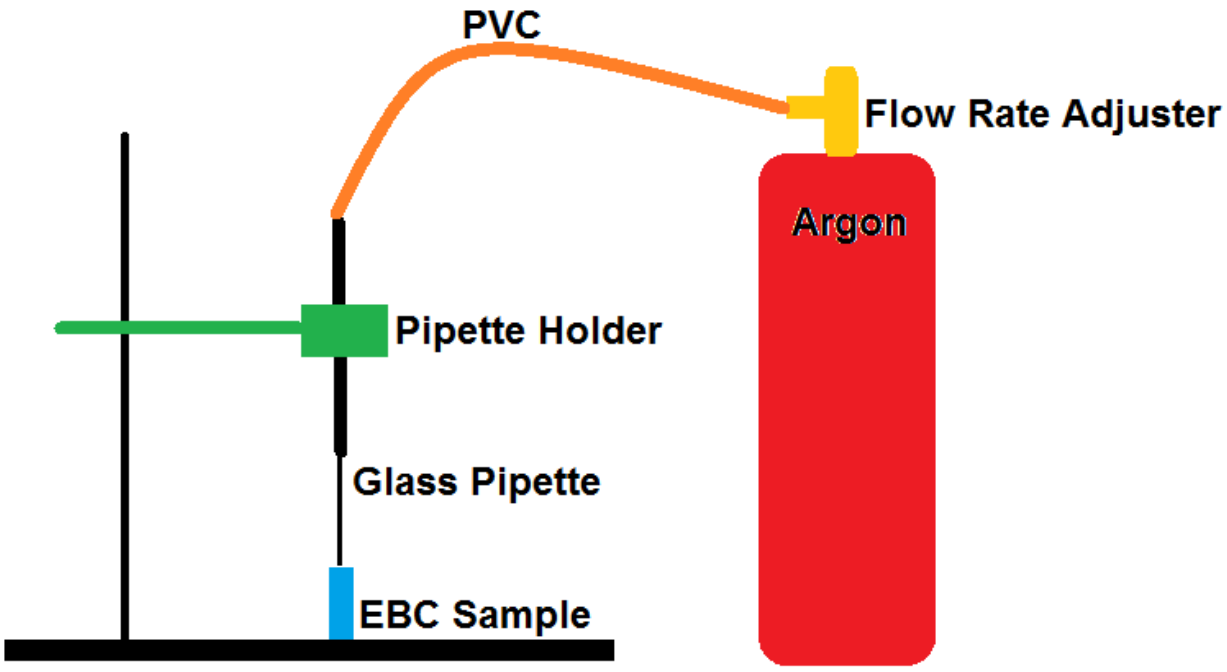

Figure 4. EBC de-aeration set up.

6. With the flow-rate of the argon tank adjusted, the glass pipette was placed in the EBC sample for 10 minutes to allow for the bubbling process to occur. The pipette was placed in the center of the sample such that the surface tension of the sample was broken.
7. The measurement of pH in the samples was performed once steps 1 through 5 were completed.
8. The EBC samples were de-aerated with argon at 350 mL/min for 10 mins or until pH stabilized.

3.4.5.2 pH measurement procedure

A PHE-2131 Omega ® KCl electrode with a Fisher Accumet Mini pH meter (Model 955, Waltham, MA) was calibrated prior to each EBC sample. The steps for the set-up and calibration of the pH meter are outlined below.

1. Thaw and shake EBC sample completely prior to pH measurement.
2. Calibrate the pH meter using 2 calibrating buffers of pH 4.01 and 7.00 prior to each EBC sample measurement.
3. Wash the Accumet microprobe using distilled water.
4. Blot the microprobe dry with ChemWipes.
5. Place the microprobe in the vial containing the EBC sample and stir gently.

6. Wait until the meter reading stabilizes, such that the measured pH fluctuates between two readings.
7. Record the measured pH.
8. For repeats of pH measurement, repeat steps 2 to 6.

3.5 Statistical analyses

Descriptive statistics were tabulated for continuous and categorical variables; counts and bar graphs were done for categorical variables while means, standard deviations, ranges and frequency distributions were computed for continuous variables. Normality was assessed for the dependent variables.

Analyses were performed for each independent categorical variable against 8-isoprostane and pH levels using a linear mixed effects model. In order to avoid too many statistical tests and generating significant results just by random chance, effect modification was only investigated when the overall ANOVA p-value from the single covariate model was lower than 0.1.

Correlations between continuous independent variables were determined using the Pearson correlation coefficient (r). A multivariate model was performed on both biomarkers using a linear mixed effects model. P- values smaller than 0.05 were used to reject the null hypothesis. All statistical descriptive and analyses were performed using SPLUS® software (version 8.0, Insightful Corp, 2007). All models were linear mixed effects models to account for repeated measured longitudinally.

3.5.1 Simple linear mixed effects model

Single convariate/multicovariate analyses were designed on the basis of the linear mixed effects model (lmem) which is described as the following:

$Y_{ij} = \beta_1 + \beta_2 X_{2ij} + \ldots + \beta_n X_{nij} + b_{i1} z_{1ij} + e_{ij}$

Where

- Y is the value of the response variable for the jth of n_i observations; the dependent variable.
- $\beta_1, \ldots, \beta_n$ are the fixed-effect coefficients, which are identical for all groups.

- $X_1, \ldots X_n$ are the fixed-effect predictors i.e. the variables influencing Y, the independent variable (i.e. sex, age).
- Note that typically, β_1 is reserved for the intercept.
- $b_{i1}z_{1ij}$ is the random effects coefficient, in this case ID being the only random term for the analyses.
- e_{ij} is the error for the observation j in group i, otherwise the term for random errors.

The above model is used to assess if any variable(s) have an influence on our outcomes of interest. In the event they do explain part of the variation in our outcome variable then they are considered for the multivariate model later on.

A model was run for each time point (0, 2, 6 and 30 hours after exposure). An example of the above for our single covariate analyses would looks as follows for baseline log 8-isoprostane levels (hereby known as logH0):
Baseline 8-isoprostane = β_1 FAP (intercept) + β_2 DEP + β_3 DEN + b_{ID} + $e_{baseline}$

For each model, treatment refers to exposures FAP, DEP and DEN; with FAP generally being the reference exposure. **Exposures are referred to as treatment 1, 2 and 3 during the statistical analyses; however are referred to by their exposure in the thesis.** Where β's are the coefficients for each of the mean pH levels at baseline at each treatment. The output looks like this:

```
Fixed effects: logH0 ~ Treatment
               Value Std.Error DF  t-value p-value
(Intercept) 3.283016 0.5522203 66 5.945121  <.0001
 Treatment2 0.543344 0.3991776 66 1.361157  0.1781
 Treatment3 0.369337 0.3991776 66 0.925246  0.3582
```

The predictor estimate (coefficients or slopes) would be interpreted as, at baseline, Treatment 2 (DEP) yields an average 0.54 increase in log 8-isoprostane levels compared to the intercept (FAP was directly classified as the reference category for the lme). Similarly, Treatment 3 (DEN) would yield an average 0.37 increase in log 8-isoprostane levels when compared to the intercept (FAP) at baseline.

3.5.2 Single covariate linear mixed effects model

An important characteristic of a categorical covariate is that, at each observed value of the response, the covariate takes on the value of one of a set of distinct levels (253). Parameters associated with the particular levels of a covariate are sometimes called the effects of the levels. If the set of possible levels of the covariate is fixed and reproducible, then we model the covariate using fixed effects parameters (253). For the single covariate models, the analyses are used in order to identify significant differences between the levels within each category. For example, if the atopy covariate significantly modifies 8-isoprostane levels, interaction terms with treatment are then identified in order to assess if there is effect modification by sex.

There are two levels to the single covariate LMEM:

1) The ANOVA overall model output
2) The individual tests output

The first typically looks as follows:

```
Analysis of Variance Table
                numDF denDF  F-value p-value
    (Intercept)     1    63 62.16644  <.0001
      Treatment     2    63  1.01093  0.3697
          Atopy     1    63  3.40479  0.0697
Treatment:Atopy     2    63  1.40476  0.2530
```

The overall p-values for the analysis of variance ("`Treatment:Atopy`" p=0.25) tells us there is no significant modification by atopic status of the relationship between treatment and log 8-isoprostane levels at baseline. However, p-value of 0.06 for "atopy" alone implies that there is a borderline-significant overall difference between atopic and non-atopic individuals in log 8-isoprostane levels at baseline. Since this difference does not interact with treatment, and because our study hypotheses do not concern the effect of atopy alone, we forgo additional analyses related to atopy.

In the event that the interaction term *was* significant, we would look at the p-values for specific contrasts to assess where the potential interaction is. The second output looks as follows:

```
logH0 ~ Atopy * Treatment
                    Value Std.Error DF   t-value p-value
    (Intercept)  2.190119 0.7108344 63  3.081054  0.0031
          Atopy  1.528541 0.6284495 63  2.432241  0.0179
     Treatment2  1.522967 0.7640922 63  1.993172  0.0506
     Treatment3  1.294525 0.7640922 63  1.694200  0.0952
AtopyTreatment2 -1.325373 0.8887614 63 -1.491259  0.1409
AtopyTreatment3 -1.251725 0.8887614 63 -1.408392  0.1639
```

- Where the intercept represents the average level of log 8-isoprostane in non-atopic subjects exposed to FAP.
- The Atopy term refers to the difference between the average levels of log 8-isoprostane in atopic subjects and non-atopic subjects with FAP at baseline (treatment1, intercept).
- Treatment 2 and 3 refer to the difference between the average levels of log 8-isoprostane in non atopic subjects with DEP and DEN compared to non atopics at FAP.
- AtopyTreatment 2 and 3 refer to the difference between the average levels of log 8-isoprostane in atopic subjects at DEP and DEN compared to atopics at FAP.

In the above example, because the overall ANOVA p-value for the interaction term was non-significant, we would normally not investigate any further. The LMEM compares DEP and DEN to FAP; however, we are more interested in how DEN compares to DEP. Thus, in the event the interaction term was significant, we would use paired t-tests as *post hoc* analyses to identify the difference between FAP vs DEP and DEP vs DEN. Also, as a rule, all covariates were included in multi-covariate analyses for the reasons described in section 2.11.2.

3.5.3 Dependent continuous variables

The biological outcomes for this thesis were EBC biomarker levels of 8-isoprostane and pH. Both biomarkers were evaluated for normality and then transformed appropriately as needed.

The DE study comprised of three exposure conditions (FAP, DEP, and DEN) with four time points of interest 0, 2, 6 and 30 hours after exposure. Biomarker levels after exposure (2, 6 and 30 hour) were corrected for baseline (0 hour) levels in order to account for within subject variability at each time point after exposure. As such, "delta" values were generated for each time point at each exposure.

For example:
Subject 8-isoprostane level 2 hours after exposure for DEP *minus* subject 8-isoprostane level at baseline for DEP = DELTA2 for DEP;
84.5pg/ml – 50.3pg/ml = 34.2pg/ml of 8-isoprostane for 2 hours after DEP exposure.

More specifically, a positive delta value would represent an increase in biomarker level from baseline while a negative delta value would represent a decrease in biomarker levels from baseline. With regards to pH, a decrease would represent an acidification. In total, four biomarker databases were created with the four different biomarker levels: log 8-isoprostane, delta 8-isoprostane, pH and delta pH. Each biomarker data set was analysed for the hypotheses listed in section 3.4.3.

3.5.4 Independent categorical variables

Independent variables will be categorical and include:

- Exposure (1=FAP, 2=DEP, 3=DEN)
- Sex (M=1, F=2)
- Methacholine-Responsive status (No=0, Yes=1)
- Atopy status (No=0, Yes=1)
- GSTP1 (No=0, Yes=1)
- Age (19-28=1, 29-38=2, 39-48=3)
    - We created three categories of age for our analyses.
- BMI (<25=1, 25-29=2, ≥30=3)
    - We created three categories of BMI for our analyses.
- Order (1,2,3)

These variables were considered due to their plausibility in having potential effect modification on biomarker levels in EBC. The background information for these factors is outlined in section 2.11.2 of the literature review chapter. Ethnicity and diet were not included in these analyses due to the inherent limits of both factors; this study is not equipped to evaluate either factor since

1. Subjects were generally Caucasian, giving very limited power to assess ethnicity-related effect modification;

2. Diet was controlled during the exposure days by asking the subjects to eat similarly on each exposure day. We made an assumption that, based on our request and general dietary habits, individuals' overall diets not would not vary significantly over the study period.

3.5.5 Hypotheses

Before any statistical analyses can be performed, null and alternative hypotheses were created using our main objectives as well as background information outlined in the literature review. They are briefly outlined below.

Correlation between pH and 8-isoprostane

Both pH and 8-isoprostane are by-products of the oxidative stress pathway, as outlined in sections 2.9 and 2.10. There is evidence that an acidified airway leads to increased oxidant injury as explained in section *2.10.1.3 acidification and oxidative stress*. As such, one might hypothesize concurrent increases in levels of 8-isoprostane and pH or, alternatively, that airway acidification will be followed by an increase in 8-isoprostane levels – we favour the latter though this study was not designed to carefully address the temporal dynamics of this relationship.

Exposure (1=FAP, 2=DEP, 3=DEN)

In general, our typical null and alternative hypotheses are shown in Table 3 below.

Table 3: Null and alternative hypotheses for exposure effect.

Variable	H_O	H_A
Exposure1 (FAP)	FAP levels of biomarker = DEP levels of biomarker = DEN levels of biomarker	An exposure modifies a biomarker level.
Exposure2 (DEP)		
Exposure3 (DEN)		

More specifically, according to our literature review, we would expect the following:

- From FAP to DEP, we expect an increase in 8-isoprostane levels while we expect a decrease in pH levels (as acidification is marked by a lowering of airway pH).
- From DEP to DEN, we expect a decrease in 8-isoprostane levels (to baseline levels) while we expect an increase in pH levels (to baseline levels).

Sex (M=1, F=2)

We would expect that males, having higher oxidative stress than females, would exhibit higher levels of EBC 8-isoprostane and slightly more acidic pH.

- H_O: biomarker levels are the same for each sex.
- H_A: biomarker levels are different for each sex.

We would also expect sex to modify the effect of exposure on biomarker levels due to this aforementioned issue.

- H_O: sex does not modify the relationship between exposure and biomarker level.
- H_A: sex does modify the relationship between exposure and biomarker level.

Methacholine Response (MR) Status (No=0, Yes=1)

We would expect that methacoline responsive individuals, having higher oxidative stress than non responsive individuals, would exhibit higher levels of EBC 8-isoprostane and slightly more acidic pH.

- H_O: biomarker levels are the same for methacoline responsive and non-responsive individuals.
- H_A: biomarker levels are different for methacoline responsive and non-responsive individuals.

We would also expect methacoline response status to modify the effect of exposure on biomarker levels due to this aforementioned issue.

- H_O: methacoline response status does not modify the relationship between exposure and biomarker level.
- H_A: methacoline response status does modify the relationship between exposure and biomarker level.

Atopic status (No=0, Yes=1)

We would expect that atopic status, having higher oxidative stress than non atopic individuals, would exhibit higher levels of EBC 8-isoprostane and slightly more acidic pH.

- H_O: biomarker levels are the same for atopic and non-atopic individuals.
- H_A: biomarker levels are different for atopic and non-atopic individuals.

We would also expect atopic status to modify the effect of exposure on biomarker levels due to this aforementioned issue.

- H_O: atopic status does not modify the relationship between exposure and biomarker level.
- H_A: atopic status does modify the relationship between exposure and biomarker level.

Genetics GSTP1 (No=0, Yes=1)

We would expect that genetic variant status, having higher oxidative stress than wildtype individuals, would exhibit higher levels of EBC 8-isoprostane and slightly more acidic pH.

- H_O: biomarker levels are the same for variant and wild type individuals.
- H_A: biomarker levels are different for variant and wild type individuals.

We would also expect genetic status to modify the effect of exposure on biomarker levels due to this aforementioned issue.

- H_O: genetic status does not modify the relationship between exposure and biomarker level.
- H_A: genetic status does modify the relationship between exposure and biomarker level.

Age (19-28=1, 29-38=2, 39-48=3)

We would expect that increasing age, being related to higher oxidative stress, would exhibit higher levels of EBC 8-isoprostane and slightly more acidic pH.

- H_O: biomarker levels are the same for all ages.
- H_A: biomarker levels are different for different age groups.

We would also expect age to modify the effect of exposure on biomarker levels due to this aforementioned issue.

- H_O: age does not modify the relationship between exposure and biomarker level.
- H_A: age does modify the relationship between exposure and biomarker level

BMI (<25=1, 25-29=2, ≥30=3)

We would expect that higher BMI, being related to higher oxidative stress, would exhibit higher levels of EBC 8-isoprostane and slightly more acidic pH.

- H_O: biomarker levels are the same for all BMI categories.
- H_A: biomarker levels are different for different BMI categories.

We would also expect BMI to modify the effect of exposure on biomarker levels due to this aforementioned issue.

- H_O: BMI does not modify the relationship between exposure and biomarker level.
- H_A: BMI does modify the relationship between exposure and biomarker level

Order (1,2,3)

We do not expect order to have a significant effect due to the washout period in the study.

Chapter 4: DE Study Results

4.1 Overview

A controlled diesel exhaust study was performed in order to assess the effect on airway oxidative stress by monitoring exhaled breath condensate levels of 8-isoprostane and pH. The following section outlines the results for this study.

4.2 Participants

Participant characteristics were outlined for both biomarkers of interest, shown in Table 4.

Table 4: Participant characteristics for 8-isoprostane and pH databases

Characteristics	**8-Isoprostane Database**		**pH database**	
Total N	23		17	
Age	N	Mean ± SD	N	Mean ± SD
- All	23	29.4 ± 6	17	28.6 ± 5.9
- 19-28	13	24.1 ± 3	9	23.0 ± 3
- 29-38	7	32.7 ± 2	6	32.0 ± 2
- 39-48	3	44.7 ± 4	2	43.4 ± 4
Height (cm ± SD)	171.3 ± 9.8		171.4 ± 8.5	
Weight (kg ± SD)	71.8 ± 10.8		70.7 ± 8.7	
BMI (± SD)	N	Mean ± SD	N	Mean ± SD
- All	23	24.3 ± 2.32	17	24.1 ± 2.01
- <25	10	21.7 ± 1.5	14	23.1 ± 2
- 25-29	10	24.8 ± 0.7	2	26.0 ± 0.5
- 30<	3	31.4 ± 3	1	34.7
% FEV_1 Predicted (%)	94.2 ± 9.8		95.4 ± 9.9	
Sex (N, %)				
- Female	10 (44)		7 (41)	
- Male	13 (56)		10 (59)	
Methacholine response status				
- ≤8mg/ml	15(65)		10 (59)	
- >8mg/ml	8(35)		7 (41)	
Atopic Status				
- Atopic	15(65)		10 (71)	
- Non Atopic	8 (35)		5 (29)	
Doctor Diagnosed Asthma				
- Yes	13(56)		8 (47)	
- No	9(44)		9 (53)	
GSTP1 status				
- Wild type	8 (35)		6 (35)	
- Variant	15 (65)		11 (65)	

The 8-isoprostane database of 23 individuals consisted mostly of young and healthy individuals with an average of 29 yrs (±6yrs) and an average BMI of 24.3 (±2.32). There was approximately the same number of males and females in the 8-isoprostane database. Most of these individuals were methacholine responsive (65%), atopic (65%) and with GSTP1 variant (65%).

The pH database of 17 individuals consisted mostly of young and healthy with an average age of 29 years (±6years) and an average BMI of 24.1 (±2.01). There was about the same number of males and females in the 8-isoprostane database; while most of these individuals were methacholine responsive (59%), atopic (71%) and with GSTP1 variant (65%).

4.2.1 EBC 8-isoprostane and pH data set characteristics

4.2.1.1 8-isoprostane

The results for the method development are shown in Appendix A. The instrumental limit of detection was found to be 1.44pg/ml with an average recovery of 100.1%, average linearity of 0.993 and intra- and inter-day coefficients of variation of 4.22 and 1.74%, respectively.

Overall, 23 patients had full exhaled breath condensate data in order to assess 8-isoprostane levels with 537 study samples analyzed, 78 samples below the instrument limit of detection (LOD), 98 quality control samples were added to the analysis for a total of 635 samples. Most samples were analyzed in duplicate, when two EBC aliquots were available. The results that had duplicate data were averaged in order to obtain the final concentration for that time point. The average coefficient of variation for all analyzed samples was 38%.

EBC 8-isoprostane sample results had 14.5% of them below the LOD. There exist several ways to treat this type of censured data, the most prominent of which is substitution. A substitution value was chosen to be LOD/sqrt2 (1.018pg/ml). Appendix B of this document explains the rationale behind the substitution value used. Briefly, statisticians mention that distribution with less than 30% censored data, geometric standard deviations over 3.0 and lognormal shapes should use the LOD/sqrt2 substitution (254).

4.2.1.2 pH

For exhaled breath condensate pH analyses, 14 subjects were available with full datasets, 6 subjects with partial data sets (only 1 or two time points missing) and 6 subjects with incomplete data sets (several times points missing over several exposures).

For the purpose of these analyses only 17 participants were analyzed, 14 with full data sets and 3 participants without missing 0 hours time points. Overall, 293 aliquots were analyzed for EBC pH.

The first three participants had all their pH samples analyzed in duplicates in order to assess coefficients of variation for each sample analyzed. These averaged 0.1% variation and as such only one duplicate was then measured per time point.

4.2.1.3 Descriptive statistics for continuous variables

The following section describes the continuous variables related to both EBC biomarkers.

Table 5: Distribution characteristics for continuous variables

Variable (N)	Min	Mean	Median	Max	SD	SEM
8-Isoprostane (pg/ml)						
Log (276)	-0.55	3.28	3.91	7.71	1.74	0.075
Baseline (69)	1.02	69.9	56.5	452	78.6	9.46
Delta2 (69)	-370	-5.43	-3.25	341	101	12.2
Delta6 (69)	-391	-8.55	-2.82	208	94.7	11.4
Delta30 (69)	-392	-7.53	-2.15	232	89.4	10.7
pH (pH units)						
All pH (200)	5.01	7.82	7.84	8.25	0.28	0.02
Baseline (51)	5.01	7.76	7.85	8.25	0.48	0.07
Delta2 (51)	-0.41	0.046	-0.003	1.09	0.27	0.038
Delta6 (51)	-0.46	0.098	-0.008	2.82	0.48	0.068
Delta30 (51)	-0.64	0.071	0.005	2.79	0.49	0.069

SD=standard deviation, SEM=standard error of mean.

The 8-isoprostane distribution, as shown in Appendix B, appeared log-normally distributed; while the delta values generated for every time point were normally distributed. pH also had a normal distribution for both its pH and delta values (Appendix B).

Bland-Altman plots were created for all participants by exposure and time point in order to identify potential outliers (Appendix C, Figures 23 and 24).

For pH, one participant's 0hr pH was 5.01; this result was deemed an outlier most likely due to ingestion of food prior to EBC collection. For subsequent analyses, DE1-51 was removed from the 0hr analyses and associated delta analyses.

Within the 8-isoprostane distribution, one subject had a markedly higher 8-isoprostane level 6 hours after FAP exposure (1141pg/ml). This time point was removed from subsequent analyses. Otherwise, outliers were typically below average 8-isoprostane levels and as such were left in the analyses since these were probably below the limit of detection.

4.2.1.4 Descriptive statistics for categorical variables

Histograms with density lines were created for categorical variables for both 8-isoprostane and pH data sets.

Table 6: Categorical variable counts for 8-isoprostane data set.

Variable	Exposure	Sex	MR	Atopy	GSTP1	Order	Age	BMI
1	23	36	45	18	24	23	39	30
2	23	33	24	51	45	23	21	30
3	23					23	9	9

Rows 1, 2 and 3 represent the amount of counts in each category. For exposure, category one is FAP, two is DEP and three is DEN. For sex, category one is males and two is females. And so on for all factors included in the analyses (Figure 5).

Table 6 shows that for Age 3 and BMI3 categories, there are fewer observations than in the other categories.

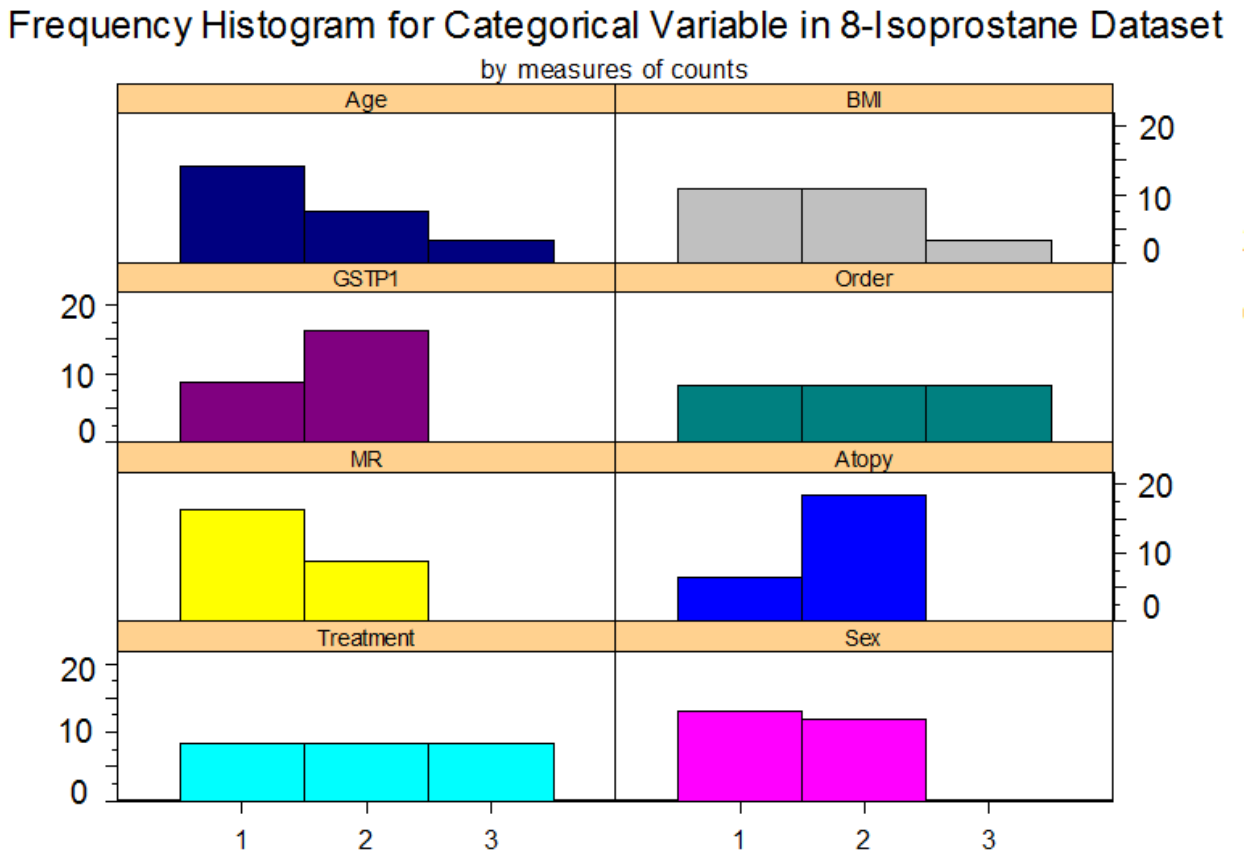

Figure 5. Histograms with density line for categorical variables in 8-isoprostane data set. "1", "2", and "3" and other terms are as previously defined.

Table 7: Categorical variable counts for pH data set.

Variable	Exposure	Sex	MR	Atopy	GSTP1	Order	Age	BMI
1	17	27	21	9	18	17	30	45
2	17	24	30	42	33	17	15	3
3	17					17	6	3

Table 7 shows that for Age 3, BMI2/3 there are few observations.

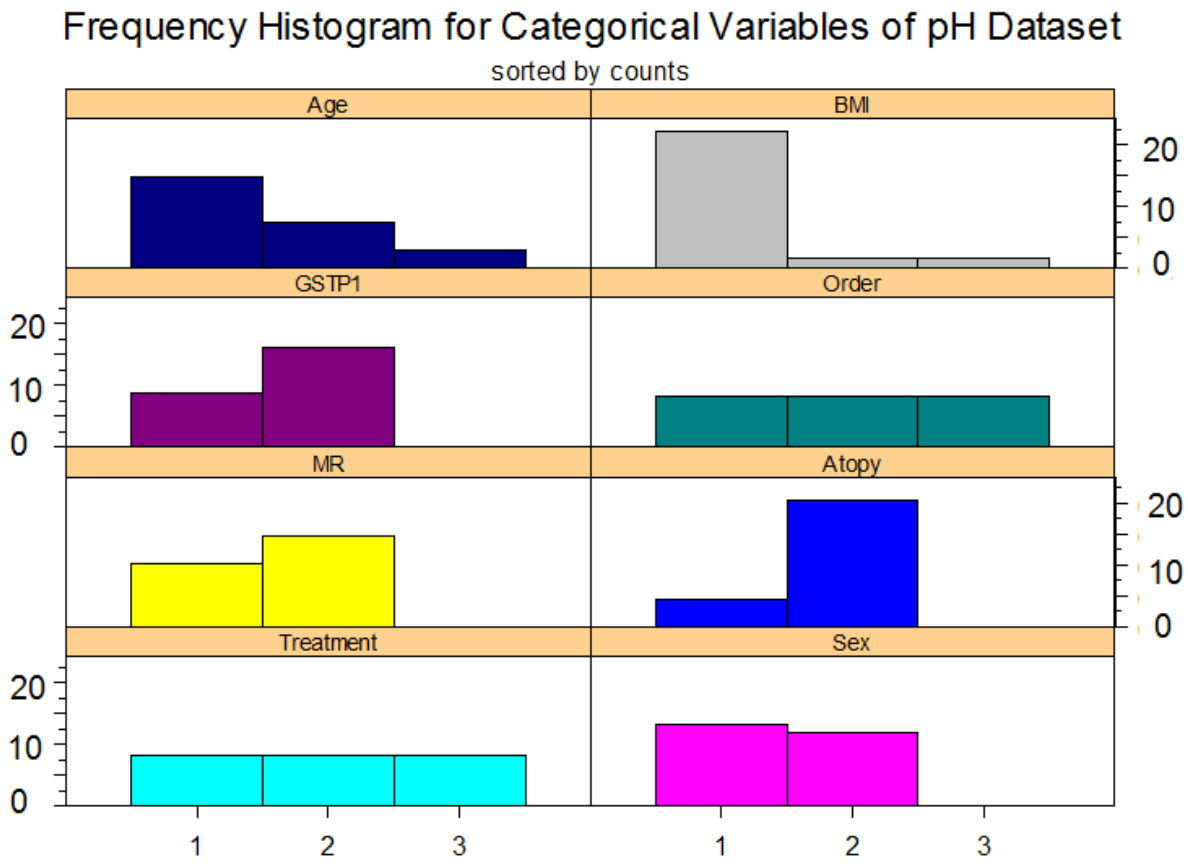

Figure 6. Histograms with density line for categorical variables in the pH data set. "1", "2", and "3" and other terms are as previously defined.

The counts in both data sets show that certain categorical variables do not have an equal number in each category, i.e. categories are unbalanced. This may play a role in having enough power in each subsequent analysis to detect differences. Categories with larger counts may bias generate significance towards the group with the most number of observations (i.e. counts).

4.2.1.5 Correlations

Both continuous variables were evaluated for correlation. Obviously, since there were discrepancies between the numbers of subjects available in each biomarker data set, only the subjects who had both biomarkers available for a specific time point were included in the evaluation.

The correlation coefficient (r) between pH and 8-isoprostane (untransformed) was 0.0287.
The correlation coefficient (r) between pH and log 8-isoprostane (transformed) was 0.0461.

We sought to correlate pH and log 8-isoprostane for each time point, for each exposure since both are related to one another with regards to oxidative stress.

Table 8: Correlation between 8-isoprostane and pH by exposure and time point

	Correlation between log 8-isoprostane (log pg/ml) and pH (pH units) (N=17)	**Linear Regression R^2** between log 8-isoprostane (log pg/ml) and pH (pH units) (N=17)	
		R^2	p-value
FAP			
0 hours after exposure	0.02	0.0004	0.94
2 hours after exposure	-0.19	0.04	0.45
6 hours after exposure	0.05	0.004	0.81
30 hours after exposure	0.62	**0.35**	**0.01**
DEP			
0 hours after exposure	0.05	0.002	0.86
2 hours after exposure	0.18	0.03	0.48
6 hours after exposure	-0.31	0.09	0.23
30 hours after exposure	-0.39	0.15	0.12
DEN			
0 hours after exposure	0.02	0.04	0.47
2 hours after exposure	-0.16	0.02	0.55
6 hours after exposure	-0.20	0.10	0.21
30 hours after exposure	-0.02	0.0003	0.95

The trends for Table 8 are shown in Figure 6. For FAP 30 hours after exposure, the correlation between log 8-isoprostane and pH is significant whereas 35% of the variation in 8-isoprostane may be explained by pH.

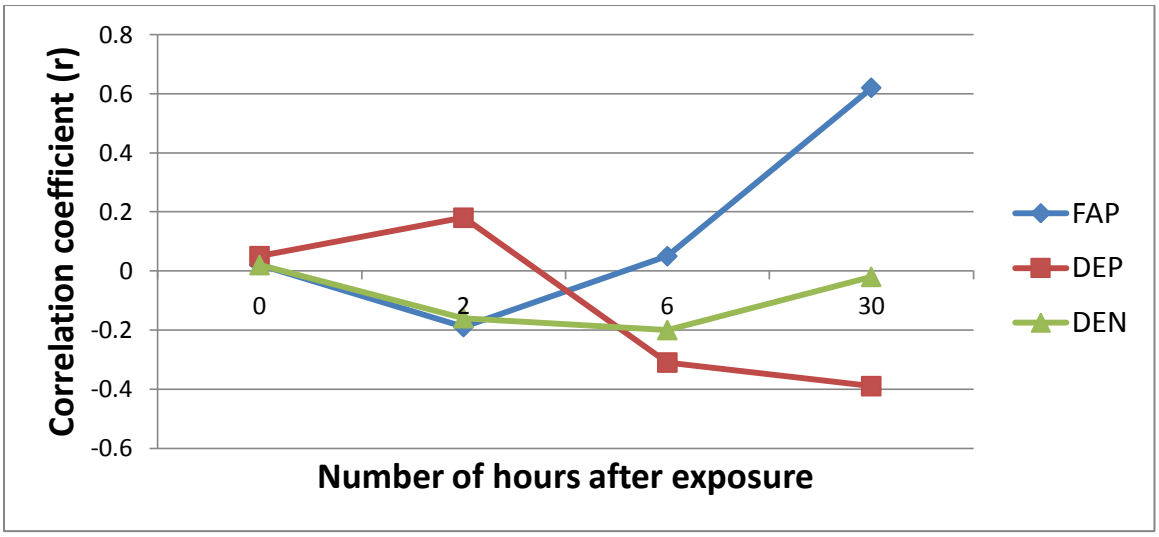

Figure 7. Correlation coefficients (r) between EBC log 8-isoprostane and pH by exposure and time point.

Correlation between the two biomarkers is non-existent when subjects are first exposed. A negative correlation depicts when one biomarker increases the other decreases.

4.2.1.6 Univariate analyses

The following sections describe the univariate analyses performed in order to evaluate our *a priori* hypotheses as well as secondary hypotheses evaluating effect modification.

1. **Primary analysis: exposure effect**

Table 8 describes the univariate results for log 8-isoprostane and pH databases; while Table 9 reflects the same for delta biomarker levels.

Table 9: Univariate analyses for exposure effect

	8-isoprostane			pH		
	β^{ϕ} (log pg/ml)	Confidence Interval[§]	p-value[ǂ]	β^{ϕ} (pH units)	Confidence Interval[§]	p-value[ǂ]
Baseline						
FAP	3.28	2.26, 4.30		7.64	7.31, 7.97	
DEP	0.54	-0.22, 1.30		0.11	-0.22, 0.44	
DEN	0.37	-0.39, 1.13	0.39	0.21	-0.12, 0.54	0.45
2 hours after exposure						
FAP	3.47	0.21, 6.72		7.86	7.74, 7.98	
DEP	-0.03	-0.97, 0.91		-0.02	-0.10, 0.06	
DEN	-0.13	-1.07, 0.81	0.96	0.0002	-0.07, 0.08	0.90

	8-isoprostane			pH		
	β[ϕ] (log pg/ml)	Confidence Interval[§]	p-value[ǂ]	β[ϕ] (pH units)	Confidence Interval[§]	p-value[ǂ]
6 hours after exposure						
FAP	3.30	1.44, 5.16		7.89	7.82, 7.97	
DEP	-0.02	-0.76, 0.72		-0.04	-0.10, 0.02	
DEN	-0.05	-0.79, 0.69	0.99	-0.06	-0.12,-0.001	0.16
30 hours after exposure						
FAP	3.18	2.08, 4.28		7.73	7.61, 7.85	
DEP	0.27	-0.49, 1.04		0.12	-0.04, 0.28	
DEN	0.004	-0.76, 0.77	0.72	0.12	-0.04, 0.28	0.26

[ϕ] Beta values represent coefficients from each term in the statistical analyses.

[§] Herein, for univariate analyses, the confidence intervals are calculated by taking 1.96*standard error from the coefficient β. For example take DEP at baseline, the coefficient 0.54 has 1.96*SE added/subtracted for the final upper and lower confidence intervals.

ǂ Herein, for the univariate analyses, the p value represents the results of a linear mixed effects model ANOVA term, unless otherwise specified.

Table 9 shows that for 8-isoprostane, levels at baseline and 30 hours are higher for DEP and DEN exposures relative to FAP; while they are lower for 2 and 6 hours after DEP and DEN exposures relative to FAP. EBC pH is more acidic after DEP relative to FAP 2 and 6 hours after exposure. EBC pH is higher after DEP and DEN 30 hours after exposure.

Univariate models were graphed in Figures 8 and 9 below.

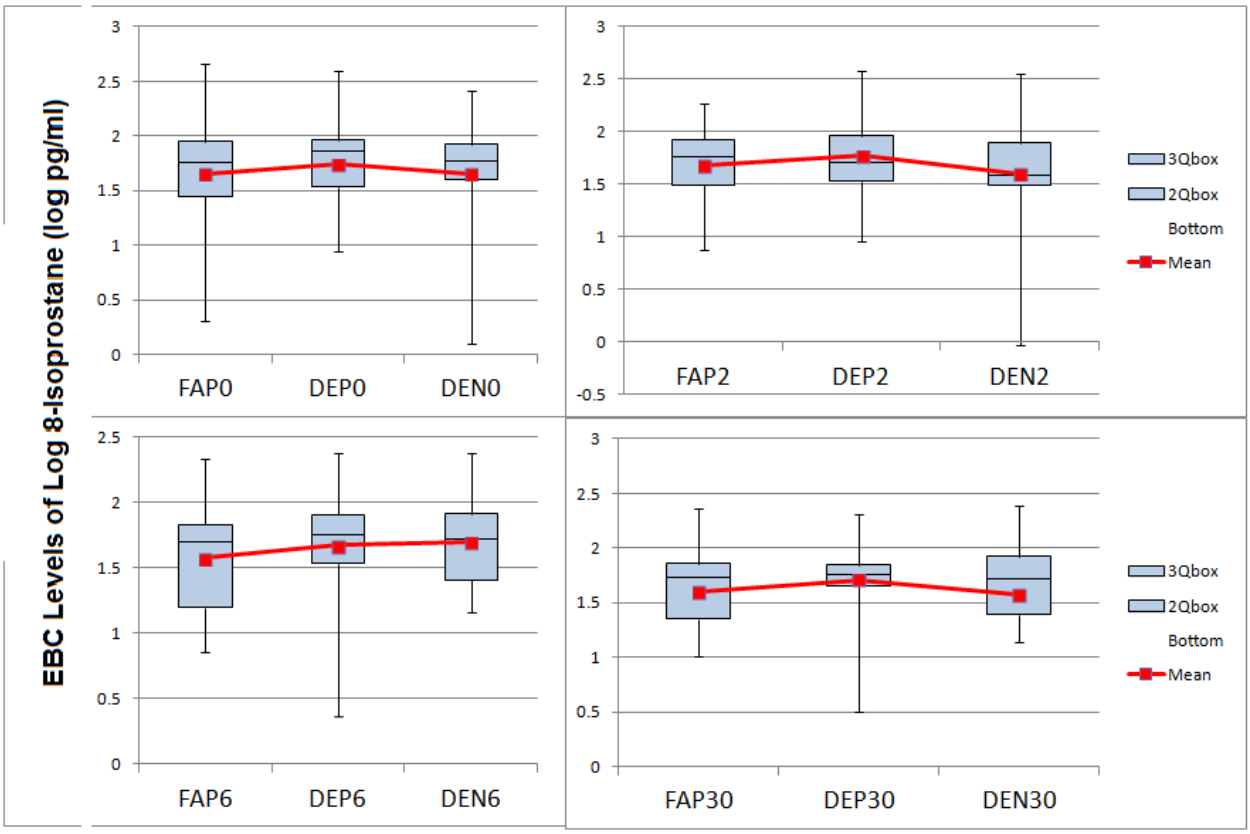

Figure 8. EBC log 8-isoprostane levels stratified by exposure for all time points (From top left, clockwise, is baseline, 2, 30 and 6 hours after exposure). The box represents 50% of the population (2Qbox=between Q1 and median, 3Qbox=between median and quartile3, Bottom=quartile1). The down whisker represents the first quartile minus the minimum and the up whisker represents the maximum minus third quartile. The line in the box represents the median log 8-isoprostane concentration for that exposure. These box plot parameters will be set for all box plot graphs herein.

There is an increase in log 8-isoprostane from FAP to DEP for all time points. There is a decrease in log 8-isoprostane from DEP to DEN for 0, 2 and 30 hours after exposure while there is an increase 6 hours after exposure.

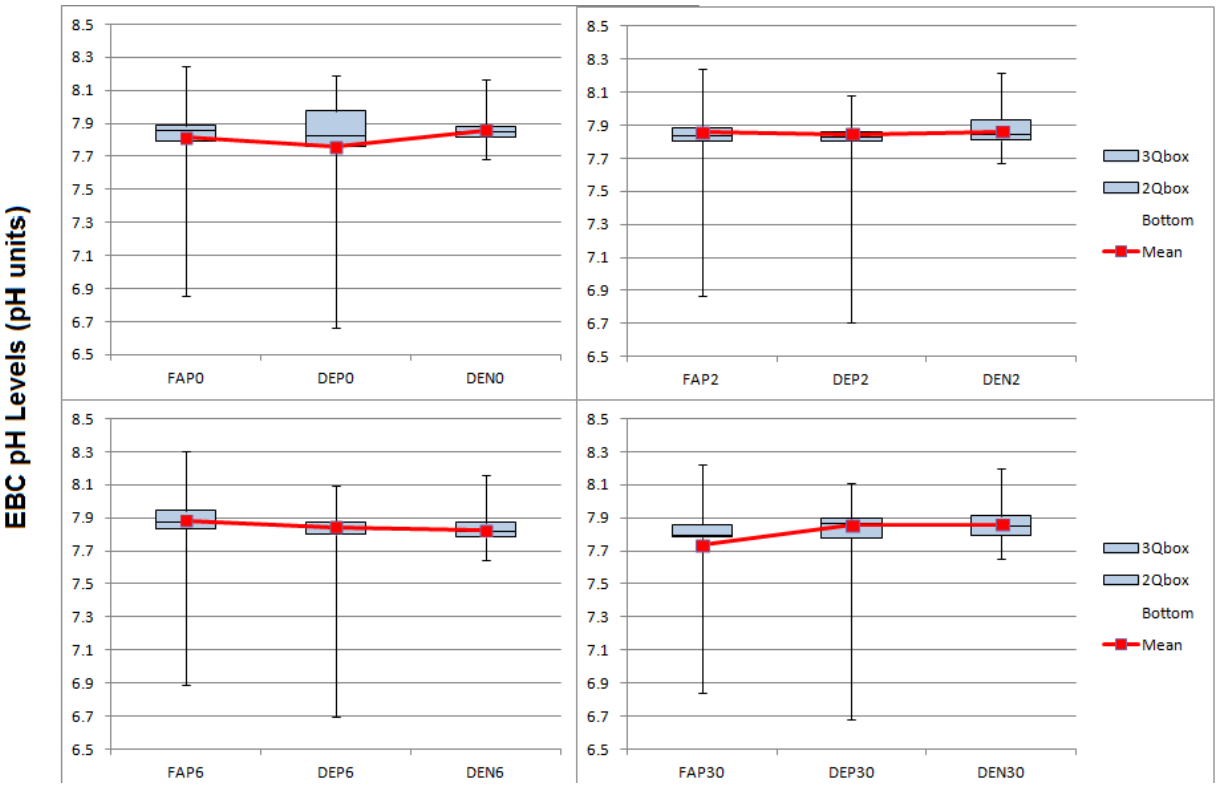

Figure 9. EBC pH levels stratified by exposure for all time points (From top left, clockwise, is baseline, 2, 30 and 6 hours after exposure).

There is no clear pattern discernible for EBC pH levels stratified by exposure. There is a small acidification from FAP to DEP for 0, 2 and 6 hours after exposure while 30 hours shows an increase in pH. There is an increase in pH from DEP to DEN 0 and 2 hours after exposure, while there is an acidification 6 hours after exposure and no change 30 hours after exposure.

Table 10: Univariate analyses for exposure effect on delta biomarker levels

	Delta 8-isoprostane			Delta pH (pH units)		
	β (pg/ml)	Confidence Interval	p-value‡	β (pH units)	Confidence Interval	p-value‡
Delta 2 hour						
FAP	-7.83	-216, 200		0.05	-0.19, 0.29	
DEP	7.64	-51.6, 66.8		0.04	-0.14, 0.22	
DEN	-0.43	-59.6, 58.8	0.96	-0.05	-0.22, 0.13	0.65
Delta 6 hour						
FAP	-8.84	-75.7, 58.0		0.25	-0.16, 0.66	
DEP	-0.96	-56.6, 54.7		-0.16	-0.50, 0.17	
DEN	1.81	-53.8, 57.5	0.99	-0.28	-0.61, 0.05	0.24
Delta 30 hour						
FAP	-7.48	-72.6, 57.6		0.09	-0.32, 0.50	
DEP	4.03	-48.3, 56.4		0.02	-0.31, 0.35	
DEN	-4.19	-56.5, 48.1	0.95	-0.07	-0.42, 0.28	0.88

‡ Herein, for the single covariate analyses, the p value represents the results of a linear mixed effects model ANOVA term, unless otherwise specified.

Table 10 shows a negative delta levels which depicts a lower biomarker level after exposure relative to before the exposure, generating a negative difference. For delta 8-isoprostane levels 2 hours after exposure, levels are lowest after FAP and DEP while almost at 0 after DEP exposure (i.e. no change in 8-isoprostane).

For delta levels 6 hours after exposure, levels are lowest after DEP exposure and are less negative after FAP and DEN exposures. For delta levels 30 hours after exposure, levels are least negative after DEP exposure, while most negative for FAP and DEN exposures. For delta pH, there is an acidification compared to FAP after DEN exposure after all time points. pH levels are typically higher than FAP levels after DEP exposure except 6 hours after exposure.

These results are shown in Figures 10 and 11.

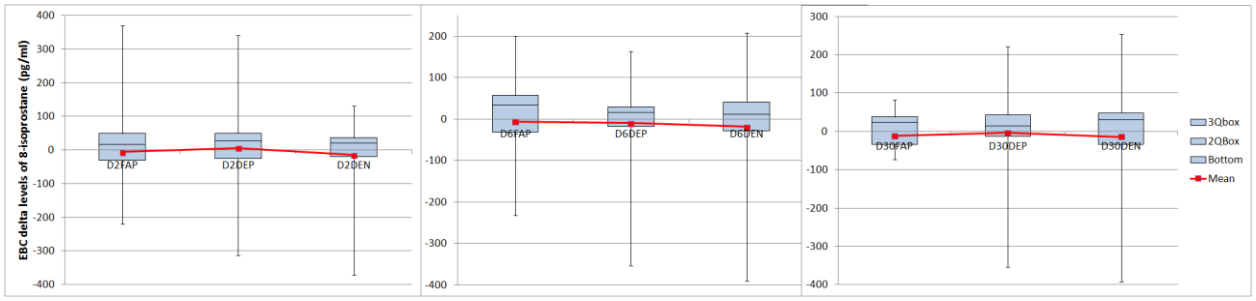

Figure 10. EBC delta 8-isoprostane levels stratified by exposure for 2, 6 and 30 hours after exposure (left, middle and right portion of figure).

EBC delta 8-isoprostane 2 and 30 hours after exposure goes from a negative value at FAP, to no difference at DEP, back to a negative value at DEN. EBC delta 8-isoprostane 6 hours after exposure remain unchanged for FAP and DEP exposures while become negative for after DEN exposure. Overall, exposure affects 8-isoprostane by increasing 8-isoprostane levels after DEP exposure while decreasing 8-isoprostane levels after FAP and DEN exposures.

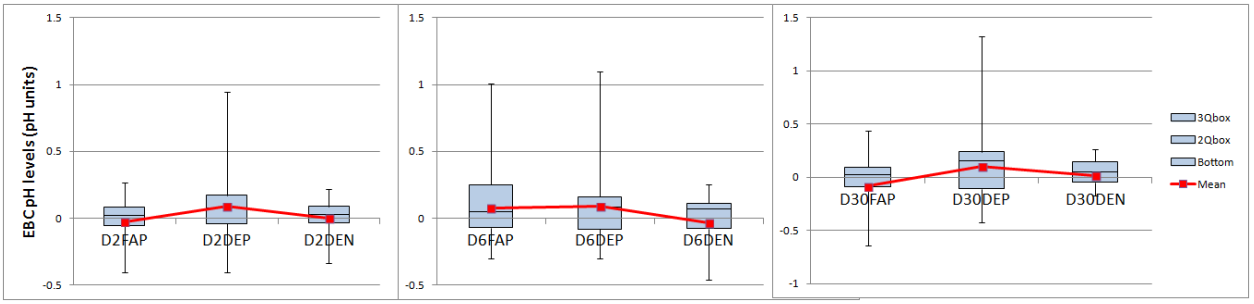

Figure 11. EBC delta pH levels stratified by exposure for 2, 6 and 30 hours after exposure (left, middle and right portion of figure).

A positive delta value with regards to EBC pH demonstrates that pH levels were higher after exposure than prior to exposure; this is opposite to an acidification of pH. Thus for delta 2, EBC pH shows no change after FAP and DEN while has an increase in pH after DEP exposure. For delta 6, EBC pH remains positive after FAP and DEP exposures while shows no change after DEN exposure. For delta 30, there is an acidification after FAP exposure, an increase in pH after DEP exposure and no change after DEN exposure.

2. Secondary analysis: effect modification by sex

The following section describes exposure effect on log and delta 8-isoprostane levels as well as pH and delta pH stratified by exposure.

Table 11: Exposure effect on EBC log and delta8-isoprostane stratified by sex.

	Males			Females		
	β (log pg/ml)	Confidence Interval	p-value	β (log pg/ml)	Confidence Interval	p-value
Baseline						
FAP	1.59	1.1, 2.08	<0.0001	1.75	1.22, 2.28	<0.0001
DEP	0.18	-0.19, 0.55	0.35	-0.05	-0.48, 0.38	0.83
DEN	0.02	-0.35, 0.39	0.91	-0.03	-0.44, 0.38	0.88
2 hours after exposure						
FAP	1.62	1.25, 1.99	<0.0001	1.77	1.20, 2.34	<0.0001
DEP	0.18	-0.09, 0.45	0.20	-0.02	-0.45, 0.41	0.92
DEN	0.03	-0.24, 0.30	0.85	-0.20	-0.63, 0.23	0.37
6 hours after exposure						
FAP	1.51	1.04, 1.98	<0.0001	1.43	0.98, 1.88	<0.0001
DEP	0.22	-0.11, 0.55	0.21	-0.06	-0.37, 0.25	0.69
DEN	0.20	-0.13, 0.53	0.24	0.01	-0.30, 0.33	0.93
30 hours after exposure						
FAP	1.49	0.98, 2.00	<0.0001	1.71	1.16, 2.26	<0.0001
DEP	0.19	-0.20, 0.58	0.32	-0.01	-0.46, 0.44	0.96
DEN	0.04	-0.35, 0.43	0.83	-0.13	-0.58, 0.32	0.57
Delta2						
FAP	-27.9	-156, 100	0.67	-46.2	-148, 55.9	0.38
DEP	-5.45	-99.3, 88.4	0.91	-0.81	-69.6, 68.0	0.98
DEN	-5.33	-99.2, 88.5	0.91	7.75	-61.0, 76.6	0.83
Delta6						
FAP	16.8	-61.8, 95.4	0.68	-61.8	-206, 82.3	0.41
DEP	-8.15	-69.1, 52.8	0.79	-22.6	-121, 75.8	0.66
DEN	-4.71	-65.7, 56.3	0.88	-14.5	-113, 83.9	0.77
Delta 30						
FAP	7.23	-36.1, 50.5	0.75	-15.3	127, 96.8	0.79
DEP	12.8	-19.3, 45.9	0.44	-16.2	-104, 71.8	0.75
DEN	-6.27	-38.4, 25.9	0.70	-5.19	-93.2, 82.8	0.91

For the non corrected tests (not deltas), there does seem to be a difference between the sexes. Males have typically lower log 8-isoprostane levels in EBC compared to females. While males

typically have higher log 8-isoprostane levels after DEP exposure. Females typically have lower delta values than males for 6 and 30 hours after exposureFemales show a larger decrease in 8-isoprostane levels than males after exposures. Also, females show larger negative changes after DEP exposure when compared to FAP; while these changes are lessened after DEN exposure. Males do not show consistent delta changes across time points. Figures 25 and 28 in Appendix C show the results from Table 11 above.

Table 12: Exposure effect on EBC pH and delta pH stratified by sex

	Males			Females		
	β (pH units)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Baseline						
FAP	7.76	7.15, 8.37	<0.0001	8.04	7.67, 8.41	<0.0001
DEP	-0.01	-0.34, 0.32	0.94	-0.09	-0.32, 0.15	0.43
DEN	0.06	-0.27, 0.39	0.70	0.04	-0.20, 0.28	0.77
2 hours after exposure						
FAP	7.84	7.70, 7.98	<0.0001	8.10	7.92, 8.27	<0.0001
DEP	-0.05	-0.13, 0.03	0.31	0.02	-0.10, 0.14	0.77
DEN	0.003	-0.08, 0.08	0.94	-0.005	-0.12, 0.11	0.93
6 hours after exposure						
FAP	7.91	7.83, 7.99	<0.0001	7.97	7.83, 8.11	<0.0001
DEP	**-0.11**	**-0.19, -0.03**	**0.007**	0.04	-0.04, 0.12	0.41
DEN	**-0.07**	**-0.15, 0.008**	**0.07**	-0.05	-0.13, 0.03	0.26
30 hours after exposure						
FAP	7.65	7.16, 8.14	<0.0001	7.84	7.70, 7.98	<0.0001
DEP	0.16	-0.15, 0.47	0.33	0.10	0.002, 0.20	0.07
DEN	0.20	-0.13, 0.53	0.25	0.06	-0.04, 0.16	0.29
Delta2						
FAP	0.14	-0.57, 0.85	0.71	0.03	-0.21, 0.27	0.80
DEP	0.07	-0.28, 0.42	0.71	-0.03	-0.21, 0.15	0.75
DEN	-0.19	-0.54, 0.16	0.30	-0.08	-0.28, 0.12	0.41
Delta6						
FAP	0.19	-0.32, 0.70	0.46	0.01	-0.46, 0.48	0.96
DEP	-0.07	-0.44, 0.30	0.74	-0.004	-0.32, 0.31	0.98
DEN	-0.05	-0.42, 0.32	0.79	0.01	-0.30, 0.32	0.94

	Males			Females		
	β (pH units)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Delta 30						
FAP	0.003	-0.12, 0.12	0.96	-0.02	-0.22, 0.18	0.85
DEP	-0.01	-0.09, 0.07	0.75	-0.07	-0.21, 0.07	0.35
DEN	-0.004	-0.10, 0.09	0.92	0.03	-0.11, 0.17	0.69

For non corrected values there isn't a clear trend in the results. However, males have a significantly more acidic pH 6 hours after DEP exposure when compared to FAP, effect modification by sex should be investigated at that time point. For delta pH, trends were also unclear, no significant results were found. Females showed little to no change in delta pH levels, while males showed an acidification 6 and 30 hours after DEP exposure. Delta pH levels after DEN exposure were typically more acidic for both males and females. Figure 26 and 27 in Appendix C display results from Table 12. Interaction between sex and exposure was investigated for EBC pH 6 hours after exposure in Table 13.

Table 13: Interaction between exposure and sex for EBC pH 6 hours after exposure

	Value (β) (pH units)	Confidence Interval	p-value	ANOVA p-value
Intercept	7.93	7.83,8.03	<0.0001	<0.0001
Sex	-0.03	-0.11,0.05	0.49	0.32
DEP	-0.11	-0.19, -0.03	0.01	
DEN	0.04	-0.04,0.12	0.09	0.13
SexDEP	**-0.07**	**-0.15,0.01**	**0.02**	
SexDEN	**-0.05**	**-0.13,0.03**	**0.71**	**0.03**

Sex significantly interacts with exposure modifying EBC pH 6 hours after exposure. Males show a significant drop in pH 6 hours after DEP exposure

Table 13 interaction terms compare to the intercept which, in this case, represents two terms which are not easily identifiable. A such, paired t-tests were performed between for FAP vs. DEP and DEP vs. DEN for EBC pH 6 hours after exposure for males and females.

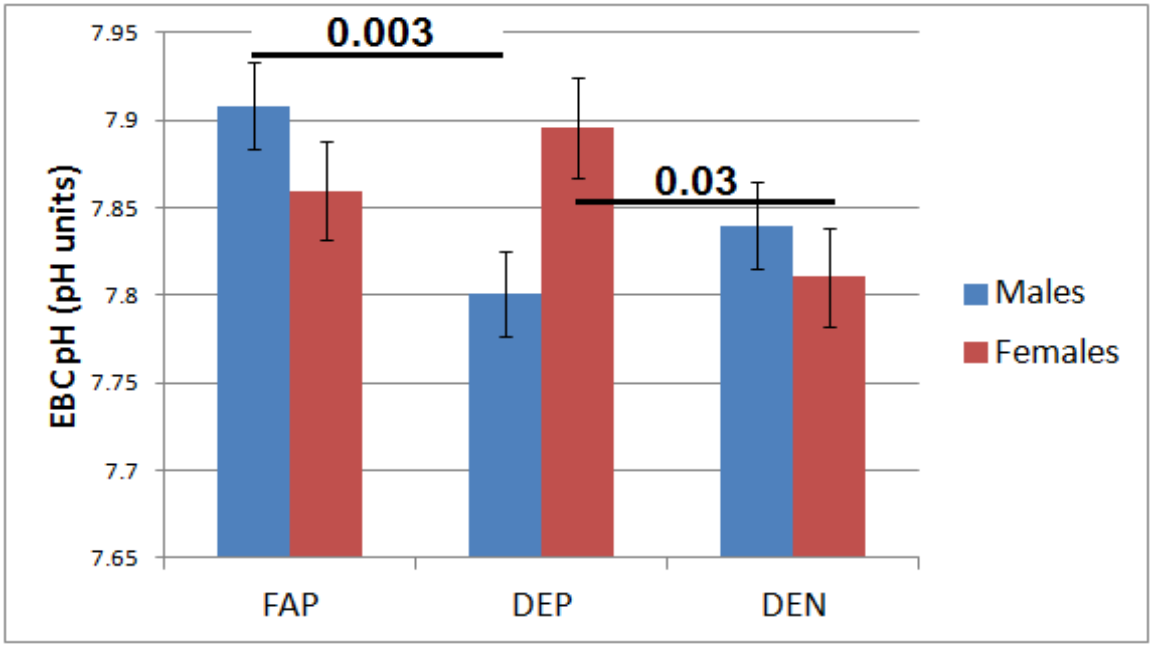

Figure 12. EBC pH levels 6 hours after exposure. EBC pH data is stratified by sex to look at effect modification between sex and exposure interaction term. The error bars represent standard error (standard deviation/sqrt[N]).

Figure 12 shows that males have a significant acidification from FAP to DEP; while females increase in pH non significantly. EBC pH in males increases from DEP to DEN; while, significantly acidifies from DEP to DEN in females.

Lastly, Table 37 and 38 in Appendix C show univariate analyses between males and females at each time point. Sex modified log 8-isprostane borderline significantly and pH significantly 2 hours after exposure and did not change delta biomarker levels.

3. Secondary analysis: effect modification by methacholine response status

The following section represents analyses on methacholine responsive status (MRS) on EBC biomarker levels.

Table 14: Exposure effect on EBC log and delta8-isoprostane stratified by MRS

	Non-Responsive (N=8)			Responsive (N=15)		
	β (log pg/ml)	Confidence Interval	p-value	β (log pg/ml)	Confidence Interval	p-value
Baseline						
FAP	1.54	0.70, 2.38	0.002	1.70	0.99, 2.41	<0.0001
DEP	0.22	-0.33, 0.77	0.44	0.06	-0.25, 0.37	0.72
DEN	-0.01	-0.56, 0.54	0.97	0.03	-0.28, 0.34	0.87
2 hours after exposure						
FAP	1.69	1.22, 2.16	<0.0001	1.65	1.26, 2.04	<0.0001
DEP	0.14	-0.21, 0.49	0.44	0.11	-0.22, 0.44	0.52
DEN	-0.10	-0.45, 0.25	0.59	-0.04	-0.35, 0.27	0.80
6 hours after exposure						
FAP	1.55	0.94, 2.16	0.0001	1.46	1.05, 1.87	<0.0001
DEP	0.03	-0.40, 0.46	0.89	0.18	-0.09, 0.45	0.21
DEN	0.16	-0.29, 0.61	0.50	0.12	-0.13, 0.37	0.39
30 hours after exposure						
FAP	1.59	0.53, 2.65	0.008	1.55	0.81, 2.29	0.0002
DEP	-0.08	-0.61, 0.45	0.76	0.25	-0.10, 0.60	0.17
DEN	-0.07	-0.60, 0.46	0.80	-0.0001	-0.33, 0.33	0.99
Delta2						
FAP	14.1	-81.3, 109	0.77	-61.9	-189, 65.7	0.35
DEP	-14.2	-86.3, 57.9	0.70	-7.08	-92.9, 78.8	0.87
DEN	-16.6	-88.7, 55.5	0.66	4.93	-79.5, 89.4	0.91
Delta6						
FAP	7.93	-158, 174	0.93	-4.74	-58.8, 49.4	0.86
DEP	-37.9	-184, 108	0.62	-0.97	-39.2, 37.3	0.96
DEN	-55.4	-202, 90.8	0.47	16.3	-21.1, 53.7	0.40
Delta 30						
FAP	-12.2	-92.4, 67.9	0.77	-0.82	-148, 146	0.99
DEP	22.4	-39-3, 84.1	0.49	-13.8	-70.4, 42.8	0.64
DEN	12.5	-49.2, 74.2	0.69	-16.2	-71.7, 39.3	0.57

There does not seem to be a clear difference between responsive and non- responsive individuals.

Deltas are generally negative meaning 8-isoprostane levels are lower after exposure than they are before exposure. Larger negative deltas occur after DEP exposure in responsive subjects, there is no clear trends for non-responsive subjects.

Figures 29 and 31 in Appendix C show the results from Table 14 above.Tables 39 and 40 in Appendix3 investigate the difference between responsive and non-responsive subjects for 8-isoprostane levels. Briefly, a borderline difference was observed 30 hours after exposure between the two groups for log 8-isoprostane (p=0.07) and for delta 8-isoprostane 6 hours after exposure (p=0.06).

Table 15: Exposure effect on EBC pH and delta pH stratified by MRS

	Non-responsives (N=6)			Responsives (N=11)		
	β (pH units)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Baseline						
FAP	7.65	7.10, 8.20	<0.0001	7.89	7.65, 8.13	<0.0001
DEP	0.10	-0.31, 0.51	0.63	-0.15	-0.35, 0.05	0.15
DEN	0.18	-0.23, 0.59	0.40	-0.03	-0.23, 0.17	0.74
2 hours after exposure						
FAP	7.91	7.69, 8.13	<0.0001	7.84	7.78, 7.90	<0.0001
DEP	-0.05	-0.25, 0.15	0.64	-0.01	-0.07, 0.05	0.77
DEN	-0.02	-0.22, 0.18	0.82	0.0002	-0.06, 0.06	0.99
6 hours after exposure						
FAP	7.90	7.74, 8.06	<0.0001	7.87	7.67, 8.07	<0.0001
DEP	-0.03	-0.12, 0.11	0.70	-0.05	-0.11,0.009	0.11
DEN	-0.06	-0.20, 0.08	0.41	**-0.06**	**-0.12, -0.001**	**0.05**
30 hours after exposure						
FAP	7.58	7.09, 8.07	<0.0001	7.81	7.71, 7.91	<0.0001
DEP	0.25	-0.16, 0.66	0.25	0.05	-0.009, 0.11	0.13
DEN	0.30	-0.11, 0.71	0.18	0.02	-0.04, 0.08	0.65

	Non-responsives (N=6)			Responsives (N=11)		
	β (pH units)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Delta2						
FAP	0.25	-0.24, 0.74	0.34	-0.05	-0.29, 0.19	0.69
DEP	-0.16	-0.55, 0.23	0.44	0.14	-0.06, 0.34	0.16
DEN	-0.22	-0.61, 0.17	0.30	0.03	-0.17, 0.23	0.75
Delta6						
FAP	0.30	-0.37, 0.97	0.39	-0.01	-0.25, 0.23	0.92
DEP	-0.14	-0.49, 0.21	0.46	0.10	-0.12, 0.32	0.33
DEN	-0.25	-0.60, 0.10	0.20	-0.03	-0.25, 0.19	0.78
Delta 30						
FAP	-0.08	-0.57, 0.41	0.74	0.20	-0.47, 0.87	0.56
DEP	0.15	-0.24, 0.54	0.47	-0.08	-0.59, 0.43	0.77
DEN	0.12	-0.27, 0.51	0.57	-0.20	-0.75, 0.35	0.48

Responsive subject have a significantly lower pH 6 hours after DEN exposure for non corrected levels. Delta levels show higher positive changes for non-responsive subjects compared to responsive subjects; acidification after DEP occurred 2 and 6 hours after exposure.

Delta levels were typically negative for responsive subjects, except after DEP exposure for delta 2 and 6. Figures 30 and 32 in Appendix C show the results from Table 15 above.

Tables 39 and 40 in Appendix3 investigate the difference between responsive and non - responsive subjects for EBC pH and delta pH. No significant differences were found for EBC pH.

4. Secondary analysis: effect modification by atopic status

This section investigates the potential effect modification of atopic status on exposure interactions with biomarker levels.

Table 16: Exposure effect on EBC log and delta8-isoprostane stratified by atopic status

	Non-Atopic (N=8)			Atopic (N=15)		
	β (log pg/ml)	Confidence Interval	p-value	β (log pg/ml)	Confidence Interval	p-value
Baseline						
FAP	1.19	0.43, 1.95	0.007	1.78	0.82, 2.74	0.0008
DEP	0.25	-0.30, 0.80	0.39	-0.004	-0.30, 0.29	0.98
DEN	0.15	-0.40, 0.70	0.59	-0.08	-0.37, 0.21	0.58
2 hours after exposure						
FAP	1.50	1.11, 1.89	<0.0001	1.85	1.46, 2.42	<0.0001
DEP	0.005	-0.39, 0.40	0.98	0.14	-0.15, 0.43	0.34
DEN	0.04	-0.35, 0.43	0.83	-0.13	-0.42, 0.16	0.37
6 hours after exposure						
FAP	1.29	0.76, 1.82	0.0001	1.64	1.25, 2.03	<0.0001
DEP	0.04	-0.33, 0.41	0.83	0.12	-0.17, 0.41	0.42
DEN	0.25	-0.12, 0.62	0.19	0.05	-0.24, 0.34	0.75
30 hours after exposure						
FAP	1.26	0.52, 2.00	0.004	1.68	0.76, 2.60	0.0008
DEP	0.07	-0.46, 0.60	0.81	0.13	-0.20, 0.46	0.45
DEN	0.05	-0.48, 0.58	0.87	-0.07	-0.40, 0.26	0.66
Delta2						
FAP	-21.7	-140, 95.9	0.72	-10.8	-395, 373	0.96
DEP	8.72	-83.4, 101	0.85	26.6	-47.5, 101	0.48
DEN	5.36	-86.8, 97.5	0.91	10.6	-63.5, 84.7	0.78
Delta6						
FAP	-50.0	-147, 46.8	0.32	-1.01	-79.2, 77.2	0.98
DEP	37.4	-44.7, 120	0.38	-5.58	-75.6, 64.4	0.88
DEN	50.4	-31.9, 133	0.24	-7.85	-77.8, 62.1	0.83
Delta 30						
FAP	-50.2	-293, 193	0.69	4.91	-271, 281	0.97
DEP	64.8	-30.2, 160	0.20	-17.7	-77.7, 42.3	0.57
DEN	40.0	-55.1, 135	0.42	-17.4	-77.4, 42.6	0.57

Atopic subjects had consistently higher 8-isoprostane levels than non atopic subjects. Although exposure did not have any significant effect on log 8-isoprostane, some trends were observed. For both atopic and non-atopic subjects, levels of log 8-isoprostane increased after DEP exposure and showed either a smaller increase or decreased after DEN exposure. Delta 8-isoprostane levels for non-atopics increased after DEP exposure compared to FAP (large negative deltas for FAP became smaller negative deltas for DEP).

Delta level trends were not consistent in atopics. Figures 33 and 35 in Appendix C show the results from Table 16 above. Tables 41 and 42 in Appendix C show the difference between atopic and non-atopics 8-isoprostane levels. Briefly, atopics had borderline significantly different levels compared to non-atopics at baseline and 2 hours after exposure (p=0.07 and 0.07, respectively).

Table 17: Exposure effect on EBC pH and delta pH stratified by atopic status

	Non-Atopic (N=3)			Atopic (N=14)		
	β (pH units)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Baseline						
FAP	7.32	6.26, 8.38	<0.0001	7.92	7.70, 8.14	<0.0001
DEP	-0.02	-0.78, 0.74	0.97	-0.07	-0.23, 0.09	0.40
DEN	0.36	-0.40, 1.12	0.40	-0.02	-0.18, 0.14	0.77
2 hours after exposure						
FAP	7.85	7.60, 8.10	<0.0001	7.88	7.76, 8.00	<0.0001
DEP	-0.13	-0.33, 0.07	0.24	0.01	-0.07, 0.09	0.83
DEN	-0.01	-0.21, 0.19	0.91	0.003	-0.08, 0.08	0.95
6 hours after exposure						
FAP	7.89	7.67, 8.11	<0.0001	7.90	7.80, 8.00	<0.0001
DEP	-0.14	-0.30, 0.02	0.15	-0.02	-0.08, 0.04	0.56
DEN	-0.03	-0.19, 0.13	0.75	**-0.07**	**-0.13, -0.01**	**0.05**
30 hours after exposure						
FAP	7.38	6.16, 8.60	<0.0001	7.81	7.71, 7.91	<0.0001
DEP	0.45	-0.49, 1.39	0.38	0.05	-0.009, 0.11	0.12
DEN	0.45	-0.49, 1.39	0.38	0.05	-0.009, 0.11	0.12

	Non-Atopic (N=3)			Atopic (N=14)		
	β (pH units)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Delta2						
FAP	0.59	-0.35, 1.53	0.27	-0.02	-0.22, 0.18	0.81
DEP	-0.12	-0.79, 0.55	0.75	0.08	-0.08, 0.24	0.31
DEN	-0.37	-1.04, 0.30	0.32	0.03	-0.13, 0.19	0.69
Delta6						
FAP	0.68	-0.18, 1.54	0.17	-0.0006	-0.18, 0.18	0.99
DEP	-0.12	-0.75, 0.51	0.71	0.05	-0.11, 0.21	0.53
DEN	-0.39	-1.02, 0.24	0.26	0.04	-0.12, 0.20	0.63
Delta 30						
FAP	-0.13	-1.21, 0.95	0.82	-0.12	-0.34, 0.09	0.29
DEP	0.47	-0.35, 1.29	0.31	0.12	-0.04, 0.28	0.14
DEN	0.09	-0.73, 0.91	0.83	0.10	-0.06, 0.26	0.24

Non-atopic subjects exhibit a clearer trend than atopic subjects, they consistently non - significantly acidify after DEP exposure and recover after DEN. Furthermore, atopic individuals show consistently higher pH after FAP exposure than non-atopics.

Delta pH in non-atopic individual seems to acidify 2 and 6 hours after DEP exposure relative to FAP and even more with DEN exposure; while atopic subjects show little to no change relative to FAP. Figures 34 and 36 in Appendix C show the results from Table 17 above.

Tables 41 and 42 in Appendix C show the difference between atopic and non-atopics pH levels. Briefly, atopics had borderline significantly different levels compared to non-atopics 2 hours after exposure (p=0.09).

5. Secondary analysis: effect modification by genetic variant status

This section investigates the potential effect modification of genetic variant status on exposure interactions with biomarker levels.

Table 18: Exposure effect on EBC log and delta8-isoprostane stratified by genetic GSTP1 status

	GSTP1 wild type (N=8)			GSTP1 variant (N=15)		
	β (log pg/ml)	Confidence Interval	p-value	β (log pg/ml)	Confidence Interval	p-value
Baseline						
FAP	1.75	1.24, 2.26	<0.0001	1.56	1.07, 2.05	<0.0001
DEP	0.15	-0.24, 0.54	0.47	0.05	-0.30, 0.40	0.79
DEN	0.12	-0.27, 0.51	0.56	-0.06	-0.41, 0.29	0.73
2 hours after exposure						
FAP	1.83	1.36, 2.30	<0.0001	1.65	1.22, 2.08	<0.0001
DEP	0.19	-0.14, 0.52	0.29	0.04	-0.27, 0.35	0.79
DEN	-0.09	-0.42, 0.24	0.61	-0.06	-0.37, 0.25	0.71
6 hours after exposure						
FAP	1.64	1.13, 2.15	<0.0001	1.48	1.09, 1.87	<0.0001
DEP	0.18	-0.23, 0.59	0.40	0.05	-0.22, 0.32	0.74
DEN	0.17	-0.24, 0.58	0.43	0.09	-0.18, 0.36	0.52
30 hours after exposure						
FAP	1.58	1.19, 1.97	<0.0001	1.51	0.94, 2.08	<0.0001
DEP	0.22	-0.07, 0.51	0.17	0.05	-0.36, 0.46	0.82
DEN	0.13	-0.16, 0.42	0.40	-0.12	-0.53, 0.29	0.57
Delta2						
FAP	-18.1	-126, 90.3	0.75	-12.8	-102, 76.2	0.78
DEP	1.32	-85.7, 83.3	0.98	30.5	-44.8, 106	0.43
DEN	-8.73	-95.8, 78.3	0.85	18.1	-57.2, 93.3	0.64
Delta6						
FAP	-6.96	-225, 211	0.95	-24.0	-95.1, 47.1	0.51
DEP	-31.6	-139, 76.2	0.57	31.2	-28.8, 91.2	0.31
DEN	12.7	-95.1, 121	0.82	12.3	-47.7, 72.3	0.69
Delta 30						
FAP	-35.4	-190, 119	0.66	-2.98	-147, 141	0.97
DEP	-9.19	-9.1, 74.7	0.83	21.8	-41.7, 85.3	0.50
DEN	13.4	-70.5, 97.3	0.76	-3.17	-66.7, 60.3	0.92

Although not significant, exposure seems to affect log 8-isoprostane levels in both wild type and variant individuals. There is a consistent increase in 8-isoprostane levels after DEP exposure relative to FAP; while either a lower increase or decrease after DEN exposure. GSTP1 variant subjects consistently show lower levels of log 8-isoprostane after FAP exposure than wild type individuals.

Delta levels for GSTP1 variants consistently increase after DEP exposure and to a lesser extent after DEN exposure. Wild type individuals have larger negative deltas after DEP than after FAP while have less negative deltas after DEN exposure.

Figures 37 and 39 in Appendix C show the results from Table 18 above. The difference between both groups was assessed in Tables 43 and 44 in Appendix3, no significant differences were found.

Table 19: Exposure effect on EBC pH and delta pH stratified by genetic GSTP1 status

	GSTP1 wild type (N=6)			GSTP1 variant (N=11)		
	β (pH units)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Baseline						
FAP	7.94	7.69, 8.20	<0.0001	7.75	7.38, 8.12	<0.0001
DEP	-0.13	-0.33, 0.07	0.19	-0.03	-0.30, 0.24	0.86
DEN	-0.06	-0.26, 0.14	0.57	0.09	-0.18, 0.36	0.53
2 hours after exposure						
FAP	7.94	7.76, 8.12	<0.0001	7.85	7.69, 8.01	<0.0001
DEP	-0.02	-0.14, 0.09	0.81	-0.01	-0.11, 0.09	0.78
DEN	-0.04	-0.16, 0.08	0.52	0.02	-0.08, 0.12	0.64
6 hours after exposure						
FAP	7.94	7.78, 8.09	<0.0001	7.89	7.65, 8.13	<0.0001
DEP	-0.01	-0.13, 0.11	0.85	-0.05	-0.13, 0.03	0.14
DEN	-0.04	-0.16, 0.08	0.45	**-0.07**	**-0.15, 0.008**	**0.08**
30 hours after exposure						
FAP	7.87	7.71, 8.03	<0.0001	7.83	7.73, 7.93	<0.0001
DEP	0.03	-0.09, 0.15	0.54	0.03	-0.05, 0.11	0.53
DEN	0.02	-0.08, 0.12	0.76	0.04	-0.04, 0.12	0.42

	GSTP1 wild type (N=6)			GSTP1 variant (N=11)		
	β (pH units)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Delta2						
FAP	-0.01	-0.28, 0.26	0.93	0.09	-0.30, 0.48	0.63
DEP	0.11	-0.09, 0.31	0.32	0.01	-0.26, 0.28	0.95
DEN	0.003	-0.19, 0.20	0.98	-0.07	-0.34, 0.20	0.61
Delta6						
FAP	-0.03	-0.30, 0.24	0.85	0.15	-0.20, 0.50	0.41
DEP	0.12	-0.08, 0.32	0.26	-0.04	-0.31, 0.23	0.78
DEN	0.01	-0.19, 0.21	0.93	-0.17	-0.44, 0.10	0.23
Delta 30						
FAP	-0.11	-0.33, 0.11	0.33	-0.07	-0.50, 0.36	0.75
DEP	0.16	0.003, 0.32	0.07	0.20	-0.07, 0.47	0.17
DEN	0.13	-0.05, 0.31	0.15	0.09	-0.20, 0.38	0.55

Exposure has a borderline significant effect on EBC pH levels 6 hours after exposure for GSTP1 variant subjects. Wild type subjects seem to show acidification after DEP and DEN exposures with larger acidifications for DEN. GSTP1 variant subjects typically had more acidic pH after FAP exposure than wild type individuals.

Delta pH levels acidify after FAP exposure, then increase after DEP exposure and increase to a lesser extent after DEN exposure for wild type individuals. Delta pH in variant individuals acidifies after DEP 6 hours after exposure and acidifies even more after DEN exposure. Otherwise, variant subjects increase in pH 2 and 30 hours after exposure.

Figures 38 and 40 in Appendix C show the results from Table 19 above. The difference between both groups was assessed in Tables 43 and 44 in Appendix3, no significant differences were found.

6. Secondary analysis: effect modification by age

This section investigates the potential effect modification of age on exposure interactions with biomarker levels.

Table 20: Exposure effect on EBC log and delta8-isoprostane stratified by age

	Age 1 (N=13)			Age2 (N=7)			Age3 (N=3)		
	β (log pg/ml)	Confidence Interval	p-value	β (log pg/ml)	Confidence Interval	p-value	β (log pg/ml)	Confidence Interval	p-value
Baseline									
FAP	1.75	1.42, 2.08	<.0001	1.43	0.72, 2.14	0.001	1.80	1.25, 2.35	0.0007
DEP	0.06	-0.23, 0.35	0.68	0.24	-0.35, 0.83	0.43	-0.21	-0.64, 0.22	0.38
DEN	0.17	-0.12, 0.46	0.28	-0.27	-0.86, 0.32	0.38	-0.09	-0.52, 0.34	0.67
2 hours after exposure									
FAP	1.66	1.31, 2.01	<.0001	1.82	0.96, 2.68	0.0006	1.83	1.07, 2.59	0.003
DEP	0.17	-0.06, 0.41	0.19	0.05	-0.56, 0.66	0.89	-0.09	-0.68, 0.50	0.75
DEN	0.05	-0.19, 0.29	0.69	-0.13	-0.74, 0.48	0.69	-0.47	-1.06, 0.12	0.17
6 hours after exposure									
FAP	1.57	1.24, 1.90	<.0001	1.66	1.13, 2.19	<.0001	1.32	0.18, 2.46	0.06
DEP	0.15	-0.14, 0.44	0.31	0.06	-0.33, 0.45	0.76	-0.09	-0.95, 0.77	0.84
DEN	0.24	-0.06, 0.53	0.11	-0.14	-0.53, 0.25	0.51	0.18	-0.68, 1.04	0.70
30 hours after exposure									
FAP	1.41	0.99, 1.82	<.0001	1.87	0.99, 2.75	0.0006	1.42	1.07, 1.77	0.0002
DEP	**0.34**	**0.05, 0.63**	**0.03**	-0.43	-1.14, 0.28	0.25	**0.35**	**0.08, 0.62**	**0.05**
DEN	**0.28**	**-0.01, 0.57**	**0.07**	**-0.66**	**-1.37, 0.05**	**0.09**	0.09	-0.18, 0.36	0.55
Delta2									
FAP	-32.2	-130, 65.8	0.52	24.7	-177, 227	0.81	12.9	-119, 144	0.85
DEP	20.3	-52.8, 93.4	0.59	23.4	-91.3, 138	0.69	13.7	-88.0, 115	0.80
DEN	-7.27	-80.4, 65.8	0.84	59.6	-55.1, 174	0.32	-40.3	-142, 61.6	0.47
Delta6									
FAP	-29.2	-304, 245	0.84	16.6	-64.3, 97.6	0.69	-50.3	-174, 73.2	0.46
DEP	3.88	-81.8, 89.5	0.93	-0.45	-64.3, 63.4	0.98	56.1	-36.8, 149	0.28
DEN	12.4	-73.3, 98.1	0.78	1.09	-62.8, 64.9	0.97	39.1	-53.8, 132	0.44

		Age 1 (N=13)			Age2 (N=7)			Age3 (N=3)		
		β (log pg/ml)	Confidence Interval	p-value	β (log pg/ml)	Confidence Interval	p-value	β (log pg/ml)	Confidence Interval	p-value
Delta 30										
	FAP	-45.1	132, 41.9	0.62	61.9	-87.5, 211	0.43	-58.4	-151, 34.1	0.26
	DEP	42.3	-31.8, 116	0.27	-75.1	-153, 2.91	0.08	76.4	3.68, 149	0.09
	DEN	22.1	-51.9, 96.2	0.56	-51.5	-130, 26.5	0.21	44.1	-29.6, 117	0.28

Exposure was significant and borderline significant for Age1, 2 and 3subjects 30hours after DEP and DEN relative to FAP exposures. This exposure effect was also seen delta 30 hours after exposure for Age 2 and 3 subjects. For Age2 subjects, delta levels are lower after DEN and DEP exposures than they were at baseline; while for Age 3 subjects, levels are higher after exposure than they were at baseline. Effect modification by age was investigated for log 8-isoprostane levels 30 hours after exposure. Age 1 subjects have mostly negative delta levels compared to Age 2 and 3 subjects which are mostly positive deltas. Age1 and 3 subjects show an increase in delta levels after DEP followed by a decrease after DEN exposure. Figures 41 and 43 in Appendix C show the results from Table 20 above. Difference between the three groups was investigated in Tables 45 and 46 of the Appendix. A significant difference was found for log 8-isoprostane levels (p=0.002) at baseline as well as for delta 2 levels (p=0.06).

Table 21: Interaction between exposure and age for EBC log 8-isoprostane 30 hours after exposure

	Value (β) (log pg/ml)	Confidence Interval	p-value	ANOVA p-value
Intercept	3.19	2.03, 4.35	<0.0001	<0.0001
Age2	0.93	-0.27, 2.13	0.13	
Age3	**-1.50**	**-3.16, 0.16**	**0.08**	0.45
DEP	0.54	-0.46, 1.55	0.28	
DEN	0.49	-0.51, 1.50	0.33	0.70
Age2DEP	**-1.62**	**-3.32, 0.08**	**0.06**	
Age3DEP	1.75	-0.57, 4.07	0.14	
Age2DEN	**-2.11**	**-3.81, -0.42**	**0.02**	
Age3DEN	1.15	-1.17, 3.47	0.32	**0.03**

Age significantly interacted with exposure 30 hours after exposure on EBC log 8-isoprostane. It is not clear whether Age 2 or Age 3 are increasing or decreasing log 8-isoprostane levels, as such paired t-tests were performed to better understand the relationship between these variables (Figure 13).

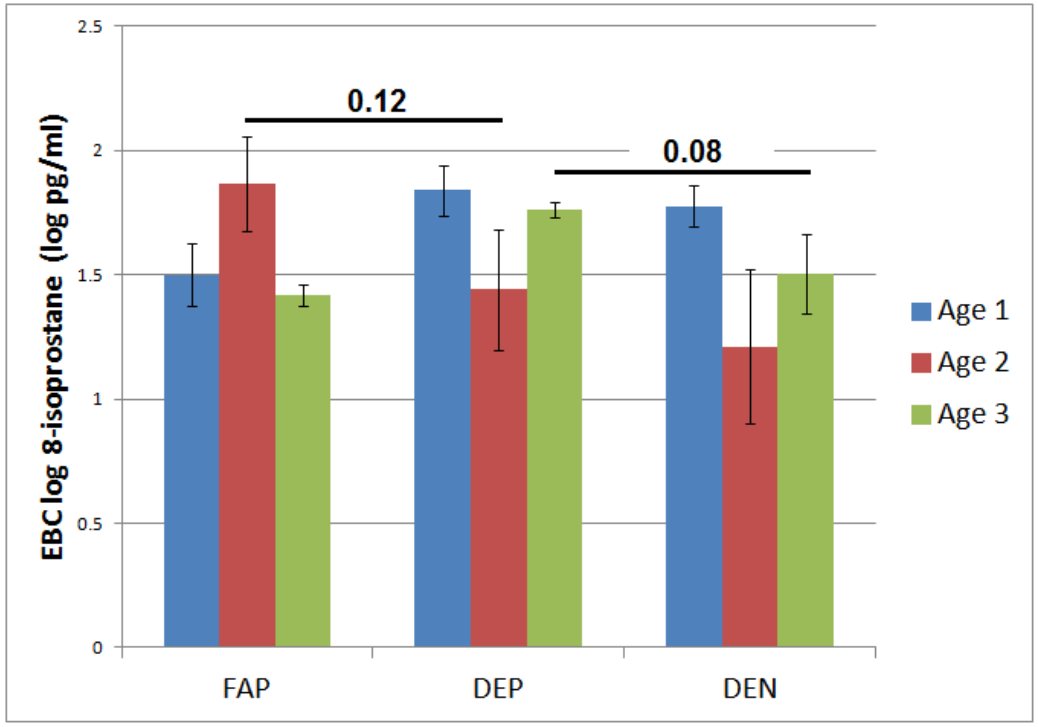

Figure 13. EBC log 8-isoprostane for three exposures stratified by Age group, lowest p-values shown.

Age 3 borderline significantly decreased from DEP to DEN, otherwise differences between exposures stratified by age was not significantly different. Age1 and Ag2 showed an increase in log 8-isoprostane levels from FAP to DEP with a subsequent decrease in levels from DEP to DEN. Age 3 showed a decrease in levels across all exposures.

Table 22: Exposure effect on EBC pH and delta pH stratified by age

	Age1 (N=10)			Age2 (N=5)			Age3 (N=2)		
	β (pH units)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Baseline									
FAP	7.86	7.61, 8.11	<.0001	7.98	7.71, 8.25	<.0001	6.96	5.49, 8.43	0.003
DEP	-0.08	-0.26, 0.09	0.39	-0.05	-0.25, 0.15	0.68	0.04	-1.02, 1.09	0.94
DEN	0.06	-0.12, 0.24	0.53	-0.16	-0.36, 0.04	0.13	0.51	-0.55, 1.57	0.41
2 hours after exposure									
FAP	7.87	7.73, 8.01	<.0001	7.87	7.75, 7.99	<.0001	7.87	7.18, 8.56	0.0002
DEP	-0.007	-0.11, 0.09	0.89	-0.02	-0.12, 0.08	0.69	-0.04	-0.57, 0.49	0.88
DEN	0.002	-0.10, 0.10	0.97	-0.02	-0.12, 0.08	0.64	0.05	-0.48, 0.58	0.85
6 hours after exposure									
FAP	7.91	7.79, 8.03	<.0001	7.94	7.78, 8.09	<.0001	7.78	7.43, 8.13	<.0001
DEP	-0.04	-0.12, 0.04	0.32	-0.08	-0.18, 0.02	0.11	0.05	-0.22, 0.32	0.73
DEN	-0.06	-0.14, 0.02	0.11	**-0.12**	**-0.22, -0.02**	**0.03**	0.11	-0.16, 0.38	0.49
30 hours after exposure									
FAP	7.85	7.73, 7.97	<.0001	7.81	7.71, 7.91	<.0001	6.95	5.34, 8.56	0.003
DEP	0.04	-0.06, 0.14	0.41	-0.01	-0.07, 0.05	0.79	0.89	-0.33, 2.11	0.25
DEN	-0.009	-0.11, 0.09	0.86	0.03	-0.03, 0.09	0.46	1.02	-19, 2.24	0.20
Delta2									
FAP	0.03	-0.22, 0.28	0.83	-0.11	-0.36, 0.15	0.45	0.73	-0.59, 2.04	0.35
DEP	0.08	-0.12, 0.28	0.45	0.02	-0.19, 0.24	0.83	-0.08	-1.04, 0.88	0.87
DEN	-0.06	-0.27, 0.14	0.58	0.14	-0.08, 0.36	0.21	-0.46	-1.42, 0.50	0.42
Delta6									
FAP	0.06	-0.12, 0.24	0.49	-0.07	-0.34, 0.20	0.63	0.86	-0.26, 1.98	0.22
DEP	0.04	-0.16, 0.24	0.69	-0.01	-0.23, 0.21	0.91	0.01	-0.78, 0.79	0.98
DEN	-0.13	-0.33, 0.07	0.22	0.07	-0.15, 0.29	0.52	-0.41	-1.19, 0.37	0.39
Delta 30									
FAP	-0.01	-0.40, 0.38	0.94	-0.17	-0.44, 0.10	0.24	-0.34	-1.98, 1.31	0.71
DEP	0.12	-0.06, 0.29	0.21	0.03	-0.19, 0.25	0.77	0.85	-0.38, 2.08	0.27
DEN	-0.05	-0.23, 0.13	0.63	0.19	-0.03, 0.41	0.10	0.50	-0.73, 1.73	0.49

For EBC pH, exposure had a significant effect 6 hours after exposure for Age 2 subjects with an increase in pH after DEN exposure relative to FAP. Otherwise, exposure does not seem to have a clear influence on EBC pH or delta pH for any age. There does not seem to be an age effect in this population. For Age 1 subjects there is an increase in pH after DEP exposure, while Age 2 subjects show an acidification and Age 3 subjects show mixed results. Most age groups show less to no change after DEN exposure.

Figures 42 and 44 in Appendix C, show the results from Table 22 above.

Tables 45 and 46 in Appendix C investigate the difference between these three groups for pH and delta pH. Delta 2 showed significant difference between age groups ($p=0.005$).

7. Secondary analysis: effect modification by body mass index

This section investigates the potential effect modification of BMI on exposure interactions with biomarker levels.

Table 23: Exposure effect on EBC log and delta8-isoprostane stratified by BMI

	BMI1 (N=17)			BMI2 (N=4)			BMI3 (N=2)		
	β (log pg/ml)	CIs‡	p-value	β (log pg/ml)	CIs‡	p-value	β (log pg/ml)	CIs‡	p-value
Baseline									
FAP	1.68	1.25, 2.11	<.0001	1.03	0.19, 1.87	0.04	7.98	2.08, 13.9	0.08
DEP	0.13	-0.22, 0.48	0.46	-0.08	-0.59, 0.43	0.77	-0.008	-0.20, 0.19	0.94
DEN	-0.04	-0.39, 0.31	0.80	0.17	-0.34, 0.68	0.54	0.03	-0.17, 0.23	0.80
2 hours after exposure									
FAP	1.66	1.29, 2.03	<.0001	1.11	0.35, 1.87	0.02	2.20	1.63, 2.77	0.005
DEP	0.16	-0.13, 0.45	0.30	0.20	-0.27, 0.67	0.44	**-0.62**	**-1.01, 0.23**	**0.06**
DEN	-0.06	-0.35, 0.23	0.70	0.17	-0.30, 0.64	0.51	**-0.66**	**-1.05, 0.27**	**0.05**
6 hours after exposure									
FAP	1.60	1.25, 1.95	<.0001	0.96	-0.04, 1.96	0.09	**15.8**	**11.3, 20.3**	**0.006**
DEP	0.06	-0.21, 0.33	0.64	0.15	-0.46, 0.76	0.64	**0.23**	**0.09, 0.37**	**0.05**
DEN	0.07	-0.20, 0.34	0.60	0.40	-0.21, 1.00	0.23	-0.06	-0.19, 0.08	0.49
30 hours after exposure									
FAP	1.65	1.19, 2.10	<.0001	0.98	0.16, 1.80	0.04	1.59	1.00, 2.18	0.01
DEP	0.06	-0.31, 0.43	0.74	0.30	-0.21, 0.81	0.27	0.09	-0.36, 0.54	0.72
DEN	-0.08	-0.45, 0.29	0.65	0.28	-0.23, 0.79	0.31	-0.19	-0.64, 0.26	0.47
Delta2									
FAP	-30.6	-428, 367	0.88	-9.48	-42.6, 23.6	0.59	-247	-1110, 615	0.61
DEP	34.3	-40.9, 110	0.38	32.2	5.94, 58.5	0.04	**-120**	**-162, -77.7**	**0.01**
DEN	26.1	-49.2, 101	0.50	0.34	-25.9, 26.6	0.98	**-121**	**-163, -78.9**	**0.01**
Delta6									
FAP	-25.1	-118, 67.4	0.59	10.6	-80.9, 102	0.82	-17.5	-80.8, 45.8	0.63
DEP	1.37	-68.0, 70.8	0.97	30.4	-39.9, 101	0.42	35.4	-12.0, 82.8	0.24
DEN	12.9	-56.5, 82.3	0.72	17.9	-52.5, 88.3	0.63	2.84	-44.6, 50.3	0.81

		BMI1 (N=17)			BMI2 (N=4)			BMI1 (N=17)		
		β (log pg/ml)	CIs‡	p-value	β (log pg/ml)	CIs‡	p-value	β (log pg/ml)	CIs‡	p-value
Delta 30										
	FAP	-13.6	-92.4, 65.2	0.74	-20.3	-60.1, 19.5	0.34	-239	-1200, 723	0.66
	DEP	5.28	-63.3, 73.9	0.88	**35.7**	**5.52, 65.9**	**0.05**	10.4	-59.2, 80.0	0.79
	DEN	1.22	-67.4, 69.8	0.97	18.6	-11.6, 48.8	0.26	-17.2	-86.8, 52.4	0.65

‡CIs=95%confidence intervals.

Exposure has a borderline significant effect on BMI3 subjects 2 and 6 hours after DEN and DEP exposures for log as well as for delta2 8-isoprostane. Trends for log 8-isoprostane decrease compared to FAP after DEP and DEN exposures 2 hours after exposure while levels increase from FAP after DEP 6 hours after exposure and lower after DEN exposure.

Exposure also seems to have an effect on BMI2 subjects at delta 2 and 30 where delta 8-isoprostane significantly increases after DEP exposure at both time points. It is unclear if there is an effect modification by BMI, significant differences in BMI3 may be an artifact of N=2.

Delta 8-isoprostane levels went from largely negative to less negative from FAP to DEP for BMI1 subjects. BMI2 subjects also showed increases in 8-isoprostane after DEN exposure with lessened or no change after DEN exposure. BMI3 subjects showed the largest variations in delta levels, all negative, but still showed an increase after DEP exposure compared to FAP (i.e. large negative value to less negative value).

Figures 45 and 47 in Appendix C show the results from Table 23 above. Differences between all three groups for 8-isoprostane levels were investigated in Tables 47 and 48 in Appendix C. None were significant.

Table 24: Exposure effect on EBC pH and delta pH stratified by BMI

	BMI1 (N=15)			BM2 and 3 (N=2)		
	β (pH units)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Baseline						
FAP	7.82	7.51, 8.14	<.0001	8.66	7.15, 10.2	0.002
DEP	-0.10	-0.34, 0.14	0.38	0.17	-0.006, 0.35	0.17
DEN	0.05	-0.17, 0.27	0.65	0.006	-0.17, 0.18	0.95
2 hours after exposure						
FAP	7.87	7.75, 7.99	<.0001	8.62	7.17, 10.1	0.007
DEP	-0.06	-0.14, 0.02	0.13	0.28	0.10, 0.46	0.11
DEN	-0.02	-0.09, 0.06	0.60	0.22	0.02, 0.42	0.16
6 hours after exposure						
FAP	7.90	7.82, 7.98	<.0001	7.88	7.55, 8.21	<.0001
DEP	-0.05	-0.11, 0.009	0.11	0.02	-0.22, 0.26	0.87
DEN	**-0.06**	**-0.12, 0.001**	**0.09**	-0.08	-0.32, 0.16	0.56
30 hours after exposure						
FAP	7.73	7.42, 8.03	<.0001	8.35	7.41, 9.29	0.0004
DEP	0.14	-0.04, 0.32	0.18	0.04	-0.08, 0.16	0.49
DEN	0.11	-0.09, 0.31	0.27	**0.20**	**-0.08, 0.32**	**0.04**
Delta2						
FAP	0.05	-0.22, 0.32	0.71	-0.02	-0.14, 0.10	0.83
DEP	0.04	-0.18, 0.26	0.73	0.10	0.002, 0.20	0.16
DEN	-0.08	-0.30, 0.14	0.48	**0.20**	**0.12, 0.28**	**0.05**
Delta6						
FAP	0.08	-0.15, 0.32	0.54	-1.09	-2.80, 0.62	0.30
DEP	0.04	-0.18, 0.26	0.70	-0.15	-0.35, 0.05	0.24
DEN	-0.11	-0.33, 0.11	0.31	-0.08	-0.28, 0.12	0.45
Delta 30						
FAP	-0.09	-0.36, 0.18	0.51	-0.02	-0.22, 0.18	0.67
DEP	0.24	0.02, 0.46	0.03	-0.12	-0.26, 0.02	0.20
DEN	0.09	-0.13, 0.31	0.44	**0.19**	**0.05, 0.33**	**0.08**

Exposure has a borderline significant effect on BMI1 subjects 6 hours after exposure (significant acidification after DEN exposure) and on BMI 2/3 subjects 30 hours after exposure (significant pH increase after DEN exposure). BMI 2/3 subjects have consistently higher pH than BMI1 subjects. Furthermore, BMI 2/3 subjects show acidification after FAP exposure at 6 and 30 delta time points. BMI1 subjects show an increase in pH after DEP exposure when compared to FAP, with less or no change after DEN exposure.

Figures 46 and 48 in Appendix C show the results from Table 24 above.

Differences between the three groups in pH levels was investigated in Tables 47 and 48 in Appendix C, a significant difference was found 2 hours after exposure ($p=0.05$).

8. Secondary analysis: effect modification by order

This section investigates the potential effect modification of exposure order on exposure interactions with biomarker levels.

Table 25: Exposure effect on EBC log and delta8-isoprostane stratified by order

	Order1 (N=23)			Order2 (N=23)			Order3 (N=23)		
	β (log pg/ml)	CIs‡	p-value	β (log pg/ml)	CIs‡	p-value	β (log pg/ml)	CIs‡	p-value
Baseline									
FAP	3.17	1.39, 4.95	0.002	3.79	0.50, 7.08	0.04	2.75	1.10, 4.40	0.004
DEP	1.02	-0.55, 2.59	0.22	-0.13	-1.44, 1.18	0.85	1.03	-0.24, 2.30	0.13
DEN	0.04	-1.53, 1.61	0.96	-0.28	-1.59, 1.03	0.68	**1.46**	**-0.19, 2.73**	**0.04**
2 hours after exposure									
FAP	3.82	1.39, 6.25	0.006	2.18	0.24, 4.12	0.04	3.34	-0.35, 7.03	0.09
DEP	-0.54	-2.56, 1.48	0.61	0.33	-0.96, 1.63	0.62	0.16	-1.43, 1.75	0.85
DEN	-0.98	-3.00, 1.04	0.35	0.97	-0.44, 2.38	0.19	0.17	-1.42, 1.76	0.83
6 hours after exposure									
FAP	3.45	0.96, 5.94	0.01	3.42	1.59, 5.24	0.002	3.47	1.68, 5.25	0.001
DEP	0.24	-1.15, 1.63	0.73	-0.004	-1.36, 1.35	0.99	-0.12	-1.35, 1.11	0.84
DEN	-0.88	-2.27, 0.51	0.23	-0.37	-1.72, 0.98	0.59	0.84	-0.39, 2.07	0.19
30 hours after exposure									
FAP	3.71	2.04, 5.38	.0003	2.00	0.16, 3.84	0.05	3.62	1.80, 5.44	.0009
DEP	0.15	-1.28, 1.58	0.84	0.79	-0.43, 2.01	0.23	-0.09	-1.58, 1.40	0.89
DEN	-0.68	-2.11, 0.75	0.36	0.82	-0.52, 2.15	0.25	0.28	-1.21, 1.77	0.71
Delta2									
FAP	37.9	-121, 196	0.64	-43.9	-183, 95.1	0.54	29.3	-102, 161	0.66
DEP	7.19	-109, 123	0.90	57.5	-37.2, 152	0.25	-58.3	-160, 43.4	0.27
DEN	31.5	-84.3, 147	0.60	20.5	-74.4, 115	0.68	-56.6	-158, 45.1	0.29
Delta6									
FAP	12.2	-75.6, 100	0.79	-37.4	-166, 91.2	0.57	8.80	-228, 246	0.94
DEP	-8.24	-76.5, 60.0	0.82	42.2	-60.3, 145	0.43	-39.3	-167, 88.5	0.55
DEN	17.9	-50.3, 86.1	0.61	6.71	-95.8, 109	0.90	3.75	-124, 132	0.95

		Order1 (N=23)			Order2 (N=23)			Order3 (N=23)		
		β (log pg/ml)	CIs‡	p-value	β (log pg/ml)	CIs‡	p-value	β (log pg/ml)	CIs‡	p-value
Delta 30										
	FAP	-15.2	-94.9, 64.6	0.71	-39.3	-163, 83.9	0.54	61.6	-72.7, 196	0.38
	DEP	39.9	-26.5, 106	0.25	40.8	-56.0, 138	0.42	-91.7	-205, 21.4	0.13
	DEN	9.00	-57.4, 75.4	0.79	23.2	-74.0, 120	0.65	-74.5	-188, 38.6	0.21

Exposure significantly affected Order 3 at baseline, DEN significantly increased log 8-isoprostane levels. There is no clear pattern for order effect on log and delta 8-isoprostane levels.

Table 26: Exposure effect on EBC pH and delta pH stratified by order

		Order1 (N=23)			Order2 (N=23)			Order3 (N=23)		
		β (pH units)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Baseline										
	FAP	7.47	6.35, 8.59	<.0001	7.61	7.28, 7.94	<.0001	7.91	7.77, 8.05	<.0001
	DEP	0.14	-0.76, 1.04	0.76	0.10	-0.17, 0.37	0.47	-0.03	-0.19, 0.13	0.75
	DEN	0.45	-0.57, 1.47	0.40	0.23	-0.10, 0.56	0.19	-0.08	-0.22, 0.06	0.25
2 hours after exposure										
	FAP	7.89	7.65, 8.13	<.0001	7.82	7.72, 7.92	<.0001	7.89	7.71, 8.07	<.0001
	DEP	-0.08	-0.26, 0.10	0.42	0.04	-0.06, 0.14	0.50	-0.02	-0.18, 0.14	0.78
	DEN	-0.02	-0.22, 0.18	0.85	0.09	-0.03, 0.21	0.15	-0.06	-0.20, 0.08	0.40

	Order1 (N=23)			Order2 (N=23)			Order3 (N=23)		
	β (pH units)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
6 hours after exposure									
FAP	7.93	7.81, 8.05	<.0001	7.82	7.72, 7.92	<.0001	7.93	7.75, 8.11	<.0001
DEP	**-0.10**	**-0.20, -0.002**	**0.05**	0.01	-0.07, 0.09	0.73	-0.008	-0.17, 0.15	0.92
DEN	**-0.12**	**-0.22, -0.02**	**0.04**	0.03	-0.07, 0.13	0.58	-0.07	-0.21, 0.07	0.34
30 hours after exposure									
FAP	7.85	7.77, 7.93	<.0001	7.38	6.73, 8.03	<.0001	7.80	7.64, 7.96	<.0001
DEP	-0.03	-0.09, 0.03	0.38	0.25	-0.18, 0.68	0.27	**0.15**	**0.01, 0.29**	**0.05**
DEN	0.02	-0.08, 0.12	0.73	0.45	-0.06, 0.96	0.10	0.08	-0.04, 0.20	0.22
Delta2									
FAP	0.01	-0.62, 0.64	0.97	0.24	-0.23, 0.71	0.33	-0.02	-0.18, 0.14	0.85
DEP	0.19	-0.28, 0.66	0.43	-0.06	-0.37, 0.25	0.69	0.004	-0.15, 0.16	0.96
DEN	-0.05	-0.56, 0.46	0.84	-0.15	-0.52, 0.22	0.43	0.02	-0.12, 0.16	0.72
Delta6									
FAP	0.46	-0.72, 1.64	0.45	0.22	-0.15, 0.59	0.29	0.03	-0.19, 0.25	0.79
DEP	-0.24	-1.10, 0.62	0.59	-0.09	-0.34, 0.16	0.53	0.02	-0.16, 0.20	0.82
DEN	-0.57	-1.55, 0.41	0.27	-0.21	-0.52, 0.10	0.21	0.02	-0.14, 0.18	0.83
Delta 30									
FAP	0.38	-0.97, 1.73	0.59	-0.22	-0.51, 0.07	0.17	-0.12	-0.41, 0.17	0.45
DEP	-0.17	-1.13, 0.79	0.73	0.15	-0.03, 0.33	0.15	0.17	-0.05, 0.39	0.14
DEN	-0.35	-1.74, 1.04	0.63	0.22	-0.02, 0.46	0.09	0.15	-0.03, 0.33	0.13

Exposure had a significant effect on EBC pH levels 6 hours after exposure for Order1. DEP borderline significantly increased pH 30 hours after exposure for Order 3. DEN significantly increased delta pH 30 hours after exposure for Order 2. The trend for Order1 6 hours after exposure was stronger than the others and so interaction was investigated.

Table 27: Interaction between exposure and order for EBC pH 6 hours after exposure

	Value (β) (pH units)	Confidence Interval	p-value	ANOVA p-value
Intercept	7.82	7.55, 8.09	<.0001	<.0001
Order2	-0.27	-0.54, 0.004	0.06	
Order3	-0.06	-0.37, 0.25	0.71	0.72
DEP	-0.02	-0.29, 0.25	0.89	
DEN	0.01	-0.38, 0.40	0.95	0.26
Order2DEP	0.30	-0.09, 0.69	0.14	
Order 3DEP	0.15	-0.30, 0.60	0.52	
Order 2DEN	0.34	-0.17, 0.85	0.19	
Order 3DEN	0.03	-0.46, 0.52	0.89	0.44

There is no significant interaction between exposure and order.

4.2.1.7 Multi-covariate analyses

The following sections depict multi-covariate models for biomarker levels. For this section, all covariates that we initially hypothesized as potential effect modifiers were included regardless of the results in the single covariate models. The reasons for this were outlined in section 2.11.2 of the literature review.

Table 28: Multivariate analyses for EBC biomarkers 0 and 2 hours after exposure.

	8-isoprostane			pH		
	β (log pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Baseline						
Intercept	3.16	0.53, 5.79	0.005	7.48	6.79, 8.17	<0.0001
Exposure2	0.53	-0.22, 1.27		0.10	-0.23, 0.43	
Exposure3	0.36	-0.37, 1.09	0.34	0.17	-0.16, 0.50	0.45
Sex	0.27	-0.44, 0.98	0.12	-0.15	-0.52, 0.22	0.65
MRS	0.57	-0.25, 1.39	0.75	-0.20	-0.63, 0.23	0.78
Atopy	0.48	-0.28, 1.24	0.14	0.24	-0.37, 0.85	0.13
GSTP1	-0.41	-1.14, 0.32	0.35	0.14	-0.17, 0.45	0.50
Order 2	0.17	-0.56, 0.89		0.15	-0.18, 0.48	
Order 3	0.15	-0.58, 0.87	0.89	0.19	-0.14, 0.52	0.49
Age 2	**-1.18**	**-1.87, -0.49**		0.10	-0.25, 0.45	
Age 3	**-1.14**	**-2.36, 0.08**	**0.005**	-0.69	-1.38, -0.004	0.22
BMI 2	0.18	-0.49, 0.85		-0.27	-1.03, 0.49	
BMI 3	0.65	-0.48, 1.77	0.52	0.68	-0.30, 1.66	0.26
2 hours after exposure						
Intercept	3.22	1.57, 4.87	<0.0001	7.87	7.71, 8.03	<0.0001
Exposure2	-0.05	-0.95, 0.85		-0.02	-0.09, 0.06	
Exposure3	-0.15	-1.03, 0.73	0.95	-0.003	-0.08, 0.08	0.88
Sex	**0.89**	**0.03, 1.75**	**0.07**	**0.11**	**0.01, 0.21**	**0.01**
MRS	0.74	-0.26, 1.74	0.62	**-0.06**	**-0.18, 0.06**	**0.08**
Atopy	0.29	-0.65, 1.23	0.22	**0.03**	**-0.11, 17**	**0.02**
GSTP1	-0.99	-1.87, -0.11	0.20	-0.03	-0.11, 0.05	0.63
Order 2	0.06	-0.82, 0.94		-0.007	-0.09, 0.07	
Order 3	0.14	-0.76, 1.04	0.95	-0.02	-0.10, 0.06	0.94
Age 2	-0.36	-1.20, 0.48		0.05	-0.03, 0.13	
Age 3	-1.86	-3.31, -0.41	0.28	-0.05	-0.21, 0.11	0.66
BMI 2	**0.11**	**-0.71, 0.93**		0.16	-0.06, 0.38	
BMI 3	**1.86**	**0.53, 3.19**	**0.02**	0.12	-0.10, 0.34	0.27

For baseline multi-covariate models, age was the only significant modifier of log 8-isoprostane levels; while no covariates modified pH levels.

For the 2 hours after exposure model, log 8-isoprostane was modified by sex and BMI; while pH was modified by sex, MRS and atopy.

Due to significant and borderline significant results for log 8-isoprostane and pH 0 and 2 hours after exposure, potential effect modification was investigated for the bolded rows. These results are shown in Appendix C, Tables 50, 51 and 52.

Table 29: Multivariate analyses for EBC biomarkers 6 and 30 hours after exposure

	8-isoprostane			pH		
	β (log pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
6 hours after exposure						
Intercept	3.87	1.22, 6.52	0.006	7.90	7.70, 8.10	<0.0001
Exposure2	-0.08	-0.86, 0.70		-0.04	-0.10, 0.02	
Exposure3	-0.11	-0.89, 0.67	0.99	-0.07	-0.13, -0.01	0.18
Sex	-0.38	-1.14, 0.38	0.41	0.02	-0.06, 0.09	0.54
MRS	**-0.49**	**-1.37, 0.39**	**0.09**	-0.09	-0.19, 0.008	0.20
Atopy	0.16	-0.66, 0.98	0.53	0.07	-0.05, 0.19	0.25
GSTP1	-0.31	-1.07, 0.45	0.71	-0.005	-0.06, 0.05	0.59
Order 2	-0.26	-1.04, 0.52		-0.03	-0.09, 0.03	
Order 3	0.20	-0.58, 0.98	0.51	0.02	-0.04, 0.08	0.32
Age 2	-0.13	-0.87, 0.62		0.03	-0.03, 0.09	
Age 3	-0.006	-1.28, 1.04	0.81	-0.04	-0.18, 0.10	0.27
BMI 2	0.31	-0.41, 1.04		-0.03	-0.19, 0.13	
BMI 3	0.71	-0.47, 1.89	0.46	-0.03	-0.23, 0.17	0.88
30 hours after exposure						
Intercept	3.37	1.92, 4.82	<0.0001	7.83	7.40, 8.26	<0.0001
Exposure2	0.24	-0.58, 1.06		0.13	-0.03, 0.29	
Exposure3	-0.03	-0.85, 0.79	0.74	0.14	-0.04, 0.62	0.23
Sex	0.11	-0.69, 0.91	0.44	0.01	-0.19, 0.21	0.19
MRS	-0.30	-1.22, 0.62	0.11	-0.08	-0.30, 0.14	0.87
Atopy	0.37	-0.49, 1.23	0.29	0.05	-0.26, 0.36	0.12
GSTP1	-0.21	-1.01, 0.59	0.87	-0.006	-0.18, 0.17	0.80
Order 2	-0.14	-0.94, 0.66		-0.08	-0.26, 0.09	
Order 3	0.13	-0.69, 0.95	0.81	-0.04	-0.22, 0.14	0.65
Age 2	-0.27	-1.03, 0.49		-0.06	-0.24, 0.12	
Age 3	-0.50	-1.85, 0.85	0.75	-0.51	-0.84, 0.18	0.13
BMI 2	0.24	-0.51, 0.98		-0.10	-0.49, 0.29	
BMI 3	0.55	-0.69, 1.79	0.66	0.47	-0.45, 1.39	0.13

Sex, methacholine response status and atopy seemed to have a significant effect on pH 2 hours after exposure.

Due to significant and borderline significant results for log 8-isoprostane and pH 6 and 30 hours after exposure, potential effect modification was investigated for the rows highlighted in green. These results are shown in Appendix C, Tables 53.

Table 30: Multivariate analyses for EBC delta biomarkers

	Delta 8-isoprostane			Delta pH		
	β (pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Delta 2						
Intercept	-7.10	-115, 101	0.91	-0.01	-0.34, 0.32	0.55
Exposure2	10.5	-47.3, 68.3		0.03	-0.15, 0.21	
Exposure3	2.42	-55.2, 60.0	0.95	-0.04	-22, 0.14	0.58
Sex	5.03	-53.4, 63.4	0.85	0.08	-0.09, 0.26	0.91
MR	-65.9	-133, 1.52	0.17	0.03	-0.19, 0.25	0.20
Atopy	37.6	-23.4, 98.6	0.30	-0.02	-0.33, 0.29	0.11
GSTP1	24.7	-31.9, 81.3	0.18	0.01	-0.15, 0.17	0.69
Order 2	-39.8	-96.8, 17.2		-0.008	-0.18, 0.17	
Order 3	-36.3	-93.9, 21.3	0.33	-0.07	-0.25, 0.11	0.71
Age 2	**61.1**	**7.00, 115**		**-0.04**	**-0.22, 0.14**	
Age 3	**63.3**	**-34.7, 161**	**0.04**	**0.66**	**0.31, 1.01**	**0.02**
BMI 2	20.9	-35.7, 77.5		**0.22**	**-0.21, 0.65**	
BMI 3	36.2	-59.1, 132	0.70	**-0.62**	**-1.11, -0.13**	**0.02**
Delta 6						
Intercept	-7.68	-400, 384	0.97	0.44	-0.23, 1.11	0.62
Exposure2	-1.87	-56.4, 52.6		-0.12	-0.45, 0.21	
Exposure3	0.90	-53.6, 55.4	0.99	-0.21	-0.56, 0.14	0.24
Sex	-46.1	-98.8, 6.62	0.29	0.19	-0.18, 0.56	0.65
MR	**-79.9**	**-141, -18.9**	**0.04**	0.17	-0.28, 0.62	0.96
Atopy	31.2	-25.8, 88.2	0.32	-0.27	-0.92, 0.38	0.20
GSTP1	11.4	-42.1, 64.9	0.64	-0.17	-0.50, 0.16	0.42
Order 2	-24.6	-78.5, 29.3		-0.19	-0.50, 0.12	
Order 3	-7.04	-61.5, 47.4	0.66	-0.21	-0.54, 0.12	0.43
Age 2	**39.4**	**-11.8, 90.6**		-0.09	-0.42, 0.24	
Age 3	**96.6**	**7.62, 186**	**0.05**	0.65	-0.04, 1.34	0.28
BMI 2	11.5	-38.3, 61.3		0.31	-0.47, 1.09	
BMI 3	-1.93	-83.5, 79.6	0.87	-0.66	-1.64, 0.32	0.24

	Delta 8-isoprostane			Delta pH		
	β (pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Delta 30						
Intercept	-31.3	-131, 68.7	0.83	0.41	-0.29, 1.12	0.71
Exposure2	2.93	-50.0, 55.9		0.03	-0.30, 0.36	
Exposure3	-5.30	-58.2, 47.6	0.95	0.03	-0.34, 0.40	0.89
Sex	-10.4	-61.6, 40.8	0.99	0.19	-0.22, 0.60	0.24
MR	-59.2	-118, -0.20	0.21	0.15	-0.30, 0.60	0.69
Atopy	13.5	-41.8, 68.8	0.95	-0.23	-0.88, 0.42	0.44
GSTP1	45.9	-5.84, 97.6	0.11	-0.18	-0.53, 0.17	0.34
Order 2	-18.5	-70.6, 33.6		-0.29	-0.64, 0.06	
Order 3	-2.89	-55.8, 50.0	0.76	-0.30	-0.67, 0.07	0.23
Age 2	47.8	-1.79, 97.4		-0.17	-0.54, 0.20	
Age 3	73.9	-12.3, 160	0.10	0.17	-0.55, 0.89	0.43
BMI 2	-4.59	-52.8, 43.6		0.20	-0.63, 1.02	
BMI 3	-37.6	-117, 41.4	0.63	-0.21	-1.22, 0.81	0.79

Due to significant and borderline significant results for delta biomarker levels, potential effect modification was investigated for the rows highlighted in green. These results are shown in Appendix C, Tables 54, 55 and 56.

CHAPTER 5: Discussion

5.1 Overview

This section will address each goal as well as the limitations associated with this study.
The goals of this study were as follows:

1. To investigate the effects of controlled diesel exhaust exposure on human airways' oxidative stress by analysis of pH and 8-isoprostane in exhaled breath condensate.
2. To characterize the effects of antioxidant exposure on potential DE-associated airway oxidative stress in terms of EBC pH and 8-isoprostane.

5.2 Study hypotheses

5.2.1 Correlation between biomarkers

Both pH and 8-isoprostane are biomarkers of oxidative stress. There is evidence that increasing airway pH causes increased oxidative stress in rats as mentioned in section 2.10.1.3of the literature review. Thus, it would be logical to check if this is reflected in the correlation between our two biomarkers of interest after DEP exposure.

Results from Table 8 show a negative increasing correlation between pH and log 8-isoprostane levels from 0 to 30 hours after DEP exposure. This result would corroborate the idea that DE related acidification of the airways is associated with increased oxidative stress as reflected by an increase in 8-isoprostane levels.

The correlations are however low. At best, only 15% of the variation in log 8-isoprostane is explained by pH 30 hours after DEP exposure. Thus, we can only look at these trends non-conclusively. There is a correlation between biomarkers after DEP exposure; however, this is not significant.

To our knowledge, there is limited evidence regarding the correlation between 8-isoprostane levels and pH. Zhao *et al*. in 2008 identified that pH and 8-isoprostane were not correlated in mild asthmatic individuals (spearman rank correlation=-0.09, P=0.7) (255). Similar to our study,

the correlation coefficient is negative identifying that increasing 8-isoprostane is associated with decreasing pH, although not significantly.

Thus, this study would corroborate the idea that airway acidity may not parallel oxidative stress. However, our study was not explicitly designed to address this question.

5.2.2 Exposure effect

We hypothesized that exposure would have an effect on biomarker levels by increasing EBC 8-isoprostane and decreasing EBC pH levels.

Levels of EBC 8-isoprostane were generally higher after DEP than FAP exposure, although no significant trends were identified. 8-isoprostane levels after DEN exposures were generally associated with lower levels relative to FAP. For delta 8-isoprostane levels, all post exposure levels were lower than at baseline. There were typically smaller changes after DEP than after FAP and DEN. Thus, biomarker levels after DEP exposure increased, but not more than baseline levels.

EBC pH was non-significantly increased 2-, 6-, and 30 hours after exposure (each relative to baseline. Antioxidant supplementation did not seem to have any effect on EBC pH. Trend in delta pH were non-significant. Antioxidant supplementation did decrease the amount of variability around the mean when compared to FAP and DEP results.

McCreanor *et al*. 2007 showed changes in EBC pH 3 and 6 hours after exposure, we did see a minor acidification of EBC pH levels 2 and 6 hours after exposure; however, this effect was reversed in delta pH results. This will be further discussed in section 5.3.4, below. More importantly, McCreanor was able to show significant acidification in 30 moderate asthmatics. This study only had 17 subjects who were either healthy or mildly asthmatic. The power required to show a significant difference was perhaps not obtained for this small of an effect size and study population.

We suggested that looking at post exposure effects as well as delta values was important in the event that baselines were biased as a result from oxidant injury from the outside world. Our post

exposure levels, particularly for FAP, identify that oxidant injury outside the study's control may have contributed to higher baseline levels of oxidative stress. This was shown from the large negative deltas observed after FAP exposure, subjects had lower oxidative stress after FAP exposure than at baseline. It is highly unlikely that this is due to circadian rhythm as our study design controls for within person variability and EBC was collected at the same time of day/time point by default. With regards to oxidative stress, post exposure levels may be a more accurate representation of the actual effects of our DE exposure versus the outside influences.

5.2.3 Sex effect

We hypothesized that since males had generally higher oxidative stress in blood than females, sex would be a significant effect modifier on the relationship between exposure and EBC biomarker levels. Results from single and multi covariate models suggest that sex does modify biomarker levels in EBC. In general, males had higher 8-isoprostane and lower pH when compared to females, particularly 2 and 6 hours after exposure.

There is evidence that males have higher systemic oxidative stress than females. There is also evidence that menstrual cycles, which are highly dependent on the woman, her age, socio-economic status, stress levels, etc may affect biological variation in females (256). Furthermore, although our study controlled for timing of exposures and collection of samples, we did not control for women's menstrual cycles. The results from this thesis may be a reflection of the multiple biological factors involved in male and female biological differences.

Due to the strength of the crossover study design, extra-experimental confounding factors should be equally distributed between the three exposures. To indirectly assess if females were categorically different from males (perhaps due to uncontrolled menstrual cycles and/or contraceptive use) we looked at the overall variation in female results compared to male EBC pH results, particularly 6 hours after exposure. Standard deviations (SDs) within the female EBC pH results are slightly higher than males 6 hours after exposure for all three exposures (Male SDs of 0.08 for FAP, 0.07 for DEP and 0.07 for DEN 6 hours after exposure while females 0.09 for FAP, 0.12 for DEP and 0.08 for DEN). This observed trend is also present in data from 2 and 30 hours after exposure. Due to the strength of the crossover study design, such extra-experimental

confounding factors should be equally distributed between the three exposures and effective bias comparisons toward null differentials.

To our knowledge, there is no evidence in the literature that sex has an effect on EBC pH levels. In 2006, Chest published a paper by Paget-Brown *et al.* that evaluated normative data for EBC pH across 404 healthy subjects (190). They briefly evaluated the pH difference among the sexes and found no significant difference between male and female pH groups (190). Hunt also confirmed that sex had no effect on pH values when his research group compiled all their research data (over 6000 samples) to determine that sex was not a significant determinant of EBC pH (192). Our results thereby contradict these findings.

Our study population was generally young and healthy. For the pH data set the average age was 28.6 yrs (±6yrs) and the average BMI was 24.1 (±2), 14 of our subjects had their BMIs lower than 25. Thus, the difference between our study results and those of Hunt and Paget-Brown may be due to the difference between our study populations. Both these studies had an age range of 0 to over 70 years old whereas ours was limited to 19-40 yrs range. Vancouver's population is generally healthier and more active than the general US population.

5.2.4 Methacholine response status

We hypothesized that a positive methacholine response status would be associated with higher levels of oxidative stress and effect modification on the interaction between exposure and biomarker level.

Results from our univariate and multivariate analyses suggest that MRS plays a role in oxidative stress but that it is not an effect modifier. Univariate analyses would suggest responsive subjects have higher levels of log 8-isoprostane while lower EBC pH when compared to non-responsive subjects. This trend remained for delta 8-isoprostane; however disappeared for EBC delta pH. MRS was not an effect modifier but as hypothesized, being positive for methacholine response did increase oxidative stress as represented by a higher 8-isoprostane level in EBC and more acidic pH after FAP exposures.

Methacholine response was set at 8mg/ml, lower than 8 subjects were classified as responsive and subjects with higher than 8mg/ml methacholine dose were classified as non-responsive. This classification was used in order to differentiate healthy subjects from mild asthmatics. Most of our study population was mildly asthmatic. This study shows that for healthy and mild asthmatics there is no significant difference between the two in terms of EBC biomarker levels.

5.2.5 Atopic effect

We hypothesized that being atopic would be associated with higher oxidative stress than in non-atopic subjects and that atopic status would modify the interaction between exposure and biomarker levels.

Our results confirmed that atopic status was not an effect modifier. However, there were differences between atopic and non-atopic biomarker levels. Atopic subjects showed consistently higher log 8-isoprostane levels when compared to non-atopics. Conversely, non-atopic subjects consistently showed lower pH levels than atopic subjects. However, this latter pH result may be a bi-product of a low sample number (N=3).

Our data suggests an opposite trend with atopic individuals having higher 8-isoprostane levels and concurrently higher pH. Due to the small sample size of non-atopic individuals, direct conclusions concerning the contradictory nature of these results cannot be made.

A study on pediatric asthma and atopic dermatitis (AD) found that pH was significantly lower in children with asthma with AD than in the control group (257). Conversely, another study found no significant difference in pH between the three study groups children with asthma, children with atopy only and normal non-atopic children (258). A study performed on adults found no significant difference between allergic asthmatics and the control group for EBC pH (259). However, a study performed on a group of Croatian adults found lower EBC pH when stricter respiratory health and atopic status criteria were used (260). Thus, further investigation of the influence atopy has on EBC pH is warranted.

Our hypotheses concerning 8-isoprostane and atopy were confirmed through borderline significant results. However, were not consistent for EBC pH.

5.2.6 Genetic GSTP1 variant

We hypothesized that genetic variant status would increase oxidative stress as well as modify the interaction between exposure and biomarker levels.

GSTP1 variant individuals had consistently lower levels of log 8-isoprostane in EBC while exhibited lower pH than wild type individuals. These differences were not significant. Our results showed that GSTP1 status was not an effect modifier.

With regards to the background literature, our 8-isoprostane results would contradict our hypotheses. These results may be due to sample size. Many population based studies require hundreds of participants in order to show genetic effects on respiratory diseases. As such, our sample size was probably not large enough to identify trends in biomarker levels with regards to background or exposure levels.

5.2.7 Age effect

We hypothesized that increasing age would be associated with higher levels of oxidative stress and would be an effect modifier for the interaction between exposure and biomarker levels.

Our results do not suggest increasing age is associated with increasing 8-isoprostane levels or more acidic EBC pH. With regards to EBC 8-isoprostane levels, there is no clear trend that with increasing age there is increasing 8-isoprostane levels. However, this may be an artifact from the systematic error embedded in our 8-isoprostane results from our analysis method discussed in section 5.3.1 of this discussion. That age did not seem a significant modifying factor may be due to the small sample size generated by separating age groups in order to perform these analyses. Indeed, for some analyses, the older age groups only had one or two individuals.

In our literature review section 2.11.2, age was a significant determinant of 8-isoprostane levels while it was not a significant determinant of EBC pH. However, we cannot compare our study to others because of the potential bias from a small sample size (189, 192, 239).

Furthermore, only two subjects were classified as being in the Age3 group and as such results are too dependent on these particular individuals. One of the subjects in the Age3 group has three

results with pH levels below 7.0 which is often associated with acid reflux from eating or drinking anything other than water (137, 149, 192, 196). Another factor is an issue, our study did not control for no eating or drinking one hour before EBC collection as recommended by the ETS/ATS guidelines (149).

Results from analyses of both biomarkers are largely dependent on a small group of subjects and may be overly biased from one individual changing the results. Thus, results cannot be generalized and compared to other studies when only a small number of subjects are available for analysis.

5.2.8 BMI effect

We hypothesized that higher BMIs would be associated with higher oxidative stress as well as that BMI may be an effect modifier for the interaction between exposure and biomarker levels.

Exposure had no significant effect on either BMI levels of 8-isoprostane. BMI3 subjects had larger log 8-isoprostane levels than the other two groups; however BMI2 subjects had lower 8-isoprostane levels than BMI1 subjects. BMI group 2/3 has higher pH than BMI1 subjects which is an opposite finding to what we hypothesized.

These findings may be artifacts of the low number of subjects in BMI groups 2 (N=4) and 3 (N=2) for the 8-isoprostane analyses, for pH analyses, groups 2 (N=1) and 3(N=1) had to be merged in order to assess trends in the data and effect modification. Results from analyses of both biomarkers are largely dependent on a small group of subjects and may be overly biased from one individual changing the results. Thus, results cannot be generalized and compared to other studies when only a small number of subjects are available for analysis.

5.2.9 Order effect

We hypothesized that order would not be a significant determinant of oxidative stress biomarker levels or that it would be related to effect modification because of the counter balanced study design.

Order had no effect on 8-isoprostane levels. However, Order1 significantly affected pH levels 6 hours after exposure; Order 1 was not related to effect modification at this time point. Thus, perhaps our study design made it so EBC 6 hours after exposure were affected. This is highly unlikely. It may be an artifact of not controlling for drink or food intake 1 hr prior to EBC collection since this time point typically occurred around or a little after lunch time.

5.2.10 General discussion

Our study comprised of many statistical analyses. With any study using multiple comparisons, it is important to note that with more statistical tests comes increased chance to find a significant results. Interpretation of the results was not solely based on statistical significance; indeed we also identified trends more so than just statistical significance. Furthermore, if results occurred in both single and multiple covariate models, strength was given to that finding versus one which did not occur in both types of tests. However, studies using this type of data may want to consider using correction methods available for multiple comparisons such as the Bonferroni correction, Tukey-Kramer method, Scheffe method, etc (261).

To our knowledge, there is no evidence in the literature concerning the time dependence of oxidative stress mechanisms involved with DE exposure. Thus, if there is indeed effect modification we might expect to see it at all time points. However, if it is time dependent then we would only see it at certain time points such as in McCreanor *et al.* 2007 for the significant acidification of EBC pH 3 and 6 hours after exposure (5). It is unclear if we were able to capture all changes associated with different exposures as in we may have missed the peak during the exposure, between measure time points, etc. Not much is known about the time dependence of oxidative stress mechanisms on different biomarkers other than pH; furthermore, it is unclear how different covariates affect this time dependence.

5.2.11 Power calculation discussion

Original power calculations for this study was based on a PC_{20} calculation based on a two-sided tests with 0.05 alpha level and 90% power for data variability prior to DE exposure. This calculation for a crossover study identified the requirement for 18 asthmatics and 12 healthy controls.

Power calculations were re-tested using data for pH and 8-isoprostane obtained in the present study at baseline, in order to better understand the sample size required in future studies to assess statistical differences in biomarker changes. The software used was developed by David Schoenfeld (262).

For pH, we assumed a 0.05 two-sided significance level with a 0.90 power. The standard deviation of the difference between FAP and DEP 6 hours after exposures was calculated to be 0.08 while the minimal detectable difference between the means of the two groups was 0.03. The sample size calculated was 77 patients.

For 8-isoprostane, we assumed a 0.05 two-sided significance level with a 0.90 power. The standard deviation of the difference between FAP and DEP 6 hours after exposures was calculated to be 50pg/ml while the minimal detectable difference between the means of the two groups was 9pg/ml. The sample size calculated was 327.

Thus, for both pH and 8-isoprostane our study was under-powered to identify a difference between effect sizes observed. This result demonstrates that future studies should attempt at increasing their sample sizes when utilizing EBC biomarkers, particularly for biomarkers with such large standard deviations.

5.3 EBC 8-isoprostane specific discussion

5.3.1 EBC 8-isoprostane analysis method development

Several methods of 8-isoprostane analysis in EBC were considered, such as GC, LC and EIA. Due to the immense complexity of GC analysis and low reliability in EIA analysis, LC was judge to be the most reliable and reproducible method to analyze over 600 study samples.

When surveying the literature, no EBC 8-isoprostane analysis was directly reproducible in our lab with available instruments. We thereby had to develop our own method of analysing EBC concentrations of 8-isoprostane. What we ended up developing, described in Appendix A, was a highly sensitive and specific to 8-isoprostane, we also delete the sample preparation step whereas EBC could be directly injected into the LC. Our method parameters are detailed in Appendix A.

Briefly, we were able to reach an average linearity of 0.99 with intra- and inter day coefficients of variation lower than 5%, and with an instrumental limit of detection of 1.44pg/ml.

The main limitations to our method were the quantitative limits of detection (LOD) and limit of quantification (LOQ). There are different ways to calculate LODs. Instrumental LODs (iLOD) use three times the standard deviation (SD) of the instrument for the LOD and 10 times the SD for the LOQ. The empirical method of assessing LOD/LOQs relies on a calibration curve's lowest concentration point detectable from background noise (eLOD) and the empirical LOQ being 10x the eLOD (eLOQ) (174). The eLOQs and eLODs thereby change every time the instrument is run because it relies on the conditions of that run to set the lowest limits it can with the background noise. It is then understood that eLODs and eLOQs are less affected by instrumental error and are a more accurate determination of measured concentration. (174).

For our study samples, the levels of 8-isoprostane in EBC samples were often below the eLOQ of each batch (average 152pg/ml); as well as below the eLOD (average 46.4pg/ml) of each batch. Like other studies, we used the iLOD for analysis of our results (i.e. 1.44pg/ml). However, if we had used the more accepted method of setting the LOD by chemists the percentage of values below the eLOD and non detects would increase to 42% (27% below LOD and 15% non detects). The average eLOD and eLOQ for the 10 batches of study samples analysed is listed in Table 47.

Table 31: EBC 8-isoprostane method development parameters

Batch No.	Sample No.	eLOD (pg/ml)	eLOQ (pg/ml)	Recovery (%)	Precision (%)	Linearity (R^2)
1	1-94	23.7	79.1	101	39.5	0.998
10	610-635	59.8	199	101	23.2	0.996
Range	1-635	23.7-102	79.1-341	91.5-105	23.2-52.0	0.968-0.999
Average of 10 batches	All 635	46.4	152	100	30	0.993

As such, it is important to note that values below the eLOD are associated with larger error margins. In our case, approximately 40% of our data was below the eLOD which signifies that the inherent variability in low concentrations makes it difficult to be able to tease out actual

differences between exposures while performing statistical analyses. Thus, although our method was highly sensitive and specific, 40% of our data was associated with higher systematic error.

5.3.2 Normative 8-isoprostane data

Baseline 8-isoprostane data showed that our mean 8-isoprostane concentration was 69.9 pg/ml; while our median concentration was 56.5 pg/ml, and standard deviation was 78.6pg/ml. These concentrations are slightly higher than normal levels for either healthy or asthmatic individuals as reported in section 2.9.3 of the literature review. In fact, the mean baseline results are similar to levels which would be observed in severe steroid native asthmatics. Because our study is comparing trends in biomarker levels, this issue is mainly relevant when comparing absolute levels to other studies.

5.4 EBC pH specific discussion

5.4.1 pH study results compared to normative data

pH results showed our study population to have a median pH value of 7.85 at baseline with a standard deviation of 0.48 pH units. This is within the normal range as reported in section 2.10.1.2 of the literature review. Thus, our results were comparable to levels previously reported.

5.4.2 Differences with McCreanor *et al.* 2007

Our study was not able to show similar effects in EBC pH as was reported by the McCreanor *et al.* 2007 study. Their study was able to show a significant acidification for EBC pH from Hyde Park to Oxford Street exposure in mild and moderate asthmatics.

The difference between their results and ours may be explained by the difference in our study populations. Our study incorporated healthy individuals and mild asthmatics while their study incorporate mild and moderate asthmatics (note: moderate asthmatics showed more acidification than mild asthmatics).

Another reason for the difference between their study and ours is the characteristics of the exposures themselves. Our study limited its exposure to that of only a diesel engine exhaust profile, i.e. we recreated diesel exhaust as found in the environment. Conversely, McCreanor *et al.* 2007 had a wide variety of exposures in their study design. Because of the ambient exposure

type of study, participants were subject to not only diesel exhaust but also to ozone, allergens, gasoline engine exhaust and other types of pollutants. There is strong evidence that concurrent exposures to ozone (72-73) and diesel as well as to diesel and allergens (231-232, 263-269) increase the respiratory health effects experienced by participants.

5.5 EBC methodological issues

Exhaled breath condensate is an easy biomarker to obtain; however, there still exists a lot of controversy around analysis of EBC for biomarkers of interest. We sought to overcome these debates by utilizing methods that were judged more reproducible as well as choosing the most reliable biomarkers in EBC: 8-isoprostane and pH.

5.5.1 EBC collection standardisation

Of general note, collection of EBC during this thesis was not uniform. Most samples prior to September 2010 were not aliquoted; as such, 2ml of condensate were collected in 15ml falcon tubes (1 per time point). This methodology was quickly changed in order to create four aliquots per time point. Thus, samples collected in 15ml aliquots had to be unfrozen, aliquot and re-frozen. Syslova et al. 2008 mentions that the degradation of 8-isoprostane depends on the amount of freeze-thaw cycles undergone (152). Thus, there may have been added error in certain 8-isoprostane results, particularly those which underwent added freeze-thaw cycles. This freeze-thaw dependence is not an issue for EBC pH (252).

5.5.2 Aliquot analysis allocation

In retrospect, the EBC aliquot analysis allocation strategy was flawed. With the smallest amount of variation within the EBC pH data, it would have been prudent to favour aliquots for EBC pH analyses in order to increase the amount of full participant data obtained. In this way, perhaps stronger trends would have been identified in the data.

5.6 Biomarker sensitivity and specificity

The two biomarkers analysed in this study had unique advantages and disadvantages as noted in the following paragraphs.

5.6.1 Biomarker variability

Firstly, overall variation in biomarker level was higher in 8-isoprostane results when compared to pH. This was notably due to the inherent variability within the 8-isoprostane method as compared to the pH method. Coefficients of variation for pH sample aliquots for the same time point at the same exposure condition were 0.1% while almost 40% for 8-isoprostane aliquots. Log transformed 8-isoprostane levels showed variations of 2 log orders between the largest and smallest values in while pH varied by 1.5 log orders.

5.6.2 Comparing to literature

Furthermore, comparing pH results to other studies is relatively straight forward since methods are somewhat constant between studies with regards to pH measurement. This is not the case for 8-isoprostane in EBC. In fact, results can only be compared to studies utilizing the same methods of 8-isoprostane analyses since most absolute 8-isoprostane values are based on the calibration curve used to set concentrations. Thus, enzyme immune assay calibration curves are typically limited by standard concentrations used and user handling; while HPLC methods offer more reliability and repeatability ensuring the potential for comparing results to other studies.

Additionally, pH levels in the general population have been more widely characterized than 8-isoprostane levels using similar methodologies. Population based studies have been able to identify a bimodal distribution for pH with a majority of the population showing pH between 7.4-8.2 and a minority of the population having their baseline pH levels around 6.5. However, 8-isoprostane population distributions vary widely depending on the method of analysis used.

5.6.3 General discussion

With regards to costs, pH is by far the least expensive biomarker to identify in EBC when compared to HPLC analysis of 8-isoprostane. Furthermore, a pH meter and KCl probe are easily obtained while HPLC analysis requires specific instruments, personnel and a higher capital.

5.7 EBC pH methodological issues

For EBC pH analyses, this method was assessed as more robust and simple to use as compared to using a CO_2 partial pressure method or other de-aeration gas. The issue with analysing EBC samples for pH is one of buffering systems. CO2 must be removed from EBC samples in order to avoid pH modification caused by the HCO3- ions present in solution when EBC left to equalize

with the surrounding atmosphere. EBC is typically de-aerated in order to standardize all pH measurements and enable them to be compared across different participants. In the event that this standardization procedure if not adequate, results may be biased. In our case, we used the method which was the most standardized and used by other research groups.

However, there still areas of contention around de-aeration with argon. Some studies suggest that not all CO2 is removed when using argon since the set up itself may let CO2 in by inadvertence or that there is a small amount of CO2 which will never ultimately be removed from the EBC samples (201). Richard M. Effros, in a letter to the ERJ editor, suggested that EBC pH was not a fair reflection of airway lining fluid pH since it did not take into account the buffer capacity of the ALF (270). Effros maintains that the buffer capacity of NH4+/NH3 and CO2/HCO3- overwhelm any effect AFL acidity can have on EBC pH (270). In another publication, Effros demonstrates that the change in pH associated with the addition of varying amounts of lactic acid to varying buffer solutions were greatest when no buffers were involved (271). How this affects our study results is unclear since we are using every subject as his or her own control thus cancelling out most biological variability associated with these types of issues. Unfortunately, it is unclear how buffer capacity changes with regards to diet, diesel exposure or even circadian rhythm. Thus, it is possible that our pH results may be confounded by the issue of the buffer capacity of each individual sample.

Certain EBC pH values were below the normal range of 7.4 or higher as described in section 2.10.1.2. These were not excluded as outliers because of their presence in other population level studies (49, 272). These subjects are thought to have lower pH because they are at the point between pKas (the logarithm to the base 10 of the proton concentration in solution at which the acid is 50% protonated) of weak acids and bases (195). This subset of individuals has been described as being biochemically unique and perhaps metabolically ideal for targeted therapy (195). However, in this study we did not have any of these individuals to observe; they would have required individual analyses since including them in our analyses may have biased results.

5.8 Conclusion

There are 2 main conclusions. First, we successfully developed a method analyzing EBC levels of 8-isoprostane, which is attractive relative to other method published in the literature. Second,

we were unable to show that diesel exhaust (with or without anti-oxidant supplementation) changed biomarkers of oxidative stress in the airways; however, we suggest that significant changes attributable to diesel exhaust might be observable in a similar study with a markedly larger sample size. Finally, we provide some evidence that sex may interact with exposure to diesel exhaust in terms of these biomarkers and we suggest that future related studies pay particular attention to this potential interaction.

A major strength of this study is the three-way crossover design which inherently controls for individual biological variability by letting every subject be his/her own control. This effectively removes any potential confounding, though the potential for effect modification remains. One limitation of this design is that investigating potential effect modification in subgroups is rendered difficult because of the smaller sample size, as such it is more difficult to get significance due to decreased power. Another limitation is the systematic error present in the 8-isoprostane data biasing results towards the null. This systematic error results from increased variability around lower 8-isoprostane concentrations; this increased variability makes it harder to identify differences in 8-isoprostane levels across concentrations and potential effect modification by sub populations.

McCreanor *et al.* 2007 exposed their subjects to ambient diesel exhaust concurrent with allergens, ozone and exhaust from other sources of pollutants (5). Controlled diesel exhaust and ozone studies have demonstrated enhanced respiratory health effects as compared to diesel exhaust alone at 100 ug/m^3 DE exposures (72-73). Controlled diesel exhaust studies have also showed increased respiratory health effects from concurrent allergens exposure with similar DE concentrations (266-267, 273-275). Future research may want to focus on these concurrent exposures using EBC pH since it is a reliable and reproducible method of collecting viable respiratory health samples and very affordable as compared to bronchoscopies and sputum inductions.

Future co-exposure studies may want to weigh-in the benefits of using EBC pH as a viable, easier, less invasive and less expensive method of assessing changes in airway health instead of

bronchoscopies or sputum inductions. However, using EBC requires careful standardization and appropriate collection and storage protocols.

Bibliography

1. McClellan RO. Health effects of exposure to diesel exhaust particles. Annu Rev Pharmacol Toxicol. 1987;27:279-300.
2. Riedl M, Diaz-Sanchez D. Biology of diesel exhaust effects on respiratory function. J Allergy Clin Immunol. 2005 Feb;115(2):221-8; quiz 9.
3. Janssen NA, Brunekreef B, van Vliet P, Aarts F, Meliefste K, Harssema H, et al. The relationship between air pollution from heavy traffic and allergic sensitization, bronchial hyperresponsiveness, and respiratory symptoms in Dutch schoolchildren. Environ Health Perspect. 2003 Sep;111(12):1512-8.
4. Goldsmith CA, Kobzik L. Particulate air pollution and asthma: a review of epidemiological and biological studies. Rev Environ Health. 1999 Jul-Sep;14(3):121-34.
5. McCreanor J, Cullinan P, Nieuwenhuijsen M, Stewart-Evans J, Malliarou E, Jarup L, et al. Respiratory effects of exposure to diesel traffic in persons with asthma. N Engl J Med. 2007;357(23):2348-58.
6. Hoek G, Brunekreef B, Goldbohm S, Fischer P, van den Brandt PA. Association between mortality and indicators of traffic-related air pollution in the Netherlands: a cohort study. Lancet. 2002 Oct 19;360(9341):1203-9.
7. Polosa R, Salvi S, Di Maria GU. Allergic susceptibility associated with diesel exhaust particle exposure: clear as mud. Arch Environ Health. 2002 May-Jun;57(3):188-93.
8. Salvi SS, Frew A, Holgate S. Is diesel exhaust a cause for increasing allergies? Clin Exp Allergy. 1999 Jan;29(1):4-8.
9. Borken J, et. al. . Health effects of transport-related air pollution. In: Krzyzanowski M, Birgit Kuna-Dibbert and Jürgen Schneider, editor. Denmark: World Health Organization; 2005. p. 190.
10. Brunekreef B, Holgate ST. Air pollution and health. Lancet. 2002 Oct 19;360(9341):1233-42.
11. Trépanier TaDOK. Road Motor Vehicles: Fuel Sales. In: Section TDMT, editor. Ottawa: Statistics Canada; 2001. p. 5.
12. Soria MBaC. Canada - road sector diesel fuel consumption. 2012 [cited 2012 December 20]; Available from: http://www.indexmundi.com/facts/canada/road-sector-diesel-fuel-consumption.
13. Soria MBaC. Canada - road sector gasoline fuel consumption. 2012 [cited 2012 December 20]; Available from: http://www.indexmundi.com/facts/canada/road-sector-gasoline-fuel-consumption-per-capita.
14. Johnson TV. Diesel emission control technology—2003 in review. . SAE Technical Paper 2004-01-0070. 2003.
15. Cutler WA. Overview of ceramic materials for diesel particulate filter applications. Ceramic Engineering and Science Proceedings. 2004;25 421–30.
16. Ghio AJ, Smith CB, Madden MC. Diesel exhaust particles and airway inflammation. Curr Opin Pulm Med. [Review]. 2012 Mar;18(2):144-50.
17. Johnson TV. Diesel Emission Control in Review. SAE Technical Paper 2006-01-0030. 2006.
18. Holguin F. Traffic, outdoor air pollution, and asthma. Immunol Allergy Clin North Am. 2008 Aug;28(3):577-88, viii-ix.

19. McCreanor J, Cullinan P, Nieuwenhuijsen MJ, Stewart-Evans J, Malliarou E, Jarup L, et al. Respiratory effects of exposure to diesel traffic in persons with asthma. N Engl J Med. 2007 Dec 6;357(23):2348-58.
20. Eder W, Ege MJ, von Mutius E. The asthma epidemic. N Engl J Med. 2006 Nov 23;355(21):2226-35.
21. Barnes PJ. Reactive oxygen species and airway inflammation. Free Radic Biol Med. 1990;9(3):235-43.
22. Hoffmeyer F, Raulf-Heimsoth M, Bruning T. Exhaled breath condensate and airway inflammation. Curr Opin Allergy Clin Immunol. 2009 Feb;9(1):16-22.
23. Nightingale JA, Maggs R, Cullinan P, Donnelly LE, Rogers DF, Kinnersley R, et al. Airway inflammation after controlled exposure to diesel exhaust particulates. Am J Respir Crit Care Med. 2000 Jul;162(1):161-6.
24. Nordenhall C, Pourazar J, Ledin MC, Levin JO, Sandstrom T, Adelroth E. Diesel exhaust enhances airway responsiveness in asthmatic subjects. Eur Respir J. 2001 May;17(5):909-15.
25. Rudell B, Ledin MC, Hammarstrom U, Stjernberg N, Lundback B, Sandstrom T. Effects on symptoms and lung function in humans experimentally exposed to diesel exhaust. Occup Environ Med. 1996 Oct;53(10):658-62.
26. Rudell B, Blomberg A, Helleday R, Ledin MC, Lundback B, Stjernberg N, et al. Bronchoalveolar inflammation after exposure to diesel exhaust: comparison between unfiltered and particle trap filtered exhaust. Occup Environ Med. 1999 Aug;56(8):527-34.
27. Nordenhall C, Pourazar J, Blomberg A, Levin JO, Sandstrom T, Adelroth E. Airway inflammation following exposure to diesel exhaust: a study of time kinetics using induced sputum. Eur Respir J. 2000 Jun;15(6):1046-51.
28. Salvi SS, Nordenhall C, Blomberg A, Rudell B, Pourazar J, Kelly FJ, et al. Acute exposure to diesel exhaust increases IL-8 and GRO-alpha production in healthy human airways. Am J Respir Crit Care Med. 2000 Feb;161(2 Pt 1):550-7.
29. Stenfors N, Nordenhall C, Salvi SS, Mudway I, Soderberg M, Blomberg A, et al. Different airway inflammatory responses in asthmatic and healthy humans exposed to diesel. Eur Respir J. 2004 Jan;23(1):82-6.
30. Nel AE, Diaz-Sanchez D, Ng D, Hiura T, Saxon A. Enhancement of allergic inflammation by the interaction between diesel exhaust particles and the immune system. J Allergy Clin Immunol. 1998 Oct;102(4 Pt 1):539-54.
31. Hiura TS, Kaszubowski MP, Li N, Nel AE. Chemicals in diesel exhaust particles generate reactive oxygen radicals and induce apoptosis in macrophages. J Immunol. 1999 Nov 15;163(10):5582-91.
32. Hiura TS, Li N, Kaplan R, Horwitz M, Seagrave JC, Nel AE. The role of a mitochondrial pathway in the induction of apoptosis by chemicals extracted from diesel exhaust particles. J Immunol. 2000 Sep 1;165(5):2703-11.
33. Whitekus MJ, Li N, Zhang M, Wang M, Horwitz MA, Nelson SK, et al. Thiol antioxidants inhibit the adjuvant effects of aerosolized diesel exhaust particles in a murine model for ovalbumin sensitization. J Immunol. 2002 Mar 1;168(5):2560-7.
34. Kumagai Y, Arimoto T, Shinyashiki M, Shimojo N, Nakai Y, Yoshikawa T, et al. Generation of reactive oxygen species during interaction of diesel exhaust particle components with NADPH-cytochrome P450 reductase and involvement of the bioactivation in the DNA damage. Free Radic Biol Med. 1997;22(3):479-87.

35. Li N, Kim S, Wang M, Froines J, Sioutas C, Nel A. Use of a stratified oxidative stress model to study the biological effects of ambient concentrated and diesel exhaust particulate matter. Inhal Toxicol. 2002 May;14(5):459-86.
36. Xiao GG, Wang M, Li N, Loo JA, Nel AE. Use of proteomics to demonstrate a hierarchical oxidative stress response to diesel exhaust particle chemicals in a macrophage cell line. J Biol Chem. 2003 Dec 12;278(50):50781-90.
37. Aruoma OI, Halliwell B, Hoey BM, Butler J. The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Radic Biol Med. 1989;6(6):593-7.
38. Romieu I, Sienra-Monge JJ, Ramirez-Aguilar M, Tellez-Rojo MM, Moreno-Macias H, Reyes-Ruiz NI, et al. Antioxidant supplementation and lung functions among children with asthma exposed to high levels of air pollutants. Am J Respir Crit Care Med. 2002 Sep 1;166(5):703-9.
39. Cepelak I, Dodig S. Exhaled breath condensate: a new method for lung disease diagnosis. Clin Chem Lab Med. 2007;45(8):945-52.
40. Vaughan J, Ngamtrakulpanit L, Pajewski TN, Turner R, Nguyen TA, Smith A, et al. Exhaled breath condensate pH is a robust and reproducible assay of airway acidity. Eur Respir J. 2003 Dec;22(6):889-94.
41. Kostikas K, Koutsokera A, Papiris S, Gourgoulianis KI, Loukides S. Exhaled breath condensate in patients with asthma: implications for application in clinical practice. Clin Exp Allergy. 2008 Apr;38(4):557-65.
42. Montuschi P. Analysis of exhaled breath condensate in respiratory medicine: methodological aspects and potential clinical applications. Ther Adv Respir Dis. 2007 Oct;1(1):5-23.
43. Horvath I, Hunt J, Barnes PJ, Alving K, Antczak A, Baraldi E, et al. Exhaled breath condensate: methodological recommendations and unresolved questions. Eur Respir J. 2005 Sep;26(3):523-48.
44. Kostikas K, Papatheodorou G, Ganas K, Psathakis K, Panagou P, Loukides S. pH in expired breath condensate of patients with inflammatory airway diseases. Am J Respir Crit Care Med. 2002 May 15;165(10):1364-70.
45. Montuschi P, Corradi M, Ciabattoni G, Nightingale J, Kharitonov SA, Barnes PJ. Increased 8-isoprostane, a marker of oxidative stress, in exhaled condensate of asthma patients. Am J Respir Crit Care Med. 1999 Jul;160(1):216-20.
46. Montuschi P, Barnes PJ, Roberts LJ, 2nd. Isoprostanes: markers and mediators of oxidative stress. FASEB J. 2004 Dec;18(15):1791-800.
47. Montuschi P, Barnes P, Roberts LJ, 2nd. Insights into oxidative stress: the isoprostanes. Curr Med Chem. 2007;14(6):703-17.
48. Van Hoydonck PG, Wuyts WA, Vanaudenaerde BM, Schouten EG, Dupont LJ, Temme EH. Quantitative analysis of 8-isoprostane and hydrogen peroxide in exhaled breath condensate. Eur Respir J. 2004 Feb;23(2):189-92.
49. Hunt JF, Fang K, Malik R, Snyder A, Malhotra N, Platts-Mills TA, et al. Endogenous airway acidification. Implications for asthma pathophysiology. Am J Respir Crit Care Med. 2000 Mar;161(3 Pt 1):694-9.
50. Morrow JD, Hill KE, Burk RF, Nammour TM, Badr KF, Roberts LJ, 2nd. A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. Proc Natl Acad Sci U S A. 1990 Dec;87(23):9383-7.

51. Longmire AW, Swift LL, Roberts LJ, 2nd, Awad JA, Burk RF, Morrow JD. Effect of oxygen tension on the generation of F2-isoprostanes and malondialdehyde in peroxidizing rat liver microsomes. Biochem Pharmacol. 1994 Mar 29;47(7):1173-7.
52. Richelle M, Turini ME, Guidoux R, Tavazzi I, Metairon S, Fay LB. Urinary isoprostane excretion is not confounded by the lipid content of the diet. FEBS Lett. 1999 Oct 8;459(2):259-62.
53. Burk RF. Glutathione-dependent protection by rat liver microsomal protein against lipid peroxidation. Biochim Biophys Acta. 1983 May 4;757(1):21-8.
54. Roberts LJ, Morrow JD. Measurement of F(2)-isoprostanes as an index of oxidative stress in vivo. Free Radic Biol Med. 2000 Feb 15;28(4):505-13.
55. Marnett LJ, Buck J, Tuttle MA, Basu AK, Bull AW. Distribution and oxidation of malondialdehyde in mice. Prostaglandins. 1985 Aug;30(2):241-54.
56. Cancer IAfRo. Diesel and Gasoline Engine Exhausts: Diesel engine exhaust (Group 2A), Gasoline engine exhaust (Group 2B). . Lyon: IARC1989.
57. Schauer JJ, Kleeman MJ, Cass GR, Simoneit BR. Measurement of emissions from air pollution sources. 5. C1-C32 organic compounds from gasoline-powered motor vehicles. Environ Sci Technol. 2002 Mar 15;36(6):1169-80.
58. Schauer JJ, Kleeman, Michael J., Cass, Glen R. , D AN, Berndrtsimonei, T. . Measurement of Emissions from Air Pollution Sources. 2. C1 through C30 Organic Compounds from Medium Duty Diesel Trucks. Environ Sci Technol 1999(33):1578-87.
59. Lloyd AC, Cackette TA. Diesel engines: environmental impact and control. J Air Waste Manag Assoc. 2001 Jun;51(6):809-47.
60. Agency EP. Particulate Matter Emissions. Cincinnati: EPA; 2009.
61. Maricq MM. Chemical characterization of particulate emissions from diesel engines: A review. Journal of Aerosol Science. 2007;38(11):1079-118.
62. Guerrassi NaD, P. A common rail injection system for high speed direct injection diesel engines. SAE Technical Paper 980803. 1998.
63. Stumpp GaR, M. Common rail—an attractive fuel injection system for passenger car DI diesel engines. SAE Technical Paper, 960870. 1996.
64. Park C, Kook, S. and Bae, C. . Effects of multiple injections in a HSDI diesel engine equipped with common rail injection system. SAE Technical Paper 2004-01-0127. 2004.
65. Johnson TV. Diesel Emission Control in Review. SAE Technical Paper 2006-01-0030. 2005.
66. Pope CA, 3rd, Thun MJ, Namboodiri MM, Dockery DW, Evans JS, Speizer FE, et al. Particulate air pollution as a predictor of mortality in a prospective study of U.S. adults. Am J Respir Crit Care Med. 1995 Mar;151(3 Pt 1):669-74.
67. Pope CA, 3rd. Respiratory disease associated with community air pollution and a steel mill, Utah Valley. Am J Public Health. 1989 May;79(5):623-8.
68. Delfino RJ, Staimer N, Tjoa T, Arhami M, Polidori A, Gillen DL, et al. Associations of primary and secondary organic aerosols with airway and systemic inflammation in an elderly panel cohort. Epidemiology. 2010 Nov;21(6):892-902.
69. Delfino RJ, Murphy-Moulton AM, Burnett RT, Brook JR, Becklake MR. Effects of air pollution on emergency room visits for respiratory illnesses in Montreal, Quebec. Am J Respir Crit Care Med. 1997 Feb;155(2):568-76.

70. Delfino RJ, Sioutas C, Malik S. Potential role of ultrafine particles in associations between airborne particle mass and cardiovascular health. Environ Health Perspect. 2005 Aug;113(8):934-46.
71. Behndig AF, Larsson N, Brown JL, Stenfors N, Helleday R, Duggan ST, et al. Proinflammatory doses of diesel exhaust in healthy subjects fail to elicit equivalent or augmented airway inflammation in subjects with asthma. Thorax. 2011 Jan;66(1):12-9.
72. Bosson J, Pourazar J, Forsberg B, Adelroth E, Sandstrom T, Blomberg A. Ozone enhances the airway inflammation initiated by diesel exhaust. Respir Med. 2007 Jun;101(6):1140-6.
73. Bosson J, Barath S, Pourazar J, Behndig AF, Sandstrom T, Blomberg A, et al. Diesel exhaust exposure enhances the ozone-induced airway inflammation in healthy humans. Eur Respir J. 2008 Jun;31(6):1234-40.
74. Behndig AF, Mudway IS, Brown JL, Stenfors N, Helleday R, Duggan ST, et al. Airway antioxidant and inflammatory responses to diesel exhaust exposure in healthy humans. Eur Respir J. 2006 Feb;27(2):359-65.
75. Sehlstedt M, Behndig AF, Boman C, Blomberg A, Sandstrom T, Pourazar J. Airway inflammatory response to diesel exhaust generated at urban cycle running conditions. Inhal Toxicol. 2010 Dec;22(14):1144-50.
76. Salvi S, Blomberg A, Rudell B, Kelly F, Sandstrom T, Holgate ST, et al. Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers. Am J Respir Crit Care Med. 1999 Mar;159(3):702-9.
77. Becker S, Soukup JM, Gilmour MI, Devlin RB. Stimulation of human and rat alveolar macrophages by urban air particulates: effects on oxidant radical generation and cytokine production. Toxicol Appl Pharmacol. 1996 Dec;141(2):637-48.
78. Bonvallot V, Baulig A, Boland S, Marano F, Baeza A. Diesel exhaust particles induce an inflammatory response in airway epithelial cells: involvement of reactive oxygen species. Biofactors. 2002;16(1-2):15-7.
79. Boland S, Bonvallot V, Fournier T, Baeza-Squiban A, Aubier M, Marano F. Mechanisms of GM-CSF increase by diesel exhaust particles in human airway epithelial cells. Am J Physiol Lung Cell Mol Physiol. 2000 Jan;278(1):L25-32.
80. Boland S, Baeza-Squiban A, Fournier T, Houcine O, Gendron MC, Chevrier M, et al. Diesel exhaust particles are taken up by human airway epithelial cells in vitro and alter cytokine production. Am J Physiol. 1999 Apr;276(4 Pt 1):L604-13.
81. Bonvallot V, Baeza-Squiban A, Baulig A, Brulant S, Boland S, Muzeau F, et al. Organic compounds from diesel exhaust particles elicit a proinflammatory response in human airway epithelial cells and induce cytochrome p450 1A1 expression. Am J Respir Cell Mol Biol. 2001 Oct;25(4):515-21.
82. Baulig A, Garlatti M, Bonvallot V, Marchand A, Barouki R, Marano F, et al. Involvement of reactive oxygen species in the metabolic pathways triggered by diesel exhaust particles in human airway epithelial cells. Am J Physiol Lung Cell Mol Physiol. 2003 Sep;285(3):L671-9.
83. Sagai M, Saito H, Ichinose T, Kodama M, Mori Y. Biological effects of diesel exhaust particles. I. In vitro production of superoxide and in vivo toxicity in mouse. Free Radic Biol Med. 1993 Jan;14(1):37-47.
84. Pronk A, Coble J, Stewart PA. Occupational exposure to diesel engine exhaust: a literature review. J Expo Sci Environ Epidemiol. 2009 Jul;19(5):443-57.

85. Davis ME, Smith TJ, Laden F, Hart JE, Blicharz AP, Reaser P, et al. Driver exposure to combustion particles in the U.S. Trucking industry. J Occup Environ Hyg. 2007 Nov;4(11):848-54.
86. NIOSH. NIOSH Health Hazard Evaluation Report 88-388-2155. Cincinnati, OH: National Institute for Occupational Safety and Health1991a.
87. NIOSH. NIOSH Health Hazard Evaluation Report 88-108-2146. Cincinnati, OH: National Institute for Occupational Safety and Health1991b.
88. NIOSH. NIOSH Health Hazard Evaluation Report 88-389-2147. Concinnati, OH: National Institute for Occupational Safety and Health1991c.
89. Woskie SR, Kalil A, Bello D, Virji MA. Exposures to quartz, diesel, dust, and welding fumes during heavy and highway construction. AIHA J (Fairfax, Va). 2002 Jul-Aug;63(4):447-57.
90. Gamble J, Jones, W. and Minshall, S. Epidemiological-environmental study of diesel bus garage workers: chronic effects of diesel exhaust on the respiratory system. Environ Res. 1987 Oct;44(1):6-17.
91. Zaebst D, et. al. Quantitative Determination of Trucking Industry Workers' Exposures to Diesel Exhaust Particles. American Industrial Hygiene Association Journal. 1991;52(12):529-41.
92. Dockery DW, Speizer FE, Stram DO, Ware JH, Spengler JD, Ferris BG, Jr. Effects of inhalable particles on respiratory health of children. Am Rev Respir Dis. 1989 Mar;139(3):587-94.
93. Garshick E, Laden F, Hart JE, Rosner B, Smith TJ, Dockery DW, et al. Lung cancer in railroad workers exposed to diesel exhaust. Environ Health Perspect. 2004 Nov;112(15):1539-43.
94. Steenland K, Silverman D, Zaebst D. Exposure to diesel exhaust in the trucking industry and possible relationships with lung cancer. Am J Ind Med. 1992;21(6):887-90.
95. Sheppard L, Levy D, Norris G, Larson TV, Koenig JQ. Effects of ambient air pollution on nonelderly asthma hospital admissions in Seattle, Washington, 1987-1994. Epidemiology. 1999 Jan;10(1):23-30.
96. Dockery DW. Health effects of particulate air pollution. Ann Epidemiol. 2009 Apr;19(4):257-63.
97. Hoppin JA, Umbach DM, London SJ, Alavanja MC, Sandler DP. Diesel exhaust, solvents, and other occupational exposures as risk factors for wheeze among farmers. Am J Respir Crit Care Med. 2004 Jun 15;169(12):1308-13.
98. Wade JF, 3rd, Newman LS. Diesel asthma. Reactive airways disease following overexposure to locomotive exhaust. J Occup Med. 1993 Feb;35(2):149-54.
99. Christopher McLeod, Terry Bogyo, Paul Demers, Demet Edeer, Clyde Hertzman, Susan Kennedy, et al. Asthma in British Columbia. Vancouver, BC: University of British Columbia2007.
100. Hiltermann TJ, Stolk J, van der Zee SC, Brunekreef B, de Bruijne CR, Fischer PH, et al. Asthma severity and susceptibility to air pollution. Eur Respir J. 1998 Mar;11(3):686-93.
101. Kay AB. Asthma and inflammation. J Allergy Clin Immunol. 1991 May;87(5):893-910.
102. Riedl MA, Nel AE. Importance of oxidative stress in the pathogenesis and exposure of asthma. Curr Opin Allergy Clin Immunol. 2008 Feb;8(1):49-56.
103. Nel A. Atmosphere. Air pollution-related illness: effects of particles. Science. 2005 May 6;308(5723):804-6.

104. Li N, Hao M, Phalen RF, Hinds WC, Nel AE. Particulate air pollutants and asthma. A paradigm for the role of oxidative stress in PM-induced adverse health effects. Clin Immunol. 2003 Dec;109(3):250-65.
105. Dozor AJ. The role of oxidative stress in the pathogenesis and exposure of asthma. Ann N Y Acad Sci. 2010 Aug;1203:133-7.
106. Henricks PA, Nijkamp FP. Reactive oxygen species as mediators in asthma. Pulm Pharmacol Ther. 2001;14(6):409-20.
107. Kinnula VL, Crapo JD. Superoxide dismutases in the lung and human lung diseases. Am J Respir Crit Care Med. 2003 Jun 15;167(12):1600-19.
108. Powell CV, Nash AA, Powers HJ, Primhak RA. Antioxidant status in asthma. Pediatr Pulmonol. 1994 Jul;18(1):34-8.
109. Chow CW, Herrera Abreu MT, Suzuki T, Downey GP. Oxidative stress and acute lung injury. Am J Respir Cell Mol Biol. 2003 Oct;29(4):427-31.
110. Chow CK. Vitamin E and oxidative stress. Free Radic Biol Med. 1991;11(2):215-32.
111. Sies H. Oxidative stress: from basic research to clinical application. Am J Med. 1991 Sep 30;91(3C):31S-8S.
112. Hulsmann AR, Raatgeep HR, den Hollander JC, Stijnen T, Saxena PR, Kerrebijn KF, et al. Oxidative epithelial damage produces hyperresponsiveness of human peripheral airways. Am J Respir Crit Care Med. 1994 Feb;149(2 Pt 1):519-25.
113. Lehtonen ST, Markkanen PM, Peltoniemi M, Kang SW, Kinnula VL. Variable overoxidation of peroxiredoxins in human lung cells in severe oxidative stress. Am J Physiol Lung Cell Mol Physiol. 2005 May;288(5):L997-1001.
114. Rahman Q, Norwood J, Hatch G. Evidence that exposure of particulate air pollutants to human and rat alveolar macrophages leads to differential oxidative response. Biochem Biophys Res Commun. 1997 Nov 26;240(3):669-72.
115. Rahman I, Morrison D, Donaldson K, MacNee W. Systemic oxidative stress in asthma, COPD, and smokers. Am J Respir Crit Care Med. 1996 Oct;154(4 Pt 1):1055-60.
116. Nadeem A, Chhabra SK, Masood A, Raj HG. Increased oxidative stress and altered levels of antioxidants in asthma. J Allergy Clin Immunol. 2003 Jan;111(1):72-8.
117. Sagai M, Furuyama A, Ichinose T. Biological effects of diesel exhaust particles (DEP). III. Pathogenesis of asthma like symptoms in mice. Free Radic Biol Med. 1996;21(2):199-209.
118. Lim HB, Ichinose T, Miyabara Y, Takano H, Kumagai Y, Shimojyo N, et al. Involvement of superoxide and nitric oxide on airway inflammation and hyperresponsiveness induced by diesel exhaust particles in mice. Free Radic Biol Med. 1998 Oct;25(6):635-44.
119. Li N, Wang M, Oberley TD, Sempf JM, Nel AE. Comparison of the pro-oxidative and proinflammatory effects of organic diesel exhaust particle chemicals in bronchial epithelial cells and macrophages. J Immunol. 2002 Oct 15;169(8):4531-41.
120. Banerjee A, Trueblood MB, Zhang X, Manda KR, Lobo P, Whitefield PD, et al. N-acetylcysteineamide (NACA) prevents inflammation and oxidative stress in animals exposed to diesel engine exhaust. Toxicol Lett. 2009 Jun 22;187(3):187-93.
121. Romieu I, Sienra-Monge JJ, Ramirez-Aguilar M, Moreno-Macias H, Reyes-Ruiz NI, Estela del Rio-Navarro B, et al. Genetic polymorphism of GSTM1 and antioxidant supplementation influence lung function in relation to ozone exposure in asthmatic children in Mexico City. Thorax. 2004 Jan;59(1):8-10.

122. Antczak A, Nowak D, Shariati B, Krol M, Piasecka G, Kurmanowska Z. Increased hydrogen peroxide and thiobarbituric acid-reactive products in expired breath condensate of asthmatic patients. Eur Respir J. 1997 Jun;10(6):1235-41.
123. Baldwin SR, Simon RH, Grum CM, Ketai LH, Boxer LA, Devall LJ. Oxidant activity in expired breath of patients with adult respiratory distress syndrome. Lancet. 1986 Jan 4;1(8471):11-4.
124. Hunt J, Byrns RE, Ignarro LJ, Gaston B. Condensed expirate nitrite as a home marker for acute asthma. Lancet. 1995 Nov 4;346(8984):1235-6.
125. Rothe M BG, Siemers R, et al. 2005. European Respiratory Journal. The pH-value of exhaled breath condensated mainly influenced by exhaled volatile compounds;26(49):2405.
126. Kharitonov SA, Barnes PJ. Exhaled markers of pulmonary disease. Am J Respir Crit Care Med. 2001 Jun;163(7):1693-722.
127. Wells K, Vaughan J, Pajewski TN, Hom S, Ngamtrakulpanit L, Smith A, et al. Exhaled breath condensate pH assays are not influenced by oral ammonia. Thorax. 2005 Jan;60(1):27-31.
128. Hunt J. Condensing exhaled breath into science. Chest. 2011 Jan;139(1):5-6.
129. Kietzmann D, Kahl R, Muller M, Burchardi H, Kettler D. Hydrogen peroxide in expired breath condensate of patients with acute respiratory failure and with ARDS. Intensive Care Med. 1993;19(2):78-81.
130. Nowak D, Kalucka S, Bialasiewicz P, Krol M. Exhalation of H2O2 and thiobarbituric acid reactive substances (TBARs) by healthy subjects. Free Radic Biol Med. 2001 Jan 15;30(2):178-86.
131. Sznajder JI, Fraiman A, Hall JB, Sanders W, Schmidt G, Crawford G, et al. Increased hydrogen peroxide in the expired breath of patients with acute hypoxemic respiratory failure. Chest. 1989 Sep;96(3):606-12.
132. Carpenter CT, Price PV, Christman BW. Exhaled breath condensate isoprostanes are elevated in patients with acute lung injury or ARDS. Chest. 1998 Dec;114(6):1653-9.
133. Grob NM, Aytekin M, Dweik RA. Biomarkers in exhaled breath condensate: a review of collection, processing and analysis. J Breath Res. 2008 Sep;2(3):037004.
134. Vass G, Huszar E, Barat E, Valyon M, Kiss D, Penzes I, et al. Comparison of nasal and oral inhalation during exhaled breath condensate collection. Am J Respir Crit Care Med. 2003 Mar 15;167(6):850-5.
135. Do R, Bartlett KH, Chu W, Dimich-Ward H, Kennedy SM. Within- and between-person variability of exhaled breath condensate pH and NH4+ in never and current smokers. Respir Med. 2008 Mar;102(3):457-63.
136. Koczulla AR, Noeske S, Herr C, Dette F, Pinkenburg O, Schmid S, et al. Ambient temperature impacts on pH of exhaled breath condensate. Respirology. 2010 Jan;15(1):155-9.
137. Leung TF, Li CY, Yung E, Liu EK, Lam CW, Wong GW. Clinical and technical factors affecting pH and other biomarkers in exhaled breath condensate. Pediatr Pulmonol. 2006 Jan;41(1):87-94.
138. Hunt J. Exhaled breath condensate: an evolving tool for noninvasive evaluation of lung disease. J Allergy Clin Immunol. 2002 Jul;110(1):28-34.
139. Rosias PP, Robroeks CM, Kester A, den Hartog GJ, Wodzig WK, Rijkers GT, et al. Biomarker reproducibility in exhaled breath condensate collected with different condensers. Eur Respir J. 2008 May;31(5):934-42.

140. Koczulla R, Dragonieri S, Schot R, Bals R, Gauw SA, Vogelmeier C, et al. Comparison of exhaled breath condensate pH using two commercially available devices in healthy controls, asthma and COPD patients. Respir Res. 2009;10:78.
141. Rosias PP, Robroeks CM, Niemarkt HJ, Kester AD, Vernooy JH, Suykerbuyk J, et al. Breath condenser coatings affect measurement of biomarkers in exhaled breath condensate. Eur Respir J. 2006 Nov;28(5):1036-41.
142. Prieto L, Ferrer A, Palop J, Domenech J, Llusar R, Rojas R. Differences in exhaled breath condensate pH measurements between samples obtained with two commercial devices. Respir Med. 2007 Aug;101(8):1715-20.
143. Gessner C, Kuhn H, Seyfarth HJ, Pankau H, Winkler J, Schauer J, et al. Factors influencing breath condensate volume. Pneumologie. 2001 Sep;55(9):414-9.
144. Effros RM, Hoagland KW, Bosbous M, Castillo D, Foss B, Dunning M, et al. Dilution of respiratory solutes in exhaled condensates. Am J Respir Crit Care Med. 2002 Mar 1;165(5):663-9.
145. Corradi M, Pesci A, Casana R, Alinovi R, Goldoni M, Vettori MV, et al. Nitrate in exhaled breath condensate of patients with different airway diseases. Nitric Oxide. 2003 Feb;8(1):26-30.
146. Zacharasiewicz A, Wilson N, Lex C, Li A, Kemp M, Donovan J, et al. Repeatability of sodium and chloride in exhaled breath condensates. Pediatr Pulmonol. 2004 Mar;37(3):273-5.
147. Dwyer TM. Sampling airway surface liquid: non-volatiles in the exhaled breath condensate. Lung. 2004;182(4):241-50.
148. Effros RM. Saving the breath condensate approach. Am J Respir Crit Care Med. 2003 Nov 1;168(9):1129-32; author reply 30-1.
149. Horvath I, Hunt J, Barnes PJ, Alving K, Antczak A, Baraldi E, et al. Exhaled breath condensate: methodological recommendations and unresolved questions. European Respiratory Journal. [Guideline
Practice Guideline]. 2005 Sep;26(3):523-48.
150. Jobsis Q, Raatgeep HC, Schellekens SL, Hop WC, Hermans PW, de Jongste JC. Hydrogen peroxide in exhaled air of healthy children: reference values. Eur Respir J. 1998 Aug;12(2):483-5.
151. Cheah FC, Darlow BA, Winterbourn CC. Problems associated with collecting breath condensate for the measurement of exhaled hydrogen peroxide from neonates on respiratory support. Biol Neonate. 2003;84(4):338-41.
152. Syslova K, Kacer P, Kuzma M, Klusackova P, Fenclova Z, Lebedova J, et al. Determination of 8-iso-prostaglandin F(2alpha) in exhaled breath condensate using combination of immunoseparation and LC-ESI-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci. 2008 May 1;867(1):8-14.
153. Horvath I. The exhaled biomarker puzzle: bacteria play their card in the exhaled nitric oxide-exhaled breath condensate nitrite game. Thorax. 2005 Mar;60(3):179-80.
154. Janssen LJ. Isoprostanes: an overview and putative roles in pulmonary pathophysiology. Am J Physiol Lung Cell Mol Physiol. 2001 Jun;280(6):L1067-82.
155. Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A. Biomarkers of oxidative damage in human disease. Clin Chem. 2006 Apr;52(4):601-23.
156. Montuschi P, Collins JV, Ciabattoni G, Lazzeri N, Corradi M, Kharitonov SA, et al. Exhaled 8-isoprostane as an in vivo biomarker of lung oxidative stress in patients with COPD and healthy smokers. Am J Respir Crit Care Med. 2000 Sep;162(3 Pt 1):1175-7.

157. Montuschi P, Kharitonov SA, Ciabattoni G, Corradi M, van Rensen L, Geddes DM, et al. Exhaled 8-isoprostane as a new non-invasive biomarker of oxidative stress in cystic fibrosis. Thorax. 2000 Mar;55(3):205-9.
158. Montuschi P, Ciabattoni G, Paredi P, Pantelidis P, du Bois RM, Kharitonov SA, et al. 8-Isoprostane as a biomarker of oxidative stress in interstitial lung diseases. Am J Respir Crit Care Med. 1998 Nov;158(5 Pt 1):1524-7.
159. Battaglia S, den Hertog H, Timmers MC, Lazeroms SP, Vignola AM, Rabe KF, et al. Small airways function and molecular markers in exhaled air in mild asthma. Thorax. 2005 Aug;60(8):639-44.
160. Montuschi P, Barnes PJ, Ciabattoni G. Measurement of 8-isoprostane in exhaled breath condensate. Methods Mol Biol. 2010;594:73-84.
161. Baraldi E, Carraro S, Alinovi R, Pesci A, Ghiro L, Bodini A, et al. Cysteinyl leukotrienes and 8-isoprostane in exhaled breath condensate of children with asthma exacerbations. Thorax. 2003 Jun;58(6):505-9.
162. Baraldi E, Ghiro L, Piovan V, Carraro S, Ciabattoni G, Barnes PJ, et al. Increased exhaled 8-isoprostane in childhood asthma. Chest. 2003 Jul;124(1):25-31.
163. Patel MM. Respiratory Health Effects Associated with Exposure to Traffic-related Particulate Matter. New York: Columbia University; 2007.
164. Koutsokera A, Loukides S, Gourgoulianis KI, Kostikas K. Biomarkers in the exhaled breath condensate of healthy adults: mapping the path towards reference values. Curr Med Chem. 2008;15(6):620-30.
165. Samitas K, Chorianopoulos D, Vittorakis S, Zervas E, Economidou E, Papatheodorou G, et al. Exhaled cysteinyl-leukotrienes and 8-isoprostane in patients with asthma and their relation to clinical severity. Respir Med. 2009 May;103(5):750-6.
166. Wang CJ, Yang NH, Liou SH, Lee HL. Fast quantification of the exhaled breath condensate of oxidative stress 8-iso-prostaglandin F2alpha using on-line solid-phase extraction coupled with liquid chromatography/electrospray ionization mass spectrometry. Talanta. 2010 Sep 15;82(4):1434-8.
167. Smith KA, Shepherd J, Wakil A, Kilpatrick ES. A comparison of methods for the measurement of 8-isoPGF(2alpha): a marker of oxidative stress. Ann Clin Biochem. 2011 Mar;48(Pt 2):147-54.
168. Morrow JD, Frei B, Longmire AW, Gaziano JM, Lynch SM, Shyr Y, et al. Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers. Smoking as a cause of oxidative damage. N Engl J Med. 1995 May 4;332(18):1198-203.
169. Zanconato S, Carraro S, Corradi M, Alinovi R, Pasquale MF, Piacentini G, et al. Leukotrienes and 8-isoprostane in exhaled breath condensate of children with stable and unstable asthma. J Allergy Clin Immunol. 2004 Feb;113(2):257-63.
170. Biernacki WA, Kharitonov SA, Barnes PJ. Increased leukotriene B4 and 8-isoprostane in exhaled breath condensate of patients with exacerbations of COPD. Thorax. 2003 Apr;58(4):294-8.
171. Ho LP, Faccenda J, Innes JA, Greening AP. Expired hydrogen peroxide in breath condensate of cystic fibrosis patients. Eur Respir J. 1999 Jan;13(1):103-6.
172. Saenger AK, Laha TJ, Edenfield MJ, Sadrzadeh SM. Quantification of urinary 8-iso-PGF2alpha using liquid chromatography-tandem mass spectrometry and association with elevated troponin levels. Clin Biochem. 2007 Nov;40(16-17):1297-304.

173. Proudfoot J, Barden A, Mori TA, Burke V, Croft KD, Beilin LJ, et al. Measurement of urinary F(2)-isoprostanes as markers of in vivo lipid peroxidation-A comparison of enzyme immunoassay with gas chromatography/mass spectrometry. Anal Biochem. 1999 Aug 1;272(2):209-15.
174. Armbruster DA, Tillman MD, Hubbs LM. Limit of detection (LQD)/limit of quantitation (LOQ): comparison of the empirical and the statistical methods exemplified with GC-MS assays of abused drugs. Clin Chem. 1994 Jul;40(7 Pt 1):1233-8.
175. Song Y, Thiagarajah J, Verkman AS. Sodium and chloride concentrations, pH, and depth of airway surface liquid in distal airways. J Gen Physiol. 2003 Nov;122(5):511-9.
176. Fischer H, Widdicombe JH. Mechanisms of acid and base secretion by the airway epithelium. J Membr Biol. 2006;211(3):139-50.
177. Ricciardolo FL, Rado V, Fabbri LM, Sterk PJ, Di Maria GU, Geppetti P. Bronchoconstriction induced by citric acid inhalation in guinea pigs: role of tachykinins, bradykinin, and nitric oxide. Am J Respir Crit Care Med. 1999 Feb;159(2):557-62.
178. Ricciardolo FL, Gaston B, Hunt J. Acid stress in the pathology of asthma. J Allergy Clin Immunol. 2004 Apr;113(4):610-9.
179. Luk CK, Dulfano MJ. Effect of pH, viscosity and ionic-strength changes on ciliary beating frequency of human bronchial explants. Clin Sci (Lond). 1983 Apr;64(4):449-51.
180. Holma B, Hegg PO. pH- and protein-dependent buffer capacity and viscosity of respiratory mucus. Their interrelationships and influence on health. Sci Total Environ. 1989 Aug;84:71-82.
181. Holma B, Lindegren M, Andersen JM. pH effects on ciliomotility and morphology of respiratory mucosa. Arch Environ Health. 1977 Sep-Oct;32(5):216-26.
182. Boucher RC. Molecular insights into the physiology of the 'thin film' of airway surface liquid. J Physiol. 1999 May 1;516 (Pt 3):631-8.
183. Pilewski JM, Frizzell RA. Role of CFTR in airway disease. Physiol Rev. 1999 Jan;79(1 Suppl):S215-55.
184. Smith JJ, Travis SM, Greenberg EP, Welsh MJ. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. Cell. 1996 Apr 19;85(2):229-36.
185. Verkman AS, Song Y, Thiagarajah JR. Role of airway surface liquid and submucosal glands in cystic fibrosis lung disease. Am J Physiol Cell Physiol. 2003 Jan;284(1):C2-15.
186. Gessner C, Hammerschmidt S, Kuhn H, Lange T, Engelmann L, Schauer J, et al. Exhaled breath condensate nitrite and its relation to tidal volume in acute lung injury. Chest. 2003 Sep;124(3):1046-52.
187. Niimi A, Nguyen LT, Usmani O, Mann B, Chung KF. Reduced pH and chloride levels in exhaled breath condensate of patients with chronic cough. Thorax. 2004 Jul;59(7):608-12.
188. Borrill Z, Starkey C, Vestbo J, Singh D. Reproducibility of exhaled breath condensate pH in chronic obstructive pulmonary disease. Eur Respir J. 2005 Feb;25(2):269-74.
189. Brooks SM, Haight RR, Gordon RL. Age does not affect airway pH and ammonia as determined by exhaled breath measurements. Lung. 2006 Jul-Aug;184(4):195-200.
190. Paget-Brown AO, Ngamtrakulpanit L, Smith A, Bunyan D, Hom S, Nguyen A, et al. Normative data for pH of exhaled breath condensate. Chest. 2006 Feb;129(2):426-30.
191. Accordino R, Visentin A, Bordin A, Ferrazzoni S, Marian E, Rizzato F, et al. Long-term repeatability of exhaled breath condensate pH in asthma. Respir Med. 2008 Mar;102(3):377-81.
192. Hunt J. Exhaled breath condensate pH assays. Immunol Allergy Clin North Am. 2007 Nov;27(4):597-606; vi.

193. Antus B, Barta I, Kullmann T, Lazar Z, Valyon M, Horvath I, et al. Assessment of exhaled breath condensate pH in exacerbations of asthma and chronic obstructive pulmonary disease: A longitudinal study. Am J Respir Crit Care Med. 2010 Dec 15;182(12):1492-7.
194. Prieto L, Orosa B, Barato D, Marin J. The effect of different periods of argon deaeration on exhaled breath condensate pH. J Asthma. 2011 May;48(4):319-23.
195. Liu L, Teague WG, Erzurum S, Fitzpatrick A, Mantri S, Dweik RA, et al. Determinants of exhaled breath condensate pH in a large population with asthma. Chest. 2011 Feb;139(2):328-36.
196. Hunt J, Yu Y, Burns J, Gaston B, Ngamtrakulpanit L, Bunyan D, et al. Identification of acid reflux cough using serial assays of exhaled breath condensate pH. Cough. 2006;2:3.
197. Effros RM, Murphy C, Hacker A, Schapira RM, Bongard R. Reduction and uptake of methylene blue from rat air spaces. J Appl Physiol. 1994 Sep;77(3):1460-5.
198. Nader-Djalal N, Knight PR, 3rd, Thusu K, Davidson BA, Holm BA, Johnson KJ, et al. Reactive oxygen species contribute to oxygen-related lung injury after acid aspiration. Anesth Analg. 1998 Jul;87(1):127-33.
199. Kaminsky DA, Mitchell J, Carroll N, James A, Soultanakis R, Janssen Y. Nitrotyrosine formation in the airways and lung parenchyma of patients with asthma. J Allergy Clin Immunol. 1999 Oct;104(4 Pt 1):747-54.
200. Kanazawa H, Shoji S, Yamada M, Fujii T, Kawaguchi T, Kudoh S, et al. Increased levels of nitric oxide derivatives in induced sputum in patients with asthma. J Allergy Clin Immunol. 1997 May;99(5):624-9.
201. Kullmann T, Barta I, Lazar Z, Szili B, Barat E, Valyon M, et al. Exhaled breath condensate pH standardised for CO2 partial pressure. Eur Respir J. 2007 Mar;29(3):496-501.
202. Mudway IS, Stenfors N, Duggan ST, Roxborough H, Zielinski H, Marklund SL, et al. An in vitro and in vivo investigation of the effects of diesel exhaust on human airway lining fluid antioxidants. Arch Biochem Biophys. 2004 Mar 1;423(1):200-12.
203. Blomberg A, Sainsbury C, Rudell B, Frew AJ, Holgate ST, Sandstrom T, et al. Nasal cavity lining fluid ascorbic acid concentration increases in healthy human volunteers following short term exposure to diesel exhaust. Free Radic Res. 1998 Jan;28(1):59-67.
204. Nordenhall C, Pourazar J, Ledin M-C, Levin J-O, Sandstrom T, Adelroth E. Diesel exhaust enhances airway responsiveness in asthmatic subjects. Eur Respir J. 2001 May 1, 2001;17(5):909-15.
205. Nel AE, Diaz-Sanchez D, Li N. The role of particulate pollutants in pulmonary inflammation and asthma: evidence for the involvement of organic chemicals and oxidative stress. Curr Opin Pulm Med. 2001 Jan;7(1):20-6.
206. Ghio AJ, Devlin RB. Inflammatory lung injury after bronchial instillation of air pollution particles. Am J Respir Crit Care Med. 2001 Aug 15;164(4):704-8.
207. Dick CA, Brown DM, Donaldson K, Stone V. The role of free radicals in the toxic and inflammatory effects of four different ultrafine particle types. Inhal Toxicol. 2003 Jan;15(1):39-52.
208. Tao F, Gonzalez-Flecha B, Kobzik L. Reactive oxygen species in pulmonary inflammation by ambient particulates. Free Radic Biol Med. 2003 Aug 15;35(4):327-40.
209. J. Gong RJL, S. Hussain, J. Zhang, K. Kelly-McNeil, P. Ohman-Strickland, A. Gow, H. Kipen. Validation Of Exhaled Breath Condensate Measures Of Oxidative And Nitrosative Stress As Markers Of Acute Traffic Pollution Effects. American Thoracic Society; Denver, Colorado2011.

210. Laumbach RJ, Kipen HM. Acute effects of motor vehicle traffic-related air pollution exposures on measures of oxidative stress in human airways. Ann N Y Acad Sci. 2010 Aug;1203:107-12.
211. Zhang JJ, McCreanor JE, Cullinan P, Chung KF, Ohman-Strickland P, Han IK, et al. Health effects of real-world exposure to diesel exhaust in persons with asthma. Res Rep Health Eff Inst. 2009 Feb(138):5-109; discussion 11-23.
212. Schluter MCWaD. Designing experiments. The analysis of biological data. Greenwood City, Colerado: Roberts and Company Publishers; 2009. p. 358-9.
213. Gordis L. Assessing the efficacy of preventive and therapeutic measures: randomized trials. In: Hall A, editor. Epidemiology. Fourth ed. Philadelphia, PA: Saunders Elsevier; 2009. p. 130-64.
214. Gordis L. From association to causation: deriving inferences from epidemiologic studies. In: Hall A, editor. Epidemiology. Philadelphia, PA: Saunders Elsevier; 2009. p. 227-64.
215. Ide T, Tsutsui H, Ohashi N, Hayashidani S, Suematsu N, Tsuchihashi M, et al. Greater oxidative stress in healthy young men compared with premenopausal women. Arterioscler Thromb Vasc Biol. 2002 Mar 1;22(3):438-42.
216. Block G, Dietrich M, Norkus EP, Morrow JD, Hudes M, Caan B, et al. Factors associated with oxidative stress in human populations. Am J Epidemiol. 2002 Aug 1;156(3):274-85.
217. Carlsten C, Melen E. Air pollution, genetics, and allergy: an update. Curr Opin Allergy Clin Immunol. 2012 Oct;12(5):455-60.
218. Heinrich J, Wichmann HE. Traffic related pollutants in Europe and their effect on allergic disease. Curr Opin Allergy Clin Immunol. 2004 Oct;4(5):341-8.
219. Nordling E, Berglind N, Melen E, Emenius G, Hallberg J, Nyberg F, et al. Traffic-related air pollution and childhood respiratory symptoms, function and allergies. Epidemiology. 2008 May;19(3):401-8.
220. Brauer M, Hoek G, Smit HA, de Jongste JC, Gerritsen J, Postma DS, et al. Air pollution and development of asthma, allergy and infections in a birth cohort. Eur Respir J. 2007 May;29(5):879-88.
221. Morgenstern V, Zutavern A, Cyrys J, Brockow I, Koletzko S, Kramer U, et al. Atopic diseases, allergic sensitization, and exposure to traffic-related air pollution in children. Am J Respir Crit Care Med. 2008 Jun 15;177(12):1331-7.
222. Sydbom A, Dahlen SE. Experimental studies on the effects of diesel exhaust emissions. Scand J Work Environ Health. 2000;26 Suppl 1:28-38.
223. Peroni DG, Bodini A, Corradi M, Coghi A, Boner AL, Piacentini GL. Markers of oxidative stress are increased in exhaled breath condensates of children with atopic dermatitis. Br J Dermatol. 2012 Apr;166(4):839-43.
224. Ercan H, Birben E, Dizdar EA, Keskin O, Karaaslan C, Soyer OU, et al. Oxidative stress and genetic and epidemiologic determinants of oxidant injury in childhood asthma. J Allergy Clin Immunol. 2006 Nov;118(5):1097-104.
225. Gilliland FD, McConnell R, Peters J, Gong H, Jr. A theoretical basis for investigating ambient air pollution and children's respiratory health. Environ Health Perspect. 1999 Jun;107 Suppl 3:403-7.
226. Islam T, Berhane K, McConnell R, Gauderman WJ, Avol E, Peters JM, et al. Glutathione-S-transferase (GST) P1, GSTM1, exercise, ozone and asthma incidence in school children. Thorax. 2009 Mar;64(3):197-202.

227. Hayes JD, Strange RC. Potential contribution of the glutathione S-transferase supergene family to resistance to oxidative stress. Free Radic Res. 1995 Mar;22(3):193-207.
228. Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol. 1995;30(6):445-600.
229. Alexeeff SE, Litonjua AA, Wright RO, Baccarelli A, Suh H, Sparrow D, et al. Ozone exposure, antioxidant genes, and lung function in an elderly cohort: VA normative aging study. Occup Environ Med. 2008 Nov;65(11):736-42.
230. Chen TM, Gokhale J, Shofer S, Kuschner WG. Outdoor air pollution: particulate matter health effects. Am J Med Sci. 2007 Apr;333(4):235-43.
231. Gilliland FD, Li YF, Saxon A, Diaz-Sanchez D. Effect of glutathione-S-transferase M1 and P1 genotypes on xenobiotic enhancement of allergic responses: randomised, placebo-controlled crossover study. Lancet. 2004 Jan 10;363(9403):119-25.
232. Gilliland FD, Li YF, Gong H, Jr., Diaz-Sanchez D. Glutathione s-transferases M1 and P1 prevent aggravation of allergic responses by secondhand smoke. Am J Respir Crit Care Med. 2006 Dec 15;174(12):1335-41.
233. Panasevich S, Lindgren C, Kere J, Wickman M, Pershagen G, Nyberg F, et al. Interaction between early maternal smoking and variants in TNF and GSTP1 in childhood wheezing. Clin Exp Allergy. 2010 Mar;40(3):458-67.
234. Islam T, McConnell R, Gauderman WJ, Avol E, Peters JM, Gilliland FD. Ozone, oxidant defense genes, and risk of asthma during adolescence. Am J Respir Crit Care Med. 2008 Feb 15;177(4):388-95.
235. Li JS, Liu QJ, Wang P, Li HC, Wei CH, Guo CH, et al. Absence of association between two insertion/deletion coding genetic polymorphisms of TIM-1 gene and asthma in Chinese Han population. Int J Immunogenet. 2006 Dec;33(6):417-22.
236. Melen E, Bruce S, Doekes G, Kabesch M, Laitinen T, Lauener R, et al. Haplotypes of G protein-coupled receptor 154 are associated with childhood allergy and asthma. Am J Respir Crit Care Med. 2005 May 15;171(10):1089-95.
237. Davila I, Mullol J, Bartra J, Del Cuvillo A, Ferrer M, Jauregui I, et al. Effect of pollutants upon patients with respiratory allergies. J Investig Allergol Clin Immunol. 2007;17 Suppl 2:9-20.
238. Castro-Giner F, Bustamante M, Ramon Gonzalez J, Kogevinas M, Jarvis D, Heinrich J, et al. A pooling-based genome-wide analysis identifies new potential candidate genes for atopy in the European Community Respiratory Health Survey (ECRHS). BMC Med Genet. 2009;10:128.
239. Cruz MJ, Sanchez-Vidaurre S, Romero PV, Morell F, Munoz X. Impact of age on pH, 8-isoprostane, and nitrogen oxides in exhaled breath condensate. Chest. 2009 Feb;135(2):462-7.
240. Keaney JF, Jr., Larson MG, Vasan RS, Wilson PW, Lipinska I, Corey D, et al. Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham Study. Arterioscler Thromb Vasc Biol. 2003 Mar 1;23(3):434-9.
241. Vincent HK, Innes KE, Vincent KR. Oxidative stress and potential interventions to reduce oxidative stress in overweight and obesity. Diabetes Obes Metab. 2007 Nov;9(6):813-39.
242. Aligne CA, Auinger P, Byrd RS, Weitzman M. Risk factors for pediatric asthma. Contributions of poverty, race, and urban residence. Am J Respir Crit Care Med. 2000 Sep;162(3 Pt 1):873-7.
243. Litonjua AA, Carey VJ, Weiss ST, Gold DR. Race, socioeconomic factors, and area of residence are associated with asthma prevalence. Pediatr Pulmonol. 1999 Dec;28(6):394-401.

244. Schwartz J, Gold D, Dockery DW, Weiss ST, Speizer FE. Predictors of asthma and persistent wheeze in a national sample of children in the United States. Association with social class, perinatal events, and race. Am Rev Respir Dis. 1990 Sep;142(3):555-62.
245. Wissow LS, Gittelsohn AM, Szklo M, Starfield B, Mussman M. Poverty, race, and hospitalization for childhood asthma. Am J Public Health. 1988 Jul;78(7):777-82.
246. Ray NF, Thamer M, Fadillioglu B, Gergen PJ. Race, income, urbanicity, and asthma hospitalization in California: a small area analysis. Chest. 1998 May;113(5):1277-84.
247. Richelle M, Turini ME, Guidoux R, Tavazzi I, Metairon S, Fay LB. Urinary isoprostane excretion is not confounded by the lipid content of the diet. FEBS Letters. [Clinical Trial Research Support, Non-U.S. Gov't]. 1999 Oct 8;459(2):259-62.
248. Gopaul NK, Halliwell B, Anggard EE. Measurement of plasma F2-isoprostanes as an index of lipid peroxidation does not appear to be confounded by diet. Free Radic Res. 2000 Aug;33(2):115-27.
249. Gopaul NK, Zacharowski K, Halliwell B, Anggard EE. Evaluation of the postprandial effects of a fast-food meal on human plasma F(2)-isoprostane levels. Free Radic Biol Med. 2000 Mar 1;28(5):806-14.
250. Soyer OU, Dizdar EA, Keskin O, Lilly C, Kalayci O. Comparison of two methods for exhaled breath condensate collection. Allergy. 2006 Aug;61(8):1016-8.
251. Company RR. Library: R Tube and EBC. Virginia: Respiratory Research Company; 2009 [cited 2011 Feb 27]; Available from: http://www.rtube.com/scientific-library.htm.
252. Do R. Acid-Base Equilibrium in Exhaled Breath Condensate of Grain Elevator Workers. Vancouver: British Columbia; 2005.
253. Unknown. Simple, linear, mixed effects model. 2010 [cited 2012 November 22]; Available from: http://lme4.r-forge.r-project.org/book/Ch1.pdf.
254. Hornung RWaLDR. Estimation of Average Concentration in the Presence of Nondetectable Values. App Occup Environ Hyg. 1990;5:46-51.
255. Zhao JJ, Shimizu Y, Dobashi K, Kawata T, Ono A, Yanagitani N, et al. The relationship between oxidative stress and acid stress in adult patients with mild asthma. J Investig Allergol Clin Immunol. 2008;18(1):41-5.
256. Rowland AS, Baird DD, Long S, Wegienka G, Harlow SD, Alavanja M, et al. Influence of medical conditions and lifestyle factors on the menstrual cycle. Epidemiology. 2002 Nov;13(6):668-74.
257. Brunetti L, Francavilla R, Tesse R, Fiermonte P, Fiore FP, Lore M, et al. Exhaled breath condensate cytokines and pH in pediatric asthma and atopic dermatitis. Allergy Asthma Proc. 2008 Sep-Oct;29(5):461-7.
258. Ratnawati, Morton J, Henry RL, Thomas PS. Exhaled breath condensate nitrite/nitrate and pH in relation to pediatric asthma control and exhaled nitric oxide. Pediatr Pulmonol. 2006 Oct;41(10):929-36.
259. Ojoo JC, Mulrennan SA, Kastelik JA, Morice AH, Redington AE. Exhaled breath condensate pH and exhaled nitric oxide in allergic asthma and in cystic fibrosis. Thorax. 2005 Jan;60(1):22-6.
260. Varnai VM, Ljubicic A, Prester L, Macan J. Exhaled breath condensate pH in adult Croatian population without respiratory disorders: how healthy a population should be to provide normative data? Arh Hig Rada Toksikol. 2009 Mar;60(1):87-97.
261. Pagano MaKG. Analysis of Variance. In: Crockett C, editor. Principles of Biostatistics. Second ed. Belmont, CA: Brooks/Cole; 2000. p. 285-301.

262. Schoenfeld D. Statistical considerations for a cross-over study where the outcome is a measurement. [cited 2012 December 21]; Available from: http://hedwig.mgh.harvard.edu/sample_size/js/js_crossover_quant.html.
263. Diaz-Sanchez D, Rumold R, Gong H, Jr. Challenge with environmental tobacco smoke exacerbates allergic airway disease in human beings. J Allergy Clin Immunol. 2006 Aug;118(2):441-6.
264. Ritz SA, Wan J, Diaz-Sanchez D. Sulforaphane-stimulated phase II enzyme induction inhibits cytokine production by airway epithelial cells stimulated with diesel extract. Am J Physiol Lung Cell Mol Physiol. 2007 Jan;292(1):L33-9.
265. Riedl MA, Landaw EM, Saxon A, Diaz-Sanchez D. Initial high-dose nasal allergen exposure prevents allergic sensitization to a neoantigen. J Immunol. 2005 Jun 1;174(11):7440-5.
266. Diaz-Sanchez D, Penichet-Garcia M, Saxon A. Diesel exhaust particles directly induce activated mast cells to degranulate and increase histamine levels and symptom severity. J Allergy Clin Immunol. 2000 Dec;106(6):1140-6.
267. Diaz-Sanchez D, Garcia MP, Wang M, Jyrala M, Saxon A. Nasal challenge with diesel exhaust particles can induce sensitization to a neoallergen in the human mucosa. J Allergy Clin Immunol. 1999 Dec;104(6):1183-8.
268. Fujieda S, Diaz-Sanchez D, Saxon A. Combined nasal challenge with diesel exhaust particles and allergen induces In vivo IgE isotype switching. Am J Respir Cell Mol Biol. 1998 Sep;19(3):507-12.
269. Diaz-Sanchez D, Tsien A, Casillas A, Dotson AR, Saxon A. Enhanced nasal cytokine production in human beings after in vivo challenge with diesel exhaust particles. J Allergy Clin Immunol. 1996 Jul;98(1):114-23.
270. Effros RM, Casaburi R, Porszasz J, Rehan V. Why conventional exhaled breath condensate pH studies cannot provide reliable estimates of airway acidification. Chest. 2011 Oct;140(4):1099.
271. Effros RM, Casaburi R, Porszasz J, Rehan VK. Assessment of exhaled breath condensate pH. Am J Respir Crit Care Med. 2011 Apr 1;183(7):952; author reply -3.
272. Kostikas K, Papatheodorou G, Ganas K, Psathakis K, Panagou P, Loukides S. pH in expired breath condensate of patients with inflammatory airway diseases. American Journal of Respiratory & Critical Care Medicine. [Comparative Study]. 2002 May 15;165(10):1364-70.
273. Diaz-Sanchez D, Tsien A, Fleming J, Saxon A. Effect of topical fluticasone propionate on the mucosal allergic response induced by ragweed allergen and diesel exhaust particle challenge. Clin Immunol. 1999 Mar;90(3):313-22.
274. Diaz-Sanchez D, Tsien A, Fleming J, Saxon A. Combined diesel exhaust particulate and ragweed allergen challenge markedly enhances human in vivo nasal ragweed-specific IgE and skews cytokine production to a T helper cell 2-type pattern. J Immunol. 1997 Mar 1;158(5):2406-13.
275. Diaz-Sanchez D, Jyrala M, Ng D, Nel A, Saxon A. In Vivo Nasal Challenge with Diesel Exhaust Particles Enhances Expression of the CC Chemokines Rantes, MIP-1[alpha], and MCP-3 in Humans. Clinical Immunology. 2000 2000/11;97(2):140-5.
276. Milne GL, Musiek ES, Morrow JD. F2-isoprostanes as markers of oxidative stress in vivo: an overview. Biomarkers. 2005 Nov;10 Suppl 1:S10-23.
277. Basu S. F2-isoprostanes in human health and diseases: from molecular mechanisms to clinical implications. Antioxid Redox Signal. 2008 Aug;10(8):1405-34.

278. Fam SS, Morrow JD. The isoprostanes: unique products of arachidonic acid oxidation-a review. Curr Med Chem. 2003 Sep;10(17):1723-40.
279. Montuschi P, Barnes PJ. Analysis of exhaled breath condensate for monitoring airway inflammation. Trends Pharmacol Sci. 2002 May;23(5):232-7.
280. Sircar D, Subbaiah PV. Isoprostane measurement in plasma and urine by liquid chromatography-mass spectrometry with one-step sample preparation. Clin Chem. 2007 Feb;53(2):251-8.
281. Montuschi P, Ragazzoni E, Valente S, Corbo G, Mondino C, Ciappi G, et al. Validation of 8-isoprostane and prostaglandin E(2) measurements in exhaled breath condensate. Inflamm Res. 2003 Dec;52(12):502-7.
282. Dworski R, Murray JJ, Roberts LJ, 2nd, Oates JA, Morrow JD, Fisher L, et al. Allergen-induced synthesis of F(2)-isoprostanes in atopic asthmatics. Evidence for oxidant stress. Am J Respir Crit Care Med. 1999 Dec;160(6):1947-51.
283. Dworski R, Roberts LJ, 2nd, Murray JJ, Morrow JD, Hartert TV, Sheller JR. Assessment of oxidant stress in allergic asthma by measurement of the major urinary metabolite of F2-isoprostane, 15-F2t-IsoP (8-iso-PGF2alpha). Clin Exp Allergy. 2001 Mar;31(3):387-90.
284. Knoll JE. Estimation of the Limit of Detection in Chromatography. Journal of Chromatographic Science. 1985;23:422-5.
285. Carlsten C, Melen E. Air pollution, genetics, and allergy: an update. Curr Opin Allergy Clin Immunol. 2012 Aug 9.
286. Helsel DR. Fabricating data: how substituting values for nondetects can ruin results, and what can be done about it. Chemosphere. 2006 Dec;65(11):2434-9.
287. Finkelstein MM, Verma DK. Exposure estimation in the presence of nondetectable values: another look. AIHAJ. 2001 Mar-Apr;62(2):195-8.

Appendix A Method Development for the Analysis of 8-Isoprostane in Exhaled Breath Condensate

1.1 Introduction

1.2 8-isoprostane and oxidative stress

Isoprostanes are prostaglandin-like compounds formed from the free radical catalyzed peroxidation of arachidonic acid, a mechanism independent of the cyclooxygenase (50). They have been widely used as stable markers of oxidative stress in many diseases (46-47, 54, 154, 276-278).

1.3 EBC

Exhaled breath condensate (EBC) is an easy to use, portable, non-invasive method of assessing airway status in humans (22, 39-40, 42-43, 133, 138, 279). EBC can contribute to the understanding of the physiological and pathophysiological processes in the lungs in several ways (22, 39, 41-43, 133, 138, 279). For instance, EBC levels of 8-isoprostane have been shown to be a reliable marker for airway oxidative stress (45-47, 50, 54).

1.4 Isoprostanes in EBC

The most widely studied isoprostane group are the F2-isoprostanes since they are chemically stable compounds, are specific products of peroxidation and are detectable in all normal biological fluids and tissues (46, 277). Levels of 8-iso PGF2α, the most commonly characterized isoprostane of the 64 other isomers, have been characterized in exhaled breath condensate in respiratory diseases such as asthma (45), COPD (156), cystic fibrosis (157), interstitial lung disease (158) and acute lung injury/adult respiratory distress syndrome (132).

1.5 Various measurement methods (GC, ELISA, LC)

Gas chromatography and mass spectrometry (GC/MS) is the analytical method of reference for isoprostane analysis in biological fluids; however, this method is time consuming and expensive (46, 167). Gas chromatography (GC) methods typically use solid-phase extraction (SPE) to separate the analyte of interest from the sample matrix; however, it is typically laborious and requires much sample preparation (167). GC may also be biased by artifact generation as shown by Morrow et al. 1995 where results may incorporate a mixture of four isomers rather than just

8-iso PGF2α (167-168). Analysis has also been performed using liquid chromatography with tandem mass spectrometry (LC-MS/MS), allowing a more specific measurement of 8-isoprostane when coupled with the GC SPE methodology and with less sample preparation (152, 280). Finally, 8-iso PGF2α can also be measured by immunoaffinity assays such as enzyme-linked immunoselective assay (ELISA), enzyme immunoassay (EIA) and radio immunoassays (RIA) which, although widely used, offers less reliability than the previously described methods (54, 167, 173, 281). Overall, LC-MS/MS has been shown to be a superior method for 8-iso PGF2α in biological fluids as it gives more specificity compared to ELISA methods as well as offers less intensive sample preparation than GC/MS(167).

1.6 Rationale for Method Development

Most studies using LC-MS/MS have identified 8-iso PGF2α in urine (50, 52, 54, 167, 172-173, 280, 282-283); however, to our knowledge, only two studies developed an LC-MS/MS method for 8-isoprostane analysis in EBC.

Briefly, Syslova *et al*., 2008 developed a liquid chromatography method identifying 8-iso PGF2α in EBC using an immunoaffinity separation step prior to liquid chromatography-electrospray tandem mass spectrometry (LC/ESI-MS/MS) analysis (152). Wang *et al*., 2010 used an automated one-line solid phase extraction (SPE) LC-ESI-MS/MS) method which, similar to our method (described below), utilized 8-iso-PGF2α-d4 internal standard for quantitative determination (166).

The aforementioned techniques require the use of SPE cleanup methods, which are laborious, time consuming and presents a major source of error in analytical methods (42, 47). EBC may be directly injected into a liquid chromatograph because the concentration of matrix interferents is low enough that they may be separated by LC.

In order to correctly analyse 8-isoprostane in EBC, our lab had to develop its own analysis methods using the instrumentation available. Saenger *et al*., 2007, a method analysing 8-isoprostane in urine, was used as an initial analysis method since it had similar instrument set ups and was then optimized in order to match Syslova *et al*., 2008 and Wang *et al*., 2010 EBC

analysis methods. We have, therefore, developed a faster, more sensitive and precise method of assessing 8-isoprostane levels in EBC (please see Table 4).

2.1 Materials and Method

2.1.2 Materials

8-iso-PGF2α (solid) and 8-iso-PGF2α -d4 (25 ug in 25uL methyl acetate) were purchased from Cayman Chemical Co, Ann Arbor, MI, USA and were stored at -80c. Acetonitrile used was high performance liquid chromatography grade (Fisher, Fair Lawn, NJ, USA) and 18ohm water was generated by a Barnstead Nanopure Ultrapure Water System.

2.1.3 Standard prep and calibration procedures

Calibration curves were prepared fresh each day of analysis. The stock solution for samples 1-337 was 2800 pg/mL 8-iso-PGF2α and for all samples thereafter the stock concentration was 2666 pg/mL. 10 point calibration curves were prepared by the standard dilutions method with a 1:2 dilution ratio (with the highest calibration point being pure stock), and this curve was supplemented by 5 additional points on the low end of the calibration curve, resulting in an overall 15 point calibration curve. 25 uL internal standard solution was then added to each 500 uL calibration point.

2.1.4 Sample preparation

EBC samples were stored at -80°C and thawed in room temperature water prior to processing. LC/MS/MS samples were prepared in the following way: 15uL internal standard solution (400pg/mL 8-iso-PGF2alpha-d4 in water) was pipetted into LC vials with 350uL inserts and then 300uL exhaled breath condensate was added.

2.1.5 LC-MS/MS instrumentation and analysis

An Agilent Technologies (Mississauga, ON Canada) 1200 Series liquid chromatograph performed the separations on an Agilent Zorbax SB-C18 2.1x50mm column with 1.8uM packing heated to 60C. A frit protected the column. Detection was performed by an Agilent Technologies 6430 triple quadrupole mass spectrometer operating multiple reaction monitoring (MRM) with electrospray ionization in negative mode (ESI-). The sample was injected with a neat water mobile phase and gradient to 85:15 acetonitrile: water was effected per Table 1. The flow was

0.450 ml/min throughout the method. Stop time was 8 minutes and post time was 2 minutes. The mass transitions monitored by the triple quadrupole were 353.2→193.2 for 8-*iso*-prostaglandin F2αand 357.3→197.2 for 8-*iso*prostaglandin F2α-d4. The qualifier mass transition for 8-*iso*-prostaglandin F2α was 353.2→309.3. Delta Electron Multiplier Voltage (EMV -) was 600 and EMV+ was 0 V. Dwell time was 200 ms, fragmentor was 45V and collision energy was 16 V for all mass transitions. Nebulizing gas temperature was 350C, gas flow was 12 l/min, nebulizer pressure was 55 psi and capillary voltage was 5000+/6000- V.

Table 32: Effected water conditions

Time (min)	MeCN %
0.00	0.0
2.00	0.0
5.00	85.0
6.00	85.0
7.00	0.0

2.1.6 LC-MS/MS method validation

The precision, linearity, limit of detection and recovery were all evaluated to assess the performance characteristics of the method. Inter-assay precision was assessed for 8-iso-PGF2α using a minimum of 9 controls over multiple runs per day. Intra-assay precision utilized the same controls with a minimum of 15-20 times within a run. Assay linearity was determined by serially diluting spiked exhaled breath condensate samples with deuterated 8-iso PGF2α and comparing the results with theoretical values. 10 replicate measurements of blanks were used to determine the lower limit of detection, defined as the mean of the blank plus 3SD. The limit of quantification was defined as the mean of the blank plus 10SD. Analytical recovery was determined by spiking 8-iso-PGF2α in EBC at high and low concentrations (700 and 350 pg/ml) and analyzing the samples.

2.1.7 Human Study

15 atopic and 8 non atopic individuals completed a crossover study designed to assess the mechanism of diesel exhaust's effects on the human airway. Non-smoking males and females aged 19-49 years of age are eligible to participate. Atopic was defined as a positive result (welt larger than 3mm from the positive histamine control) during a skin prick test. Exclusion criteria include: 1) pregnancy/ breastfeeding; 2) using inhaled corticosteroids, 3) using their

bronchodilator medication more than 3 times per week, 4) taking any vitamins A, C, or E, 5) co-existing medical conditions, and 6) taking part in another study involving taking medications.

EBC was obtained from subjects before and 2-, 6- and 30 hours post-exposure using standard methodology. Briefly, seated participants breathed normally for 15 min into an R Tube™ (EBC collection system, Respiratory Research, Inc, Charlottesville, Virginia, USA). Aliquoted 500µL samples were temporarily stored at -20°C and subsequently transferred to a -80°C freezer until further analysis. 8-isoprostane levels were log normally distributed; they were log-transformed in order to perform parametric statistics.

The objective of the human study was to assess airway oxidative stress in a controlled diesel exhaust exposure. For the purpose of this study, only one baseline result was reported; all other results will be submitted for future publication.

3.1 Results and discussion

3.1.1 Method development parameters

10 batches of samples were run using this method. Table 2 shows batches 1 and 10 with the overall average and range of method parameters for all batches run.

Table 2 limit of detections (LOD) and limits of quantification (LOQ) refer to the empirical method of assessing limits. The empirical method of assessing LOD/LOQs takes a highly concentrated sample and serial dilutes it until the sample peak can no longer be discerned from the background noise (174, 284). This is opposed to the statistical method which typically yields the instrument LOD/LOQ which refers to multiplying the blank standard deviations by 3 for the LOD and 10 for the LOQ. Statistical LOD/LOQs are more often reported in papers while they do not necessarily reflect the empirical LOD/LOQs which are vital in quantifying actual study samples. To our knowledge, we are the first to describe empirical limits for quantifying 8-isoprostane levels in EBC by liquid chromatography.

Table 33 Developed method parameters

Batch No.	Sample No.	LOD (pg/ml)	LOQ (pg/ml)	Recovery (%)	Precision (%)	Linearity (R^2)
1	1-94	23.7	79.1	101	39.5	0.998
10	610-635	59.8	199	101	23.2	0.996
Range	1-635	23.7-102	79.1-341	91.5-105	23.2-52.0	0.968-0.999
Average	----	46.4	152	100	30	0.993

LOD=limit of detection, LOQ=limit of quantification.

Linearity and sensitivity of the method are two important factors in method development. Calibration curves were constructed using the least square regression of analyte concentration versus the peak area ratio. The calibration curves had average correlation coefficients of 0.993. Comparison of the slopes and intercepts showed no evidence of a significant matrix effect, as the slopes and intercepts, were similar. Indeed, slopes were maintained between 4.31 and 5.45 in magnitude, less than Wang et al. 2010. Our instrument LOD was 1.44pg/ml (statistical methodology of LOD assessment).

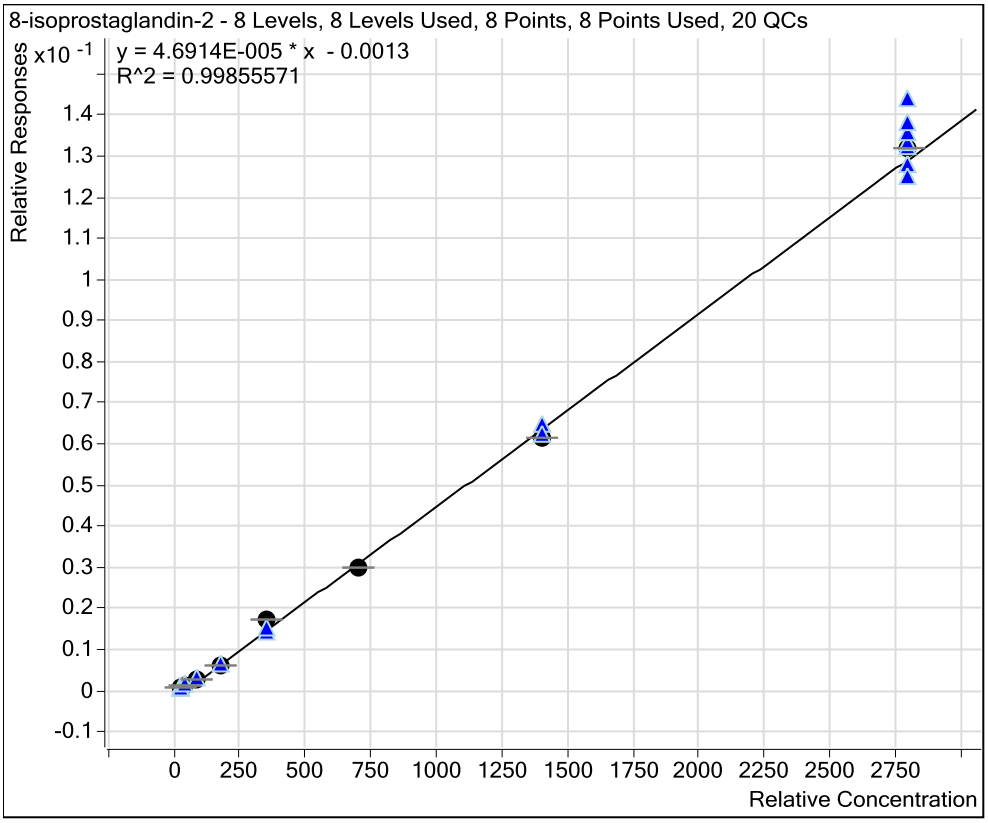

Figure 14. Calibration curve for present method.

3.1.2 Reliability

Over the span of 90 days, the method yielded a 5% loss of 8-isoprostane standard at -20°C. At -80°C over the span of 90 days, samples 1-337 had an 0.8% gain while samples 338-635 had a 4.5% loss.

Intra- and inter-day coefficients of variance were low, as shown in Table 3, mean intra-day coefficients of variations of 5.7 and 2.8. Mean inter-day coefficients of variation of 2.2 and 1.4. Recoveries were generally higher than observed in Syslova et al. 2008 and Wang et al. 2010 methods, given 96.3 and 98.6 mean % recoveries for the two concentrations, respectively.

After 337 samples, a new stock solution was prepared. The two concentrations used were 2800pg/ml for samples 1-337 and 2666pg/ml for samples 338-635.

Table 34: Intra and inter-day reproducibility of the LC-MS/MS quantitation of 8-iso-PGF2α in EBC

Concentration (pg/ml)		Intraday (n=9)		Interday (n=25)	
Expected	Measured Means (SD)	Mean CV (%)	Recovery (%)	Mean CV (%)	Recovery (%)
2800	2697 (152)	5.7	96.3	2.2	96.3
2666	2645 (93.1)	2.8	99.2	1.4	98.6

CV=coefficient of variation, SD=standard deviation.

This method reconfirms the strength of LC-MS/MS methods over immunoaffinity assays with better reproducibility and reliability. Indeed, ELISA kits by Cayman Inc offer intra-assay coefficient of variation (CV) of between 6.4% and 34.8% and an inter-assay CV of 9.6-39.1% (measured between 0.8-500pg/ml) (167); which are higher than the ones presented in this method.

3.1.3 Environmental matrix method development

Example chromatograms of spiked EBC samples are shown in Figure 1A to 1D. The LC-MS method was originally developed and optimized for 8-iso in neat water, but spiked EBC samples showed that the EBC matrix resulted in split peaks: this is shown in 1A and 1B. The organic phase was switched from methanol to acetonitrile and the gradient was re-optimized. Ion source

and triple quadrupole (QQQ) settings were manually optimized to maximize the signal: dwell time was optimized from 80 ms to 200 ms, fragmentor from 125 to 45 and collision energy from 12 to 16. Previously the source and QQQ settings were optimized by Agilent Optimizer software. EBC presents a difficult challenge chromatographically because, though a protective frit was used and replaced when necessary, backpressure from the column increased slowly throughout the six months of study samples. It is thought that perhaps precipitation of proteins upon gradient with acetonitrile could be the cause of this slow plugging of the column, though acetonitrile usually precipitates larger proteins (larger than ~20 KDa), which would generally not be present in EBC due to their low volatility. Chromatography did not suffer throughout the runs, though signal decreased and LOD rose.

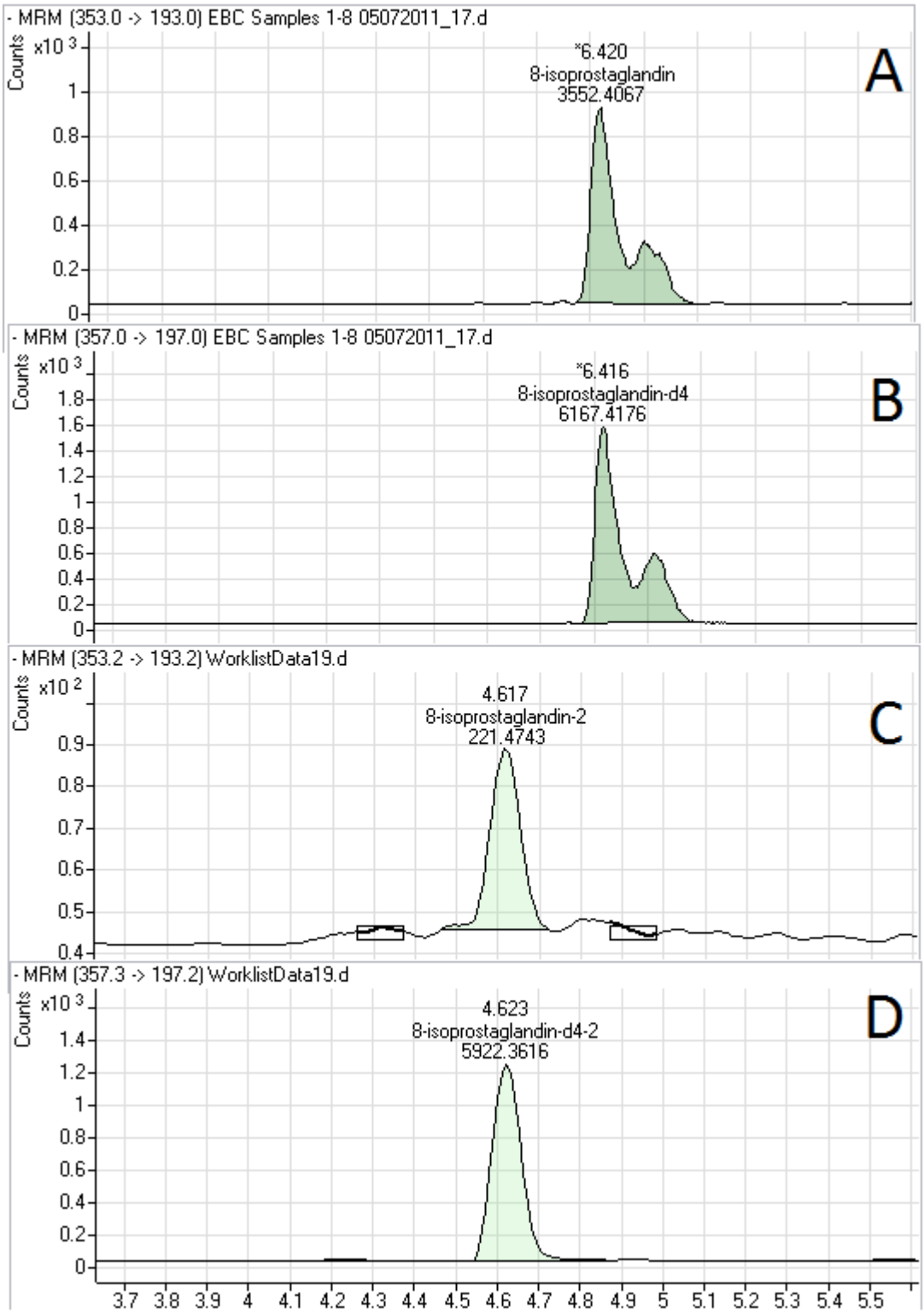

Figure 15. LC-MS/MS chromatogram of an exhaled breath condensate sample spiked with A) 1011.6 pg/ml standard solution of **8**-iso-PGF2α, B) 2012.3 pg/ml internal standard 8-iso-PGF2α-d4, using the pre-optimized method. LC-MS/MS chromatogram of an exhaled breath condensate sample spiked with C) 744.5 pg/ml standard solution of **8**-iso-PGF2α, D) internal standard 8-iso-

PGF2α-d4, using the optimized method. Concentrations of standard were 2800 pg/mL for sample 1-337 and 2666pg/ml for samples 338-635 of 8-iso-PGF2α, internal solutions was 400 pg/mL of 8-iso-PGF2α-d4 in water.

3.1.4 Human study

The developed method was applied to the analysis of the EBC sample from a larger human clinical study. Our method compares well with the two other liquid chromatography methods application to identifying 8-isoprostane in EBC, as shown in Table 4.

Table 35: Methods for analyzing 8-isoprostane in EBC using liquid chromatography

Study	Method Specification	LOD (pg/ml)	Intra-day CV (%)	Inter-day CV (%)	Recovery (%)	Linearity	Ref.
Syslova *et al.*2008	LC-MS/MS	1	N/A	N/A	90.4-95.6	0.9988 [a]	(152)
Wang *et al.*2010	On-line SPE-LC-MS/MS	1	6.5-8.0	5.2-6.3	98.9-99.9	0.998	(166)
Present method		1.4	2.8-5.7	1.4-2.2	96.3-99.2	0.993	

[a] note: this linearity was derived by using pooled EBC versus individuals samples.

The development of this method was used in a clinical study evaluating the effects of diesel exhaust exposure in humans; with particular emphasis on exhaled breath condensate marker of oxidative stress, 8-isoprostane.

Allergic status is important to discuss, as a recent review paper synthesized the most recent evidence linking air pollution to sensitization (285). There has been increasing evidence on the role of air pollution with respect to allergic sensitization in children (218). Indeed, the European birth cohort studies have suggested that exposure to air pollution during the first years of life or prior to may incur allergic sensitization (219-221). These studies have also suggested the link between air pollution and childhood asthma. Experimental studies have identified diesel exhaust particles (DEPs) to induce IgE responses (222). Thus, through these ideas, we hypothesize that a positive atopic status will be associated with increased 8-isprostane levels at baseline.

Both groups, atopic and non-atopic individuals, were of similar average age (31±8yrs and 28±6yrs, respectively) and similar gender distribution (8 males, 7 females; 5 males, 3 females, respectively).

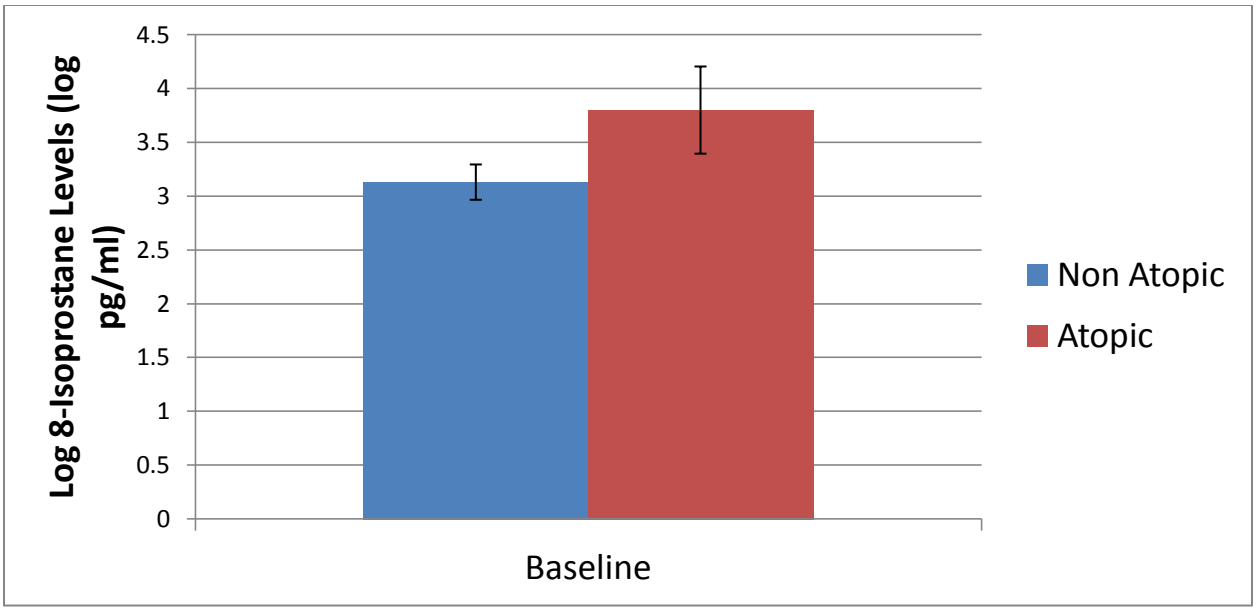

Figure 16. EBC concentration of log 8-isoprostane from atopic and non atopic subjects at baseline. Error bars represent standard error of the group, (standard deviation/sqrt[N], p=0.07).

Borderline significance was found for baseline levels of log 8-isoprostane with a p-value of 0.07. This result suggests that atopic individuals may have increased oxidative stress as compared to non atopic individuals perhaps reflecting a characteristic of underlying biological condition.

3.1.5 Limitations

There may be loss of sensitivity and specificity due to the direct injection of EBC into the instrument for the human study samples. While the SPE method, despite being more laborious, may be more appropriate for human study samples.

Syslova et al. 2008 was able to add a freeze-thaw stability assessment to their method. Syslova showed that the number of freeze thaw cycles decreased the total amount of 8-isoprostane in the EBC samples by 8-9% per cycle (152). During our study, an equipment fail lead to our samples being left at -20°C over a two day period. Unfortunately, we were unable to assess the overall damage this had on our samples. Our long term stability data suggests little effect was sustained by the samples; however, this was only for the samples which had internal standard in them. We were not able to characterize pure EBC study samples for the effect of the freeze-thaw on 8-

isoprostane levels. This freeze-thaw issue may have contributed to not seeing significance in the baseline 8-isoprostane results in atopic individuals.

The human study primary hypotheses were not shown in this publication as they will be drafter for another submission; however, the results show borderline statistical significance between atopic and non atopic individuals at baseline. This is an interesting finding since it may reflect underlying biological difference between atopic and non atopic individuals.

4.1 Conclusion

A method for the analysis of 8-isprostane in EBC sample was optimized and results were reported in this paper. This method development for 8-isoprostane detection (8-iso-PGF2α) in EBC showed comparable results to two other detection methods while having smaller sample preparation and facile method. While the lower limit of detection was slightly higher than Syslova and Wang methods, our percent recoveries and linearity were comparable while our intra and inter-day coefficients of variation were lower.

Overall, the method developed in this paper offers comparable results with less sample preparation and easier handling methods for other laboratories to follow; thus decreasing time and costs spent on laborious methods. Furthermore, this method uses less instrument time due to a relatively short chromatographic method of 7 minutes. Wang et al. 2010 utilized a total run time of 12 minutes; while most gradient methods are over ten minutes (166).

In conclusion, we present a valid method for the assessment of 8-isoprostane in EBC samples.

5. Conflicts of interest: none to declare.

6. Acknowledgements: people who funded.

7. Funding provided by WorkSafe BC.

Appendix B Rationale for Normality Assessment of 8-isoprostane Data

Censured data refers to values in a sampled data set which were evaluated as non-detectable, i.e. under the limit of detection (LOD) as defined by sampling and analytical method (254). Nondetects (NDs) are low-level concentrations of organic or inorganic chemicals with values known only to be somewhere between zero and the laboratory's detection/reporting limits.

There are many ways to treat censored data, the most prominent of which is the substitution method.
Substituting non detected values with (254, 286-287):

1. 0, mean of censored data substituted for 0 would be smaller than the true mean of the distribution.
2. LOD, mean of censored data substituted for L would be bigger than the true mean of the distribution, this is more of a conservative method to determine mean exposures and make sure they are in compliance.
3. LOD/2, follows the assumption that data below the L follow a uniform distribution, i.e. the values between 0 and L are all at equal probabilities of occurring.
4. LOD/root2, when the proportion of non detectable is such that the l is NOT bigger than the more, the general shape of the left side of the lognormal distribution is better approximated by a right triangle rather than a rectangle. Must be used when data is not highly skewed.

Excluding non detects from the data analysis (considered by Hesel to be the worst method for data analysis because it creates a strong upward bias for the distribution parameters) can also be done but is not recommended as it can bias results from subsequent analyses (286).

The method best suited for these type of analyses would be the Monte Carlo simulation. This substitution method replaces non detects with values randomly selected from a pre-existing known distribution. In our case, most values are below the instrumental limit of detection; however, it seems biological unfeasible that levels would ever reach a 0 pg/ml for 8-isoprostane. Since asthmatics typically have higher levels of 8-isoprostane than healthy volunteers in their

EBC (45), our distribution values would have been taken from healthy volunteers such as in the Montuschi *et al.* 1999 paper (45).

However, this method requires a statistical sampling methodology that was beyond the scope of this thesis, as such another substitution method was chosen.

Hornung and Reed offer that the LOD/2 substitution should only be used when the sample data is highly skewed, with a geometric standard deviation larger than 3.0 and when the proportion of nondetects in the data is larger than 30% (254).

Otherwise, the LOD/squareroot2 (LOD/sqrt2) should only be used when the data is not highly skewed, has a proportion of non detects smaller than 30% of the data, but more importantly, should be used when the proportion of nondetectables is such that the **limit of detection is not greater than the mode**.

Furthermore, Hornung and Reed explain that once you graph the detectable data as a histogram, if the frequency of the data steadily declines in every interval; then, method L/2 should be used. As opposed to using method L/root2 if the frequency in the first or second interval is less than one or more of the subsequent intervals.

Applying these concepts to our 8-isoprostane data, we come to the following rationale:

The 8-isprostane has 78 of its 537 data points below the instrumental limit of detection thereby yielding a 14.5% censored data set. For this characteristic, Hornung and Reed, 1990 would suggest the LOD/sqrt 2.

The distribution characteristics would also help understand which substitution method to use.

Table 36: 8-isoprostane distribution characteristics for non- and substituted datasets

Variable (N=569)	Min	Mean	Median	Mode	Max	SD	Variance	SE mean	KS (pval)
8-iso	0.58	83.3	58.3	34.8	2234	129	16563	5.86	0.26 (0)
8-Iso LOD/2	0.58	73.4	51.7	0.72	2234	121	14576	5.06	0.27 (0)
8-iso LOD/rt2	0.58	74.5	51.7	1.02	2234	120	14473	5.04	0.27 (0)
Log 8-iso LOD/2	-0.55	3.73	3.95	-0.14	7.71	1.17	1.37	0.05	0.11 (0)
Log 8-iso LOD/rt2	-0.55	3.77	3.95	0.02	7.71	1.15	1.32	0.05	0.13 (0)

From the log data geometric mean (GM) and geometric standard deviation (GSD) of LOD/rt2 logged data: GSD is 3.16, GM is 43.4pg/ml. In this instance both methods have their modes below the limit of detection which would not suggest the LOD/sqrt2 method.

In this respect, a GSD over 3.0 would suggest the LOD/2 method; however, looking at Figures 2, 3 and 4 will also help us assess the distribution patterns.

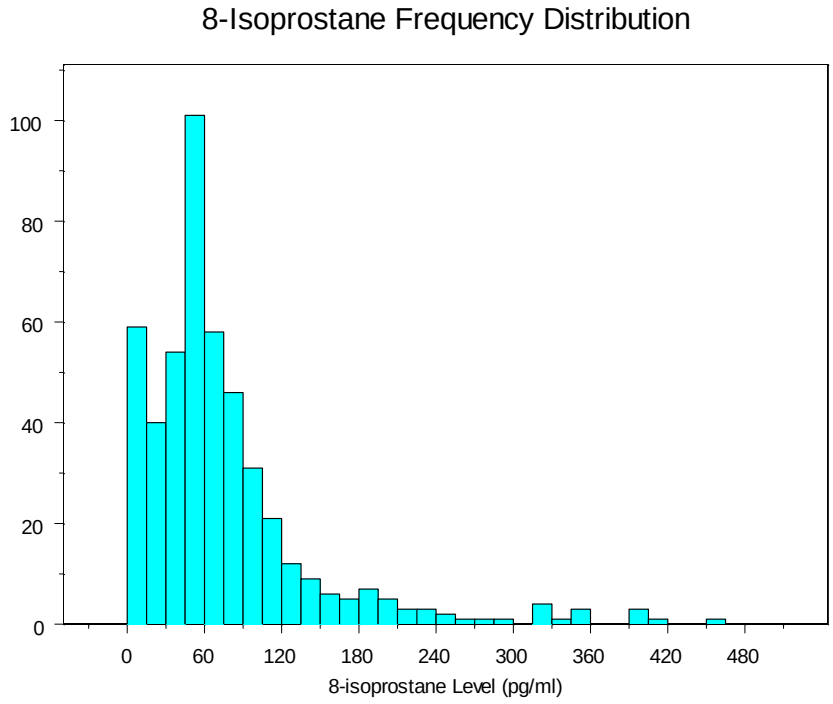

Figure 17. Frequency distribution of the 8-isoprostane dataset.

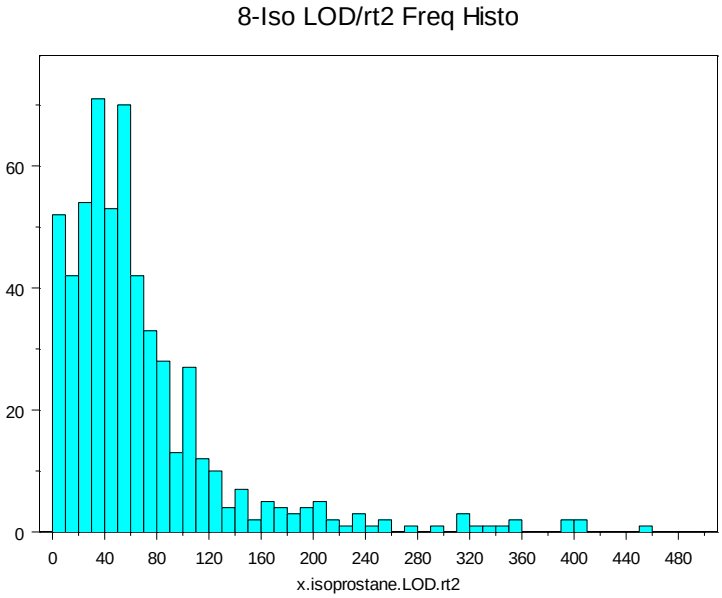

Figure 18. Frequency distribution of the LOD/2 substituted 8-isoprostane dataset.

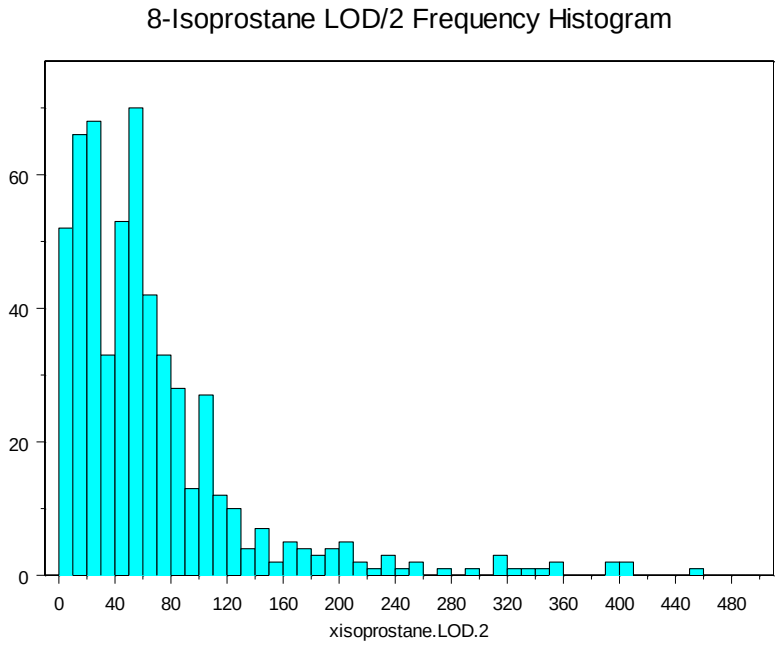

Figure 19. Frequency distribution of the LOD/sqrt2 substituted 8-isoprostane dataset.

The frequency distributions for the 8-isoprostane data and substitution methods shows a distribution slightly skewed to the left.

The results would suggest an LOD/sqrt2 substitution method should be favored. As such, all non detects were replaced with LOD/sqrt2 in order to continue and assess data normality.

Assessment of Distribution Normality:

Figures 2 and 4 show two distributions skewed to the left. In order to fully confirm the log normal distribution of our 8-isoprostane data, a log transformation was applied to the LOD/sqrt2 substituted 8-isoprostane data (see Figure 5).

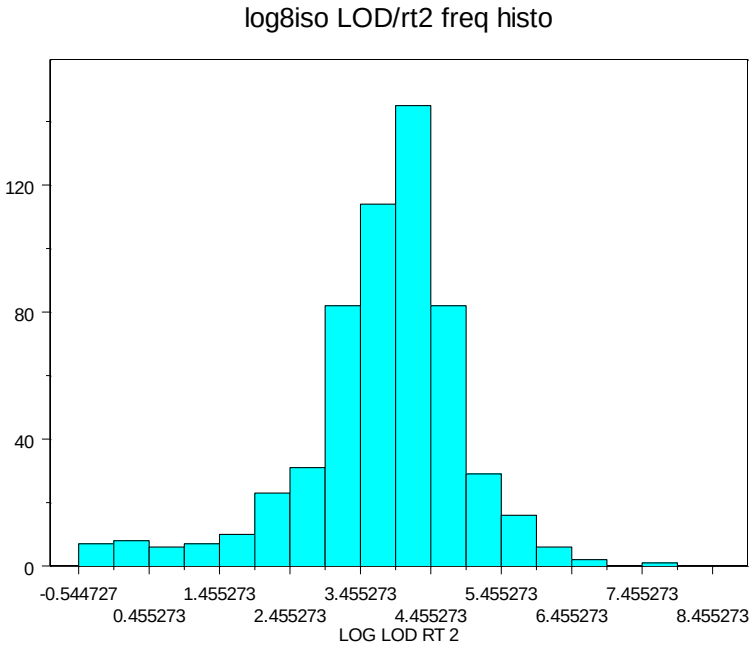

Figure 20. Frequency distribution of the log transformed LOD/sqrt2 substituted 8-isoprostane dataset.

The log transformed distributions look more normally distributed than the untransformed data. Quantile-Quantile plots were made with these two substitutions in order to determine which substitution fit the normal line the most. The quantile-quantile (QQ) plots (Figures 17 and 18) reveal the distribution about the normal line of both the 8-isoprostane and log 8-isoprostane data.

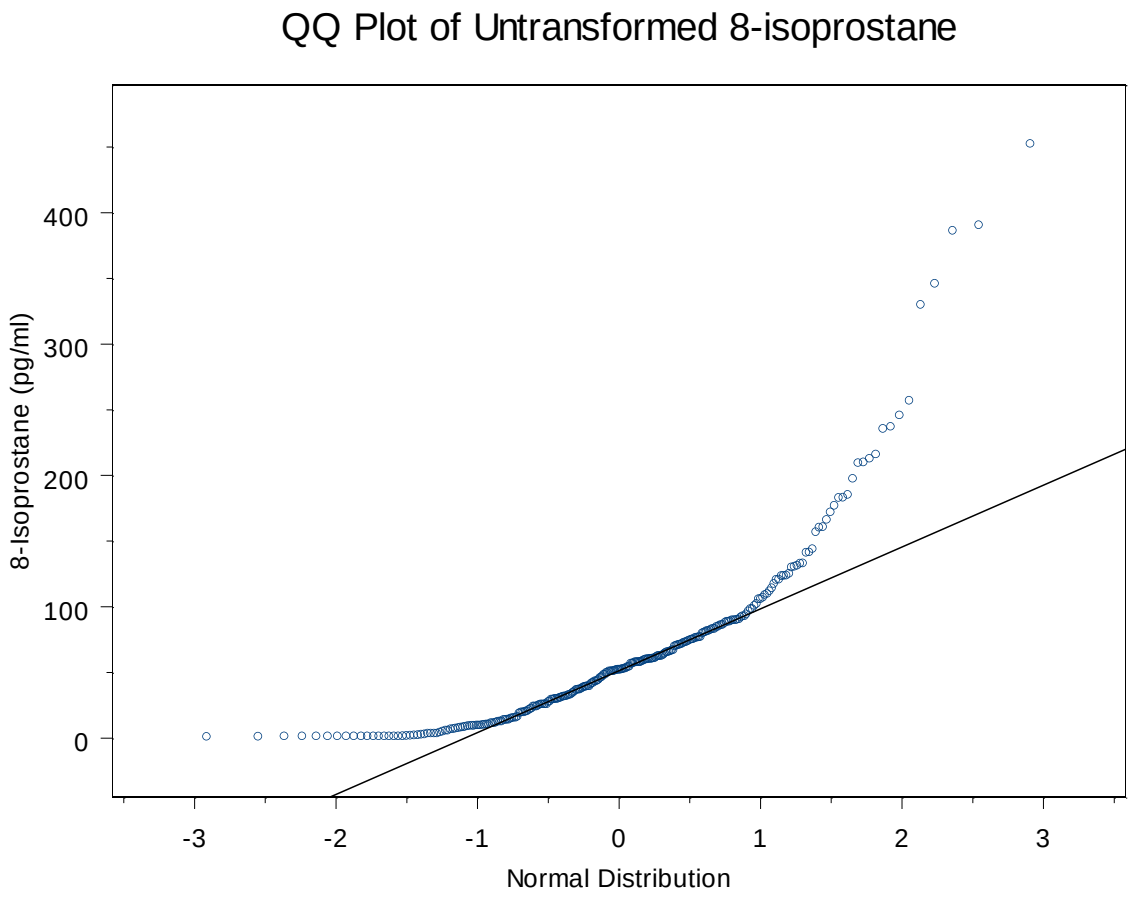

Figure 21. QQ plot for untransformed 8-isoprostane levels. The line represents what the distribution should look like if it were normal with the same mean and standard deviations of the 8-isoprostane data.

Figure 17 shows the untransformed data curving off both ends from the normal line. This depicts that values that are very high and positive as well as values close to the limit of detection for the 8-isoprostane data will differ the most away from what a normal distribution would look like.

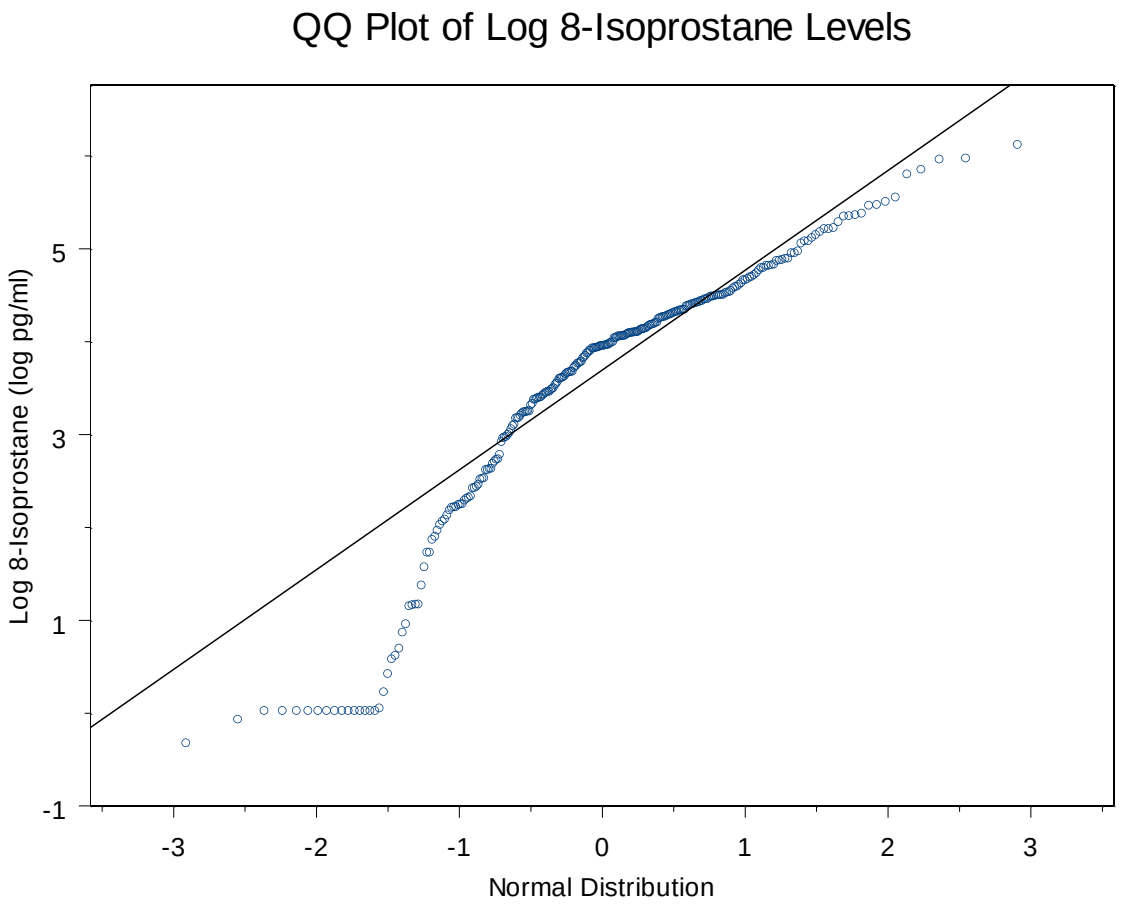

Figure 22. QQ plot for log 8-isoprostane levels. The line represents what the distribution should look like if it were normal with the same mean and standard deviations of the log 8-isoprostane data.

Compared to Figure 17, Figure 18 depicts that mostly only the values close to the limit of detection will differ from what the normal distribution would look like. This depicts a slight improvement from using untransformed data as values close to the limit of detection, inherently will have a lot more variability than higher levels of 8-isoprostane due to the method of detection. All the above information is definitely conflicting. The smaller the KS the better, it is a test which measures the difference between the normal distribution which would have the same mean and SDs with the actual distribution of data (see Table 1). The lower the KS for untransformed data the more normal it is. The logged data seems to have a smaller KS meaning the log transformed data is more "normally" distributed than the non transformed data. Thus, all signs point to a log normally distributed database. For the statistical analyses, log 8-isprostane will be used in order to assess exposure effects.

Appendix C Supplementary DE Study Result Figures

This section outlines additional information for Chapter 3 study results.

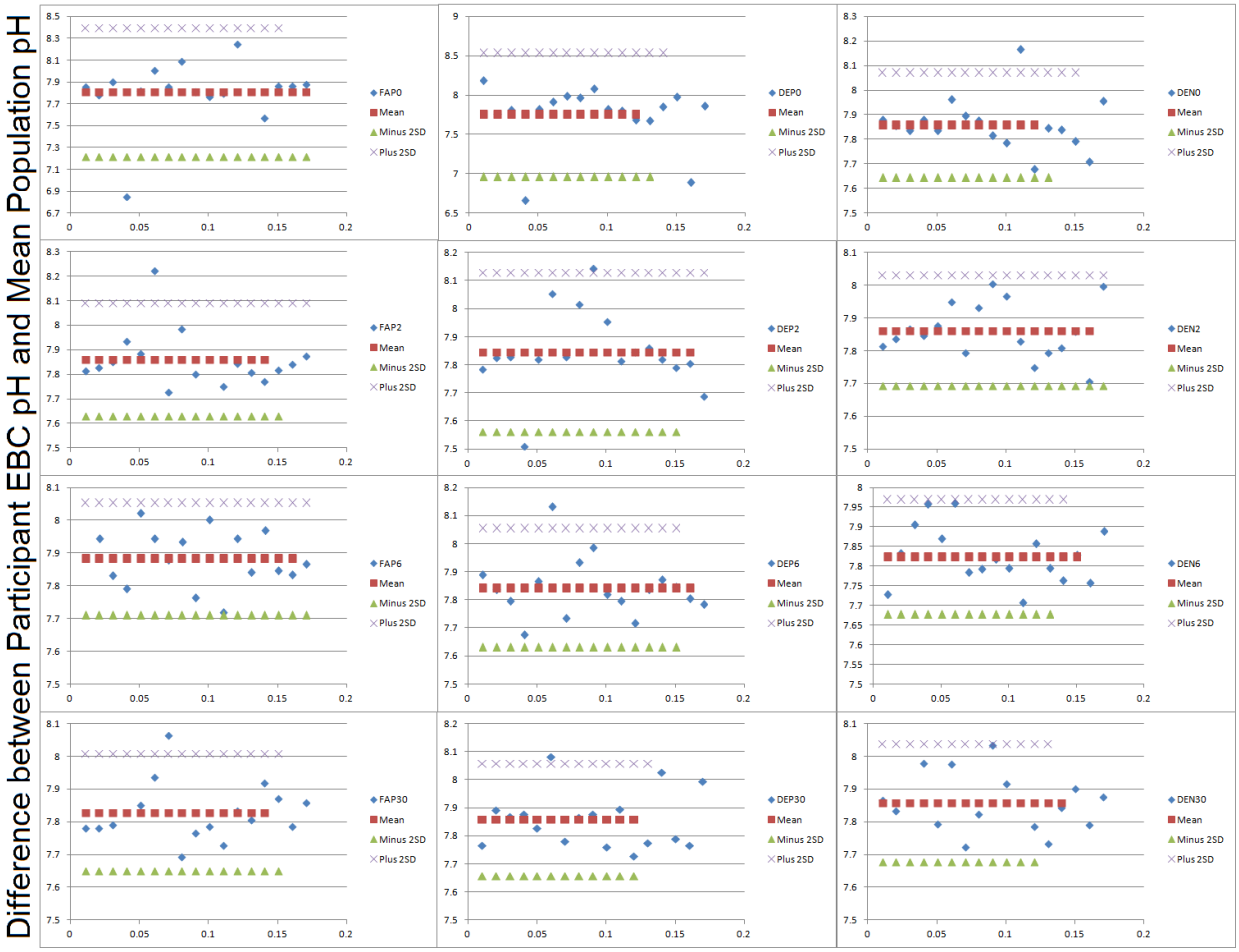

Figure 23. Bland-Altman plots for individual EBC pH level data by exposure and time point. Top right to left are exposures FAP, DEP and DEN. Top to bottom are time points 0 to 30 hours after exposure.

Figure 21 shows potential outliers for EBC pH. With regards to EBC pH, higher levels (+2SD from population mean) are of less interest since they are not biasing results. EBC pH values below -2SD may be over acidic and biasing results. Baseline FAP and DEP show two dots below -2SD and DEP 2 hours after exposure as well. Further investigation identified the two values at baseline around 6.5 and 6.8 were within the "normal" range for that particular participant and left in the analyses. Not shown in FAP baseline levels is the point at pH 5, this one is over 3SD away

from the population mean and was excluded from further analyses. Lastly, the outlier two hours after exposure for DEP is around 7.5 pH and well within the normal range.

Figure 24. Bland-Altman plots for individual EBC 8-isoprostane data by exposure and time point. Top right to left are exposures FAP, DEP and DEN. Top to bottom are time points 0 to 30 hours after exposure.

Figure 22 shows potential outliers for EBC 8-isoprostane. Note, for these results, levels lower than -2SD are typically due to the LOD/sqrt2 substitution method due to non detects. Thus, it is important to understand at what exposures non detects may be biasing results towards the null. There are three lower outliers at baseline: one at FAP and to at DEN exposures. There is one lower outlier two hours after DEN exposure. There is one lower outlier 6 hours after DEP exposure. there are four lower outliers 30 hours after exposure: one at FAP, one at DEP and two at DEN exposure. There is one outlier 6 hours after FAP exposure with a value above 1000pg/ml 8-isoprostane, more than 3SD away from the population mean. This result was excluded from further analyses.

This section goes through additional diagrams and tables for different covariate effects.

1. Secondary analysis: effect modification by sex

Table 37: Univariate analyses for effect modification by sex

	8-isoprostane			pH		
	β (log pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Baseline						
Intercept	3.40	1.21, 5.60		7.78	7.41, 8.15	
Sex	0.48	-0.15, 1.11	0.14	-0.06	-0.33, 0.21	0.65
2 hours after exposure						
Intercept	**3.09**	**2.35, 3.83**		**7.86**	**7.76, 7.96**	
Sex	**0.67**	**-0.07, 1.42**	**0.08**	**0.09**	**0.01, 0.17**	**0.01**
6 hours after exposure						
Intercept	3.35	2.39, 4.31		7.86	7.78, 7.94	
Sex	-0.41	-1.04, 0.22	0.20	0.02	-0.04, 0.08	0.39
30 hours after exposure						
Intercept	3.24	2.24, 4.24		7.77	7.60, 7.95	
Sex	0.18	-0.47, 0.83	0.59	0.09	-0.05, 0.23	0.21

Sex modified log 8-isprostane borderline significantly and pH significantly 2 hours after exposure.

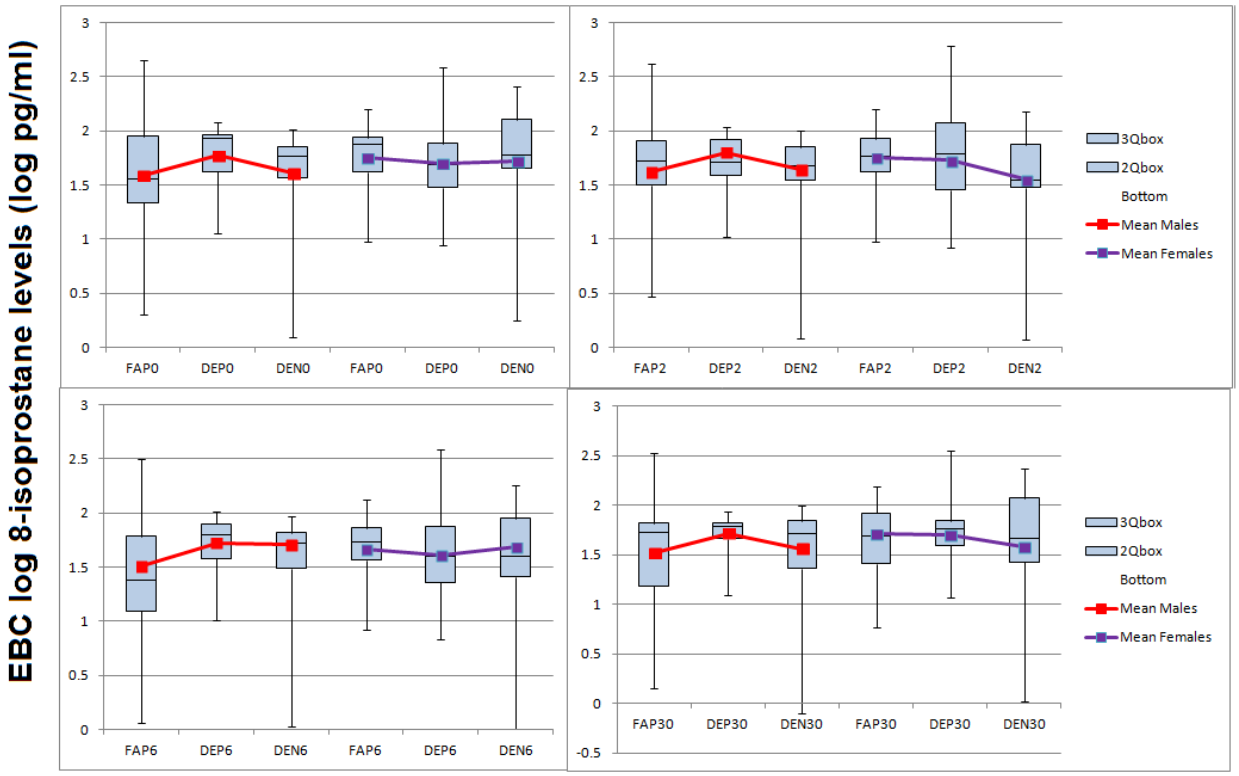

Figure 25. EBC log 8-isoprostane levels stratified by exposure for males (red) and females (purple) at all time points (From top left clockwise is baseline, 2, 30 and 6 hours after exposure).

Males consistently increase in EBC log 8-isoprostane from FAP to DEP and decrease from DEP to DEN. Female EBC log 8-isoprostane remains unchanged from FAP to DEP. EBC log 8-isoprostane decreases from DEP to DEN 2 and 30 hours after exposure in females, while remains constant at baseline and increases 6 hours after exposure from DEP to DEN.

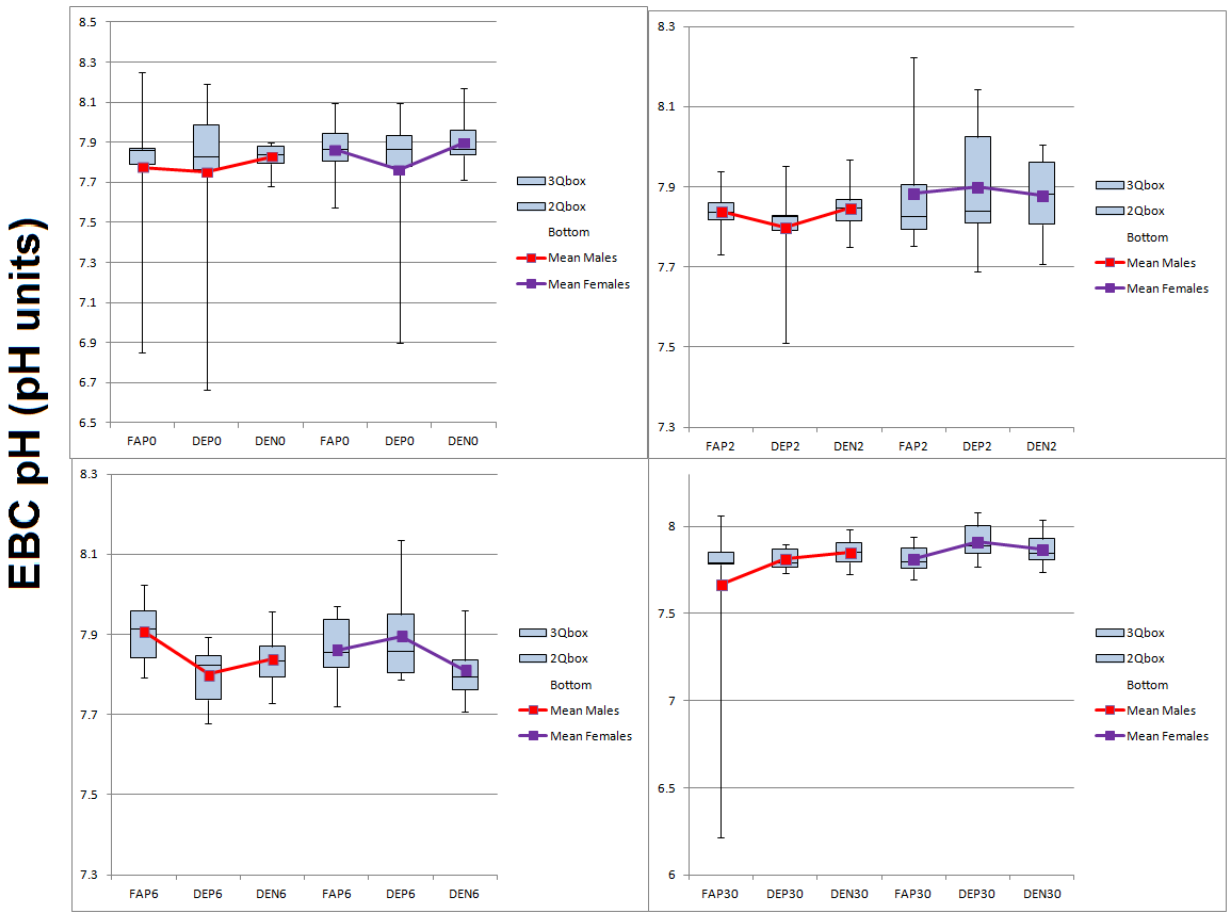

Figure 26. EBC pH levels stratified by exposure for males (red) and females (purple) for all time points (From top left clockwise is baseline, 2, 30 and 6 hours after exposure).

EBC pH in males shows acidification from FAP to DEP and recovery from DEP to DEN for baseline, 2 and 6 hours after exposure. Also in males, EBC pH 30 hours after exposure increases from FAP to DEP and from DEP to DEN. EBC pH in females show increases from FAP to DEP and decreases from DEP to DEN for 2, 6 and 30 hours after exposure. Baseline EBC pH in females acidifies from FAP to DEP and recovers from DEP to DEN.

Table 38: Univariate analyses for effect modification by sex on delta biomarker levels

	Delta 8-isoprostane			Delta pH		
	β (pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Delta 2						
Intercept	-5.64	-72.1, 60.8		0.044	0.20, -0.15	
Sex	0.66	-47.4, 48.7	0.98	0.009	0.16, -0.15	0.91
Delta 6						
Intercept	2.93	-201, 207		0.066	0.88, -0.82	
Sex	-23.9	-68.6, 20.8	0.30	0.07	0.27, -0.21	0.62
Delta 30						
Intercept	-7.45	-176, 161		-0.009	0.41, -0.42	
Sex	-0.16	-42.7, 42.4	0.99	0.17	0.27, -0.10	0.22

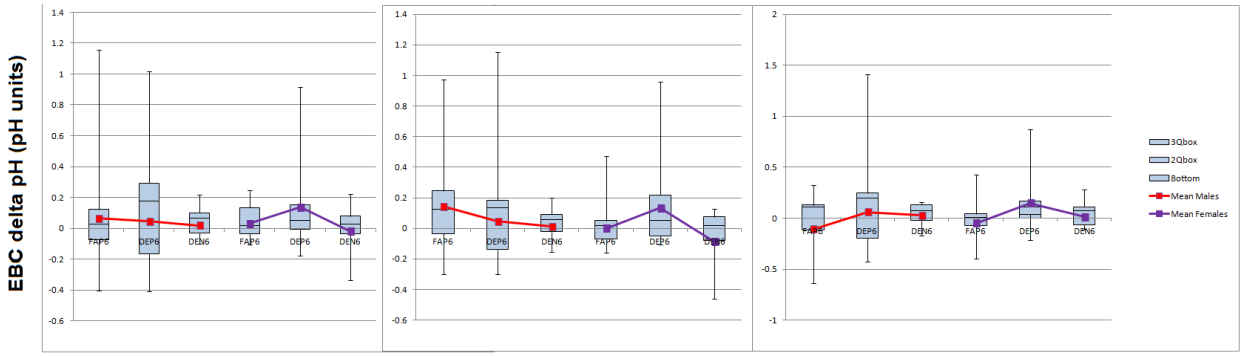

Figure 27. EBC delta pH at 2, 6 and 30 hours after exposure (in order) stratified by exposure for males (red) and females (purple).

Briefly, EBC delta 8-isoprostane in males is unchanged from FAP to DEP and DEP to DEN for all time points. Female delta 8-isoprostane remains unchanged from FAP to DEP for all time points and decreases from DEP to DEN 2 and 30 hours after exposure while remains unchanged for 6 hours after exposure.

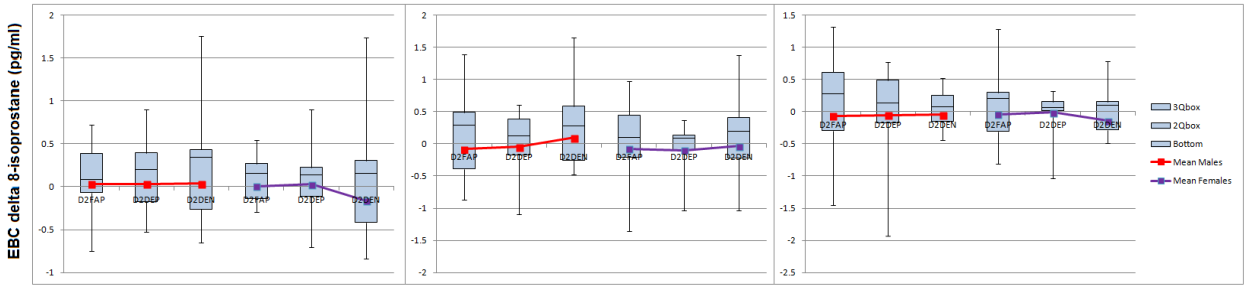

Figure 28. EBC delta 8-isoprostane at 2, 6 and 30 hours after exposure (in order) stratified by exposure for males (red) and females (purple).

EBC delta pH in males from FAP to DEP remains unchanged 2 hours after exposure, acidifies 6 hours after exposure, while increases at 30 hours after exposure. In males from DEP to DEN, EBC delta pH remains unchanged. In females, EBC delta pH increases from FAP to DEP and acidifies from DEP to DEN for all time points.

2. Secondary analysis: effect modification by methacoline response status

Table 39: Univariate analyses for effect modification by methacoline response status

	8-isoprostane			pH		
	β (log pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Baseline						
Intercept	3.55	2.51, 4.59		7.79	7.38, 8.20	
MRS	-0.16	-0.89, 0.57	0.66	-0.06	-0.33, 0.21	0.68
2 hours after exposure						
Intercept	3.22	2.14, 4.30		7.89	7.79, 7.99	
MRS	0.29	-0.49, 1.07	0.46	-0.05	-0.11, 0.01	0.12
6 hours after exposure						
Intercept	3.19	2.33, 4.05		7.87	7.69, 8.05	
MRS	0.53	-0.09, 1.16	0.11	-0.04	-0.16, 0.08	0.19
30 hours after exposure						
Intercept	**3.15**	**2.44, 3.86**		7.79	7.61, 7.97	
MRS	**0.61**	**-0.04, 1.26**	**0.07**	0.04	-0.10, 0.18	0.61

A positive methacholine response was borderline significantly associated with higher levels of log 8-isoprostane.

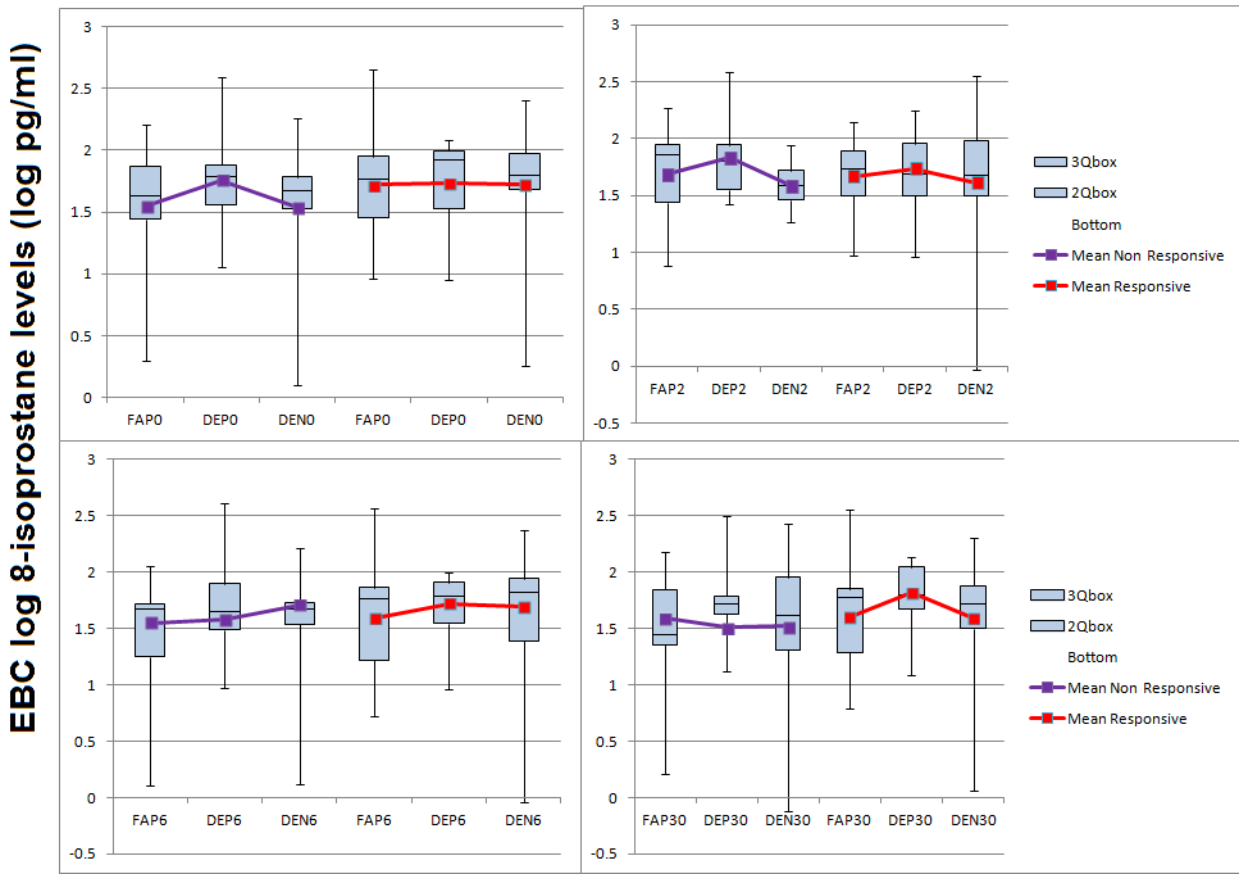

Figure 29. EBC log 8-isoprostane levels stratified by exposure for methacholine responsive (red) and non responsive (purple) individuals (From top left clockwise is baseline, 2, 30 and 6 hours after exposure).

Briefly, in non methacholine responsive subject (purple) EBC log 8-isoprostane increased from FAP to DEP at baseline, 2 and 6 hours after exposure. Levels decrease from DEP to DEN at baseline and 2 hours after exposure; while they increase 6 hours after exposure (Figure 33, Appendix C).

In methacholine responsive subjects (red), EBC log 8-isoprostane increases from FAP to DEP for 2, 6 and 30 hours after exposure while remaining unchanged at baseline. Levels decrease from DEP to DEN for 2, 6 and 30 hours after exposure while remain unchanged for baseline (Figure 33, Appendix C).

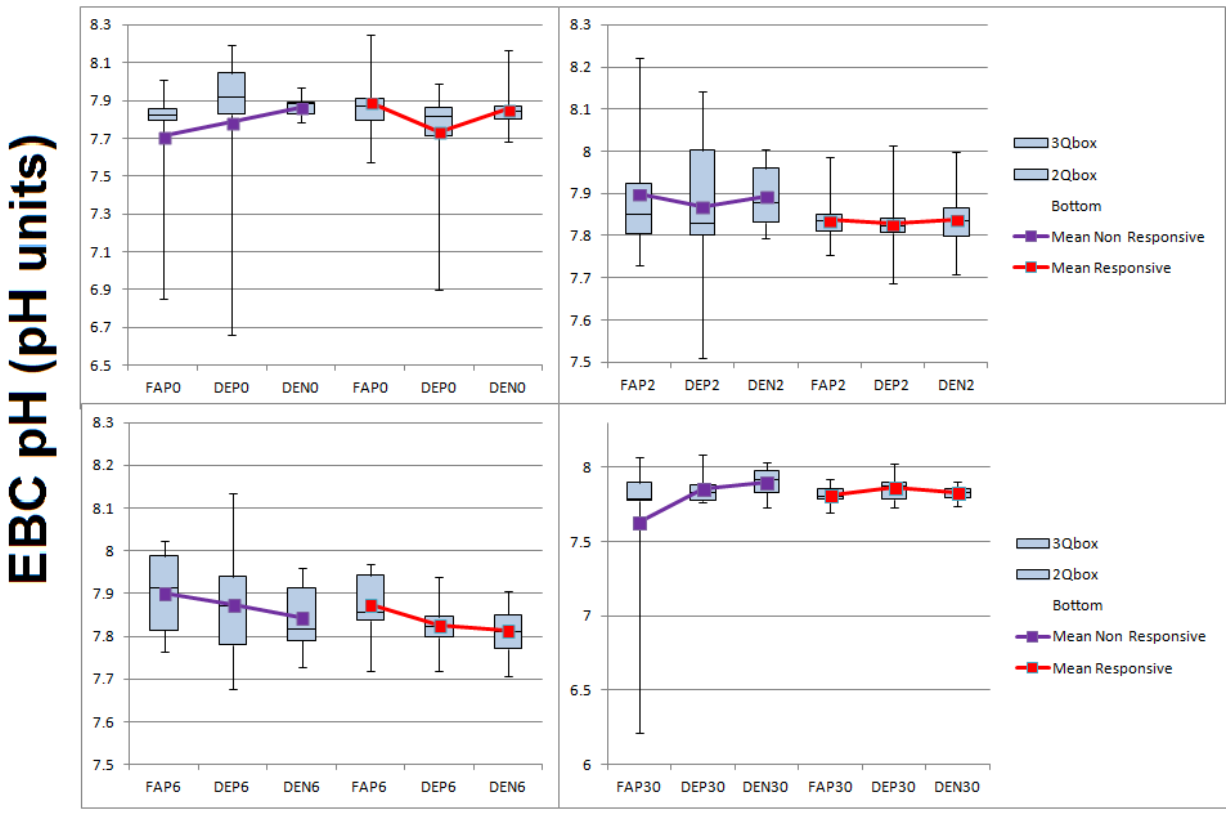

Figure 30. EBC pH stratified by exposure for methacholine responsive (red) and non responsive (purple) subjects at all time points (From top left clockwise is baseline, 2, 30 and 6 hours after exposure).

Briefly, non methacholine responsive subjects increases from FAP to DEP 0 and 30 hours after exposure; while conversely, EBC pH decreases from FAP to DEP 2 and 6 hours after exposure. EBC pH increases from DEP to DEN 0, 2 and 30 hours after exposure while decreases 6 hours after exposures.

In methacholine responsive individuals, EBC pH decreases from FAP to DEP 0, 2 and 6 hours after exposure; while it increases 30 hours after exposure. pH remains mostly unchanged 2, 6 and 30 hours after exposure from DEP to DEN, while it increases at baseline (Figure 34, Appendix C).

Table 40: Univariate analyses for effect modification by methacoline response status on delta biomarker levels

	Delta 8-isoprostane			Delta pH		
	β (pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Delta 2						
Intercept	-13.8	-90.0, 62.4		0.10	-0.12, 0.32	
MR	30.9	-23.2, 85.0	0.27	-0.09	-0.25, 0.07	0.28
Delta 6						
Intercept	**-37.4**	**-217, 143**		0.09	-0.42, 0.60	
MR	**44.4**	**-1.66, 90.5**	**0.06**	0.02	-0.25, 0.30	0.89
Delta 30						
Intercept	-26.1	-224, 172		0.005	-0.51, 0.52	
MR	28.5	-15.4, 72.4	0.21	0.11	-0.16, 0.38	0.42

A positive MR status significantly increased delta 8-isoprostane 6 hours after exposure. This trend was also observed 2 and 30 hours after exposure, however was not significant. No clear trend of MRS effect modification was observed on EBC delta pH.

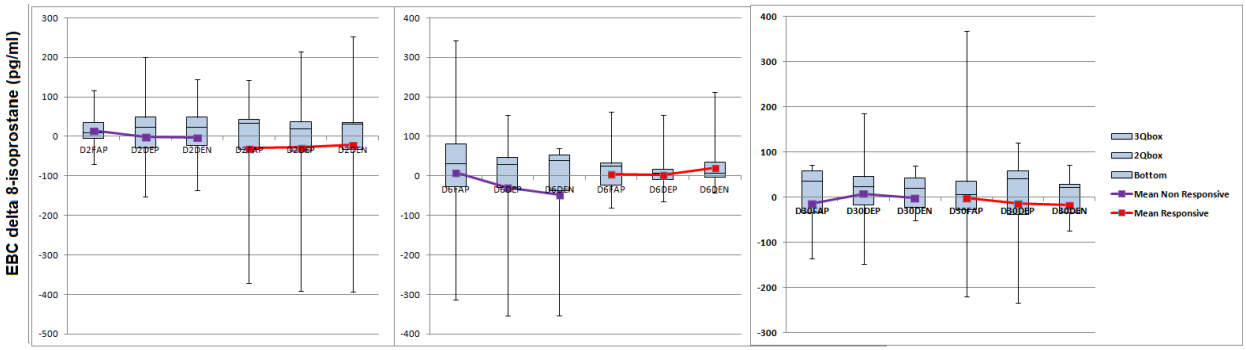

Figure 31. EBC delta 8-isoprostane stratified by exposure for methacholine responsive (red) and non responsive (purple) subjects 2, 6 and 30 hours after exposure.

Briefly, non methacholine responsive subjects show little to no change 2 and 30 hours after exposure for EBC delta 8-isoprostane. Deltas decrease from FAP to DEP and from DEP to DEN suggesting that levels after exposure with regards to baseline are becoming smaller (i.e. increasing with regards to exposure but still smaller than baseline levels). Methacholine responsive individuals show little to no change 2, 6 and 30 hours after exposure from FAP to DEP and DEP to DEN.

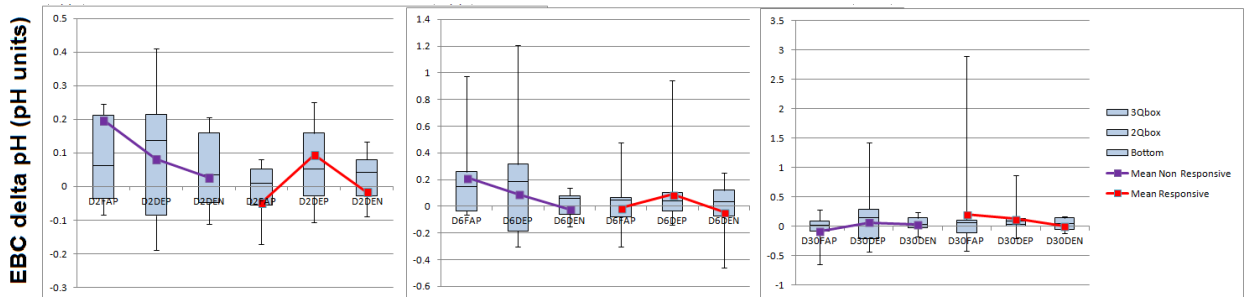

Figure 32. EBC delta ph stratified by exposure for methacholine responsive (red) and non responsive (purple) individuals 2, 6 and 30 hours after exposure.

For EBC delta pH, non methacholine responsive subjects decreased 2 and 6 hours after exposure, while showed little to no change 30 hours after exposure for FAP to DEP. A larger positive change in FAP to a smaller positive change in DEP suggests an acidification of EBC pH from FAP to DEP (Appendix C, Figure 36).

In methacholine responsive subjects, an increase in EBC delta pH was observed 2 and 6 hours after exposure from FAP to DEP; while a decrease was observed from DEP to DEN. Little to no change was observed 30 hours after exposure (Figure 36, Appendix C).

3. **Secondary analysis: effect modification by atopic status**

Table 41: Univariate analyses for effect modification by atopic status on biomarker levels

	8-isoprostane			pH		
	β (log pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Baseline						
Intercept	**3.12**	**0.71, 5.53**		7.63	7.28, 7.98	
Atopy	**0.67**	**-0.04, 1.38**	**0.07**	0.16	-0.17, 0.49	0.38
2 hours after exposure						
Intercept	**2.35**	**1.21, 3.49**		**7.81**	**7.60, 8.03**	
Atopy	**0.78**	**-0.06, 1.63**	**0.07**	**0.07**	**-0.01, 0.15**	**0.09**
6 hours after exposure						
Intercept	3.23	2.19, 4.27		7.85	7.75, 7.95	
Atopy	0.08	-0.63, 0.79	0.83	0.02	-0.06, 0.10	0.56
30 hours after exposure						
Intercept	3.02	1.16, 4.89		**7.68**	**7.45, 7.92**	
Atopy	0.51	-0.22, 1.24	0.17	**0.17**	**-0.01, 0.35**	**0.07**

Atopic subjects significantly increased 8-isoprostane 0 and 2 hours after exposure; while borderline significantly increased pH 2 and 30 hours after exposure.

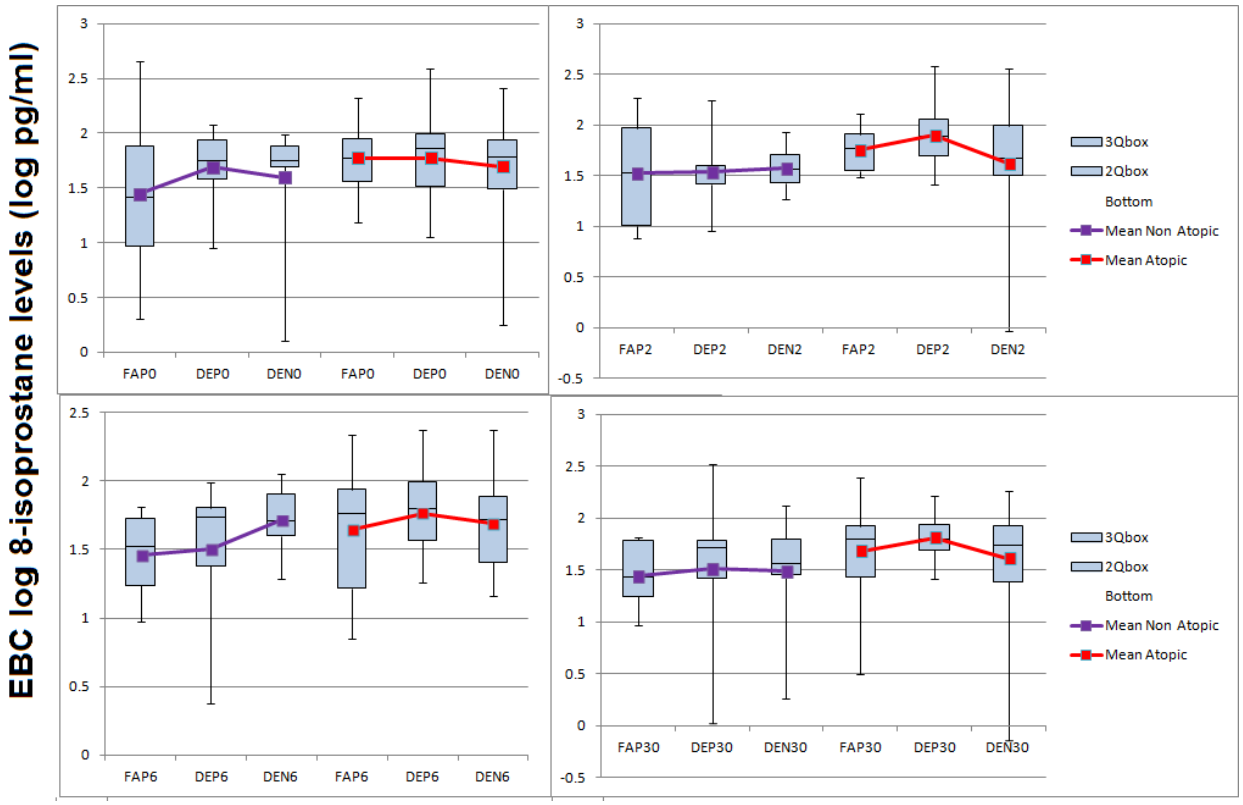

Figure 33. EBC log 8-isoprostane stratified by exposure for atopic (red) and non atopic (purple) subjects for all time points (From top left clockwise baseline, 2, 30 and 6 hours after exposure).

Briefly, in non atopic subjects, EBC log 8-isoprostane increases from FAP to DEP at baseline but remains unchanged for the other time points. Levels decrease from FAP to DEP at baseline, increase 6 hours after exposure and remain unchanged 2 and 30 hours after exposure. In atopic subjects, EBC log 8-isoprostane increases from FAP to DEP 2, 6 and 30 hours after exposure; while they decrease from DEP to DEN 2, 6 and 30 hours after exposure (Figure 37, Appendix C).

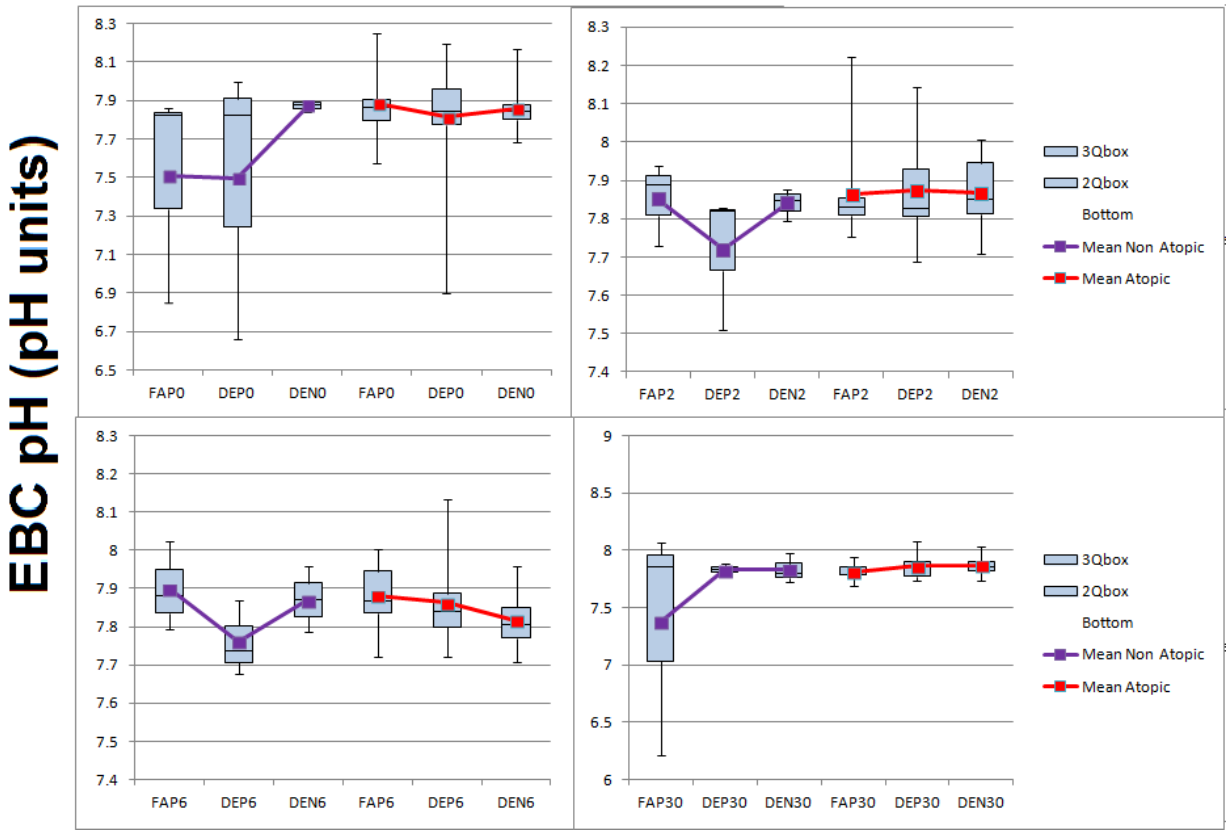

Figure 34. EBC Ph stratified by exposure for atopic (red) and non atopic (purple) individuals for all time points (From top left, clockwise baseline, 2, 30 and 6 hours after exposure).

EBC pH remains unchanged at baseline from FAP to DEP, acidifies 2 and 6 hours after exposure, increases 30 hours after exposure for non atopic subjects (Figure 38, Appendix C). Atopic subjects show a slight decrease in mean EBC pH from FAP to DEP 0 and 6 hours after exposure. They show little to no change 2 and 30 hours after exposure in mean EBC pH levels.

Table 42: Univariate analyses for effect modification by atopic status on delta biomarkers

	Delta 8-isoprostane			Delta pH		
	β (pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Delta 2						
Intercept	-23.9	-202, 154		0.18	-0.10, 0.45	
Atopy	23.1	-31.2, 77.4	0.34	-0.16	-0.36, 0.04	0.10
Delta 6						
Intercept	-23.9	-96.2, 48.4		0.22	-0.21, 0.65	
Atopy	20.8	-30.4, 72.0	0.43	-0.15	-0.50, 0.20	0.41
Delta 30						
Intercept	-11.8	-134, 110		0.06	-1.35, 1.47	
Atopy	5.75	-42.7, 54.2	0.82	0.02	-0.33, 0.37	0.92

No significant trend was observed for delta biomarker levels. A non significant increase in delta 8-isoprostane was observed at all time points; while a non significant decrease in delta pH was observed 2 and 6 hours after exposure.

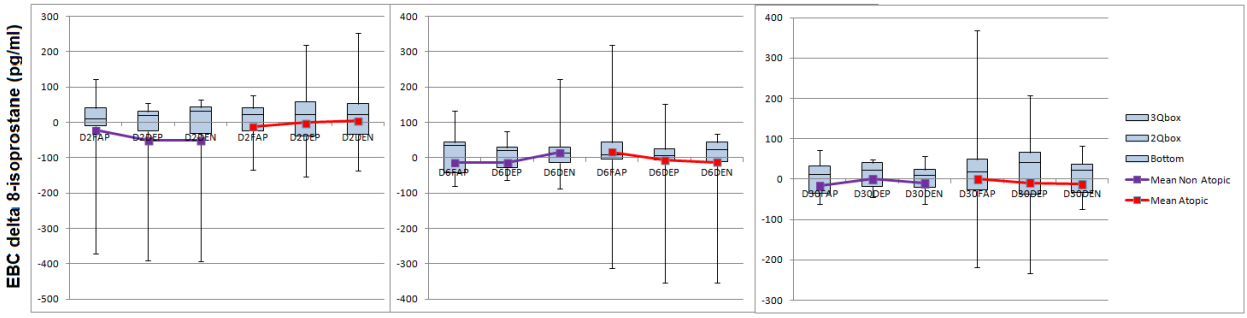

Figure 35. EBC delta 8-isoprostane stratified by exposure for atopic (red) and non atopic (purple) individuals 2, 6 and 30 hours after exposure (in order).

In non atopic subjects, little to no change is observed in delta 8-isoprostane 6 and 30 hours after exposure from FAP to DEP. 2 hours after exposure, levels decrease from FAP to DEP and remains unchanged from DEP to DEN. At all time points, exposure shows little to no variation in delta 8-isoprostane from DEP to DEN. Atopic subjects show little to no change at all time points for all exposures.

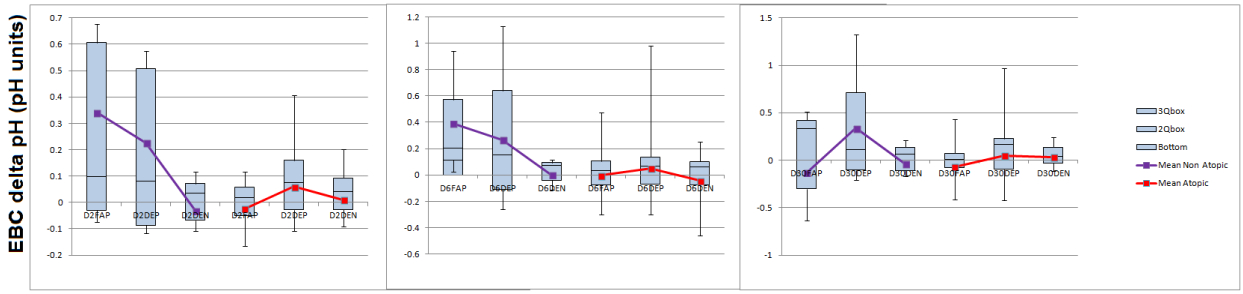

Figure 36. EBC delta pH stratified by exposure for atopic (red) and non atopic (purple) individuals 2, 6 and 30 hours after exposure (in order).

In non atopic subjects, EBC delta pH decreases from FAP to DEP 2 and 6 hours after exposure; while increases 30 hours after exposure. EBC delta pH decreases from DEP to DEN at all time points. In atopic subjects, EBC delta pH increases from FAP to DEP at all time points, decreases from DEP to DEN at 2 and 6 hours after exposure, while remains unchanged from DEP to DEN 30 hours after exposure.

4. Secondary analysis: effect modification by genetic variant status

Table 43: Univariate analyses for effect modification by GSTP1 status on biomarkers

	8-isoprostane			pH		
	β (log pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Baseline						
Intercept	3.78	1.04, 4.82	0.23	7.72	7.35, 8.09	0.68
GSTP1	-0.41	-1.08, 0.26		0.06	-0.21, 0.33	
2 hours after exposure						
Intercept	3.75	2.55, 4.97		7.86	7.70, 8.02	
GSTP1	-0.65	-1.41, 0.11	0.11	-0.007	-0.07, 0.05	0.84
6 hours after exposure						
Intercept	3.43	2.41, 4.45		7.87	7.79, 7.95	
GSTP1	-0.29	-1.00, 0.42	0.37	-0.008	-0.07, 0.05	0.76
30 hours after exposure						
Intercept	3.42	2.36, 4.48		7.85	7.71, 7.99	
GSTP1	-0.28	-0.95, 0.39	0.41	-0.05	-0.21, 0.11	0.55

Genetic variant status had no significant effect on log 8 isoprostane or on pH levels. GSTP1 variant status non-significantly decreased log 8-isoprostane levels at all time points. GSTP1 variant status non-significantly acidified EBC pH 2, 6 and 30 hours after exposure.

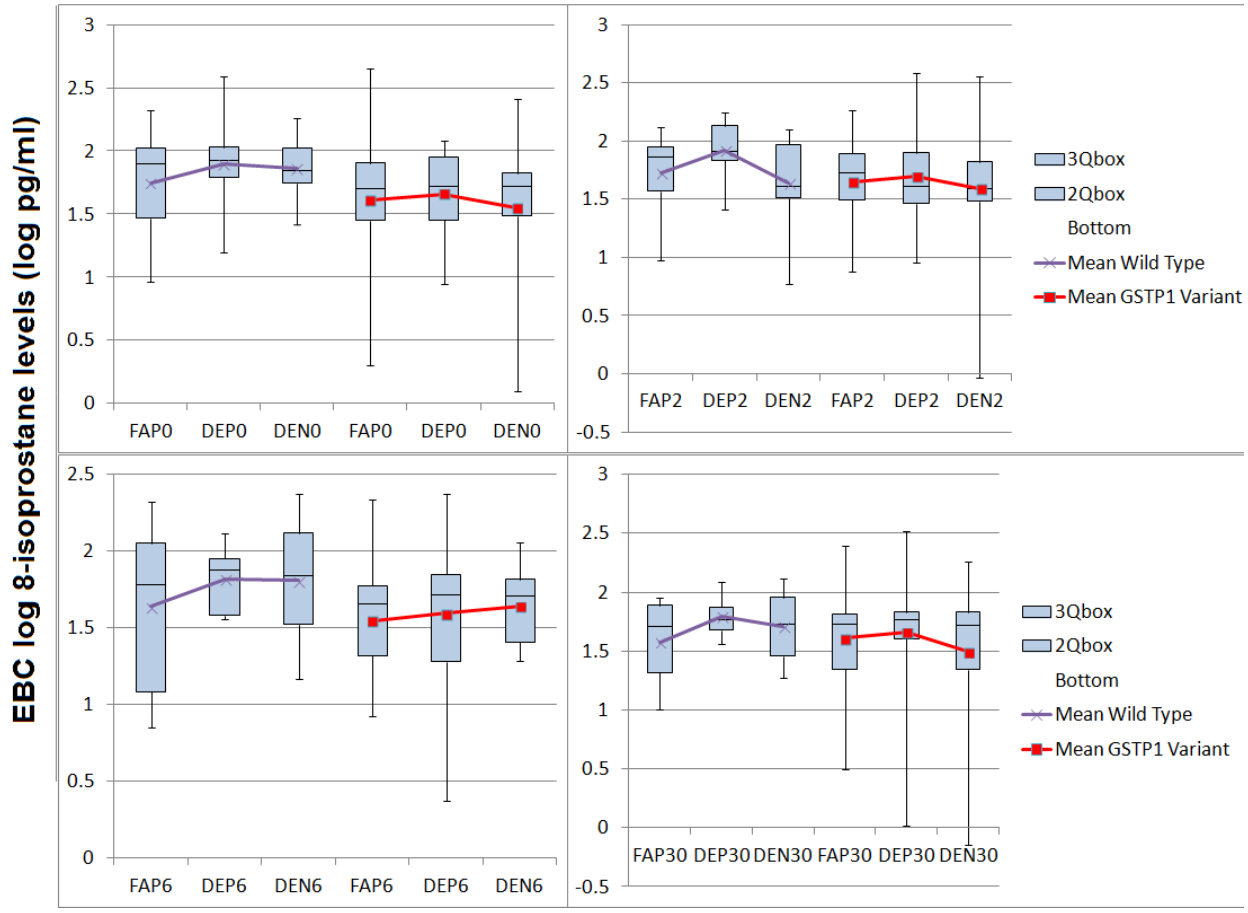

Figure 37. EBC log 8-isoprostane stratified by exposure for GSTP1 wild type (purple) and variant (red) subjects for all time points (Top left clockwise baseline, 2, 30 and 6 hours after exposure).

Briefly, wild type subjects show an increase in EBC log 8-isoprostane from FAP to DEP and a decrease from DEP to DEN at all time points. GTSP1 variant subjects show a slight increase in EBC 8-isoprostane from FAP to DEP at all time points, a decrease from DEP to DEN 0, 2 and 30 hours after exposure while it increases 6 hours after exposure (Figure 41, Appendix C).

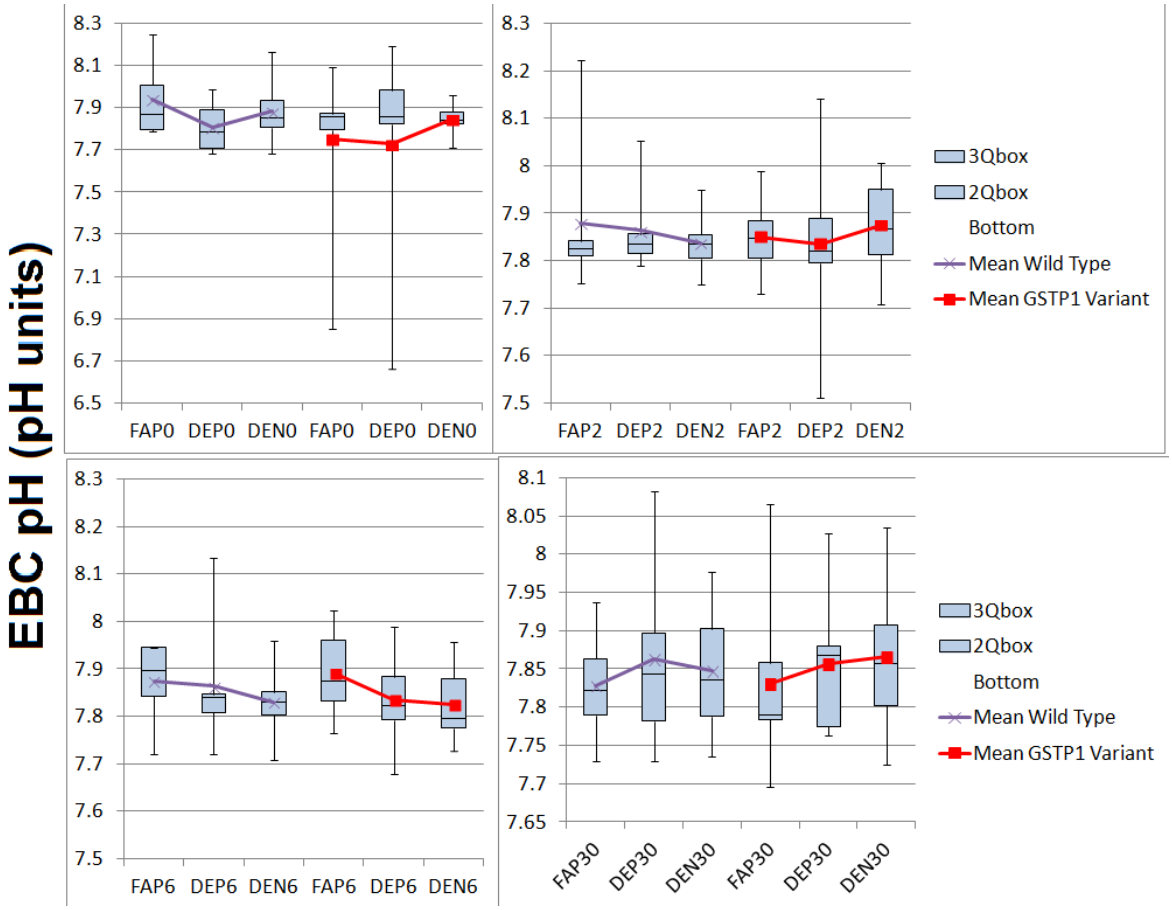

Figure 38. EBC pH stratified by exposure for GSTP1 wild type (purple) and variant (red) subjects for all time points (Top left clockwise baseline, 2, 30 and 6 hours after exposure).

For EBC pH, wild type subjects showed an acidification from FAP to DEP 0, 2 and 6 hours after exposure while it increased 30 hours after exposure; EBC pH acidified from DEP to DEN 2, 6 and 30 hours after exposure while it increased at baseline. For GSTP1 variant subjects a small acidification was noted from FAP to DEP 0, 2 and 6 hours after exposure while it increased 30 hours after exposure. From DEPT to DEN pH increased for all time points except 6 hours after exposure where is slightly decreased (Figure 42, Appendix C).

Table 44: Univariate analyses for effect modification by GSTP1 status on delta biomarkers

	Delta 8-isoprostane			Delta pH		
	β (pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Delta 2						
Intercept	-14.8	-88.5, 58.9		-0.01	-0.25, 0.23	
GSTP1	14.8	-35.6, 65.2	0.57	0.09	-0.07, 0.25	0.28
Delta 6						
Intercept	-8.11	-77.3, 61.1		0.14	-0.25, 0.53	
GSTP1	-0.78	-48.0, 46.5	0.97	-0.07	-0.34, 0.20	0.65
Delta 30						
Intercept	-27.9	-88.3, 32.5		0.16	-0.27, 0.59	
GSTP1	31.2	-12.7, 75.1	0.17	-0.13	-0.42, 0.16	0.38

Genetic variant status had no significant effect on delta biomarker levels. Genetic variant status was associated with lower changes in log 8-isoprostane levels compared to wild type individuals. GSTP1 variant subjects were associated with acidification 6 and 30 hours after exposure.

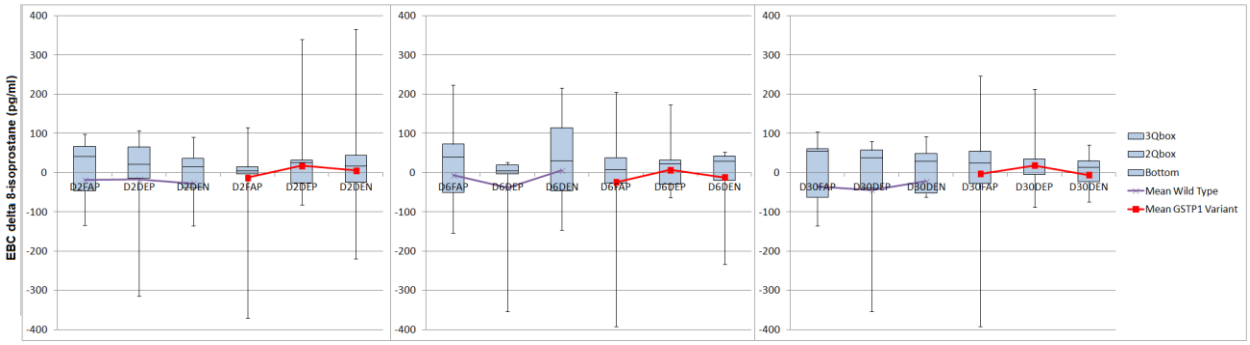

Figure 39. EBC delta 8-isoprostane stratified by exposure for GSTP1 wild type (purple) and variant (red) subjects for all time points (left to right 2, 30 and 6 hours after exposure).

Briefly, GSTP1 wild type (WT) subjects had no change in delta 8-isoprostane 2 and 30 hours after exposure, a decreased change 6 hours after exposure from FAP to DEP. From DEP to DEN, WT subjects had no change in delta 8-isoprostane 2 and 30 hours after exposure; while went from a negative change to a positive change 6 hours after exposure. GSTP1 variant subjects had their delta 8-isoprostane increase from FAP to DEP at all time points; while from DEP to DEN, all time points saw their delta 8-isoprostane decrease.

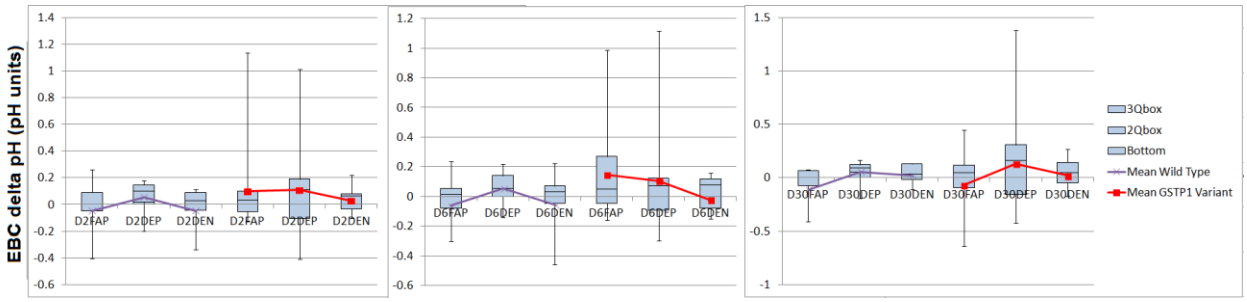

Figure 40. EBC delta pH stratified by exposure for GSTP1 wild type (purple) and variant (red) subjects for all time points (left to right 2, 30 and 6 hours after exposure).

EBC delta pH for wilt type subjects went from a small negative change in pH at FAP to a small positive change in pH at DEP depicting an increase in pH for all time points; while from DEP to DEN, changes went from small positive to small negative, depicting an acidification. For GSTP1 variant subjects no change was observed 2 hours after exposure, a decrease 6 hours after exposure and increase 30 hours after exposure from FAP to DEP. From DEP to DEN, EBC delta pH went from a small positive change to no change at all time points.

5. Secondary analysis: effect modification by age

Table 45: Univariate analyses for effect modification by age on biomarkers

	8-isoprostane			pH		
	β (log pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Baseline						
Age1	4.08	3.32, 4.84	<0.001	7.73	7.40, 8.06	<0.001
Age2	**-1.25**	**-1.92, -0.59**		0.18	-0.11, 0.47	
Age3	**-0.59**	**-1.49, 0.31**	**0.002**	-0.21	-0.62, 0.21	0.22
2 hours after exposure						
Age1	3.68	1.35, 6.01	0.005	7.85	7.65, 8.05	<0.001
Age2	-0.54	-1.38, 0.30		0.004	-0.08, 0.08	
Age3	-0.80	-1.96, 0.36	0.26	0.02	-0.08, 0.12	0.92
6 hours after exposure						
Age1	3.34	-98.6, 105	<0.001	7.86	7.78, 7.94	<0.001
Age2	-0.04	-0.75, 0.67		0.01	-0.05, 0.07	
Age3	-0.16	-1.14, 0.82	0.95	-0.02	-0.10, 0.06	0.74

	8-isoprostane			pH		
	β (log pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
30 hours after exposure						
Age1	3.56	1.68, 5.44	0.0007	7.86	7.63, 8.09	<0.001
Age2	-0.31	-1.02, 0.40		-0.05	-0.21, 0.11	
Age3	-0.54	-1.54, 0.46	0.47	-0.23	-0.45, -0.01	0.12

Age significantly modified log 8-isoprostane levels at baseline. Increasing age group was non-significantly associated with lower 8-isoprostane levels than Age 1 subjects. Conversely, increasing age group was associated with more acidic pH.

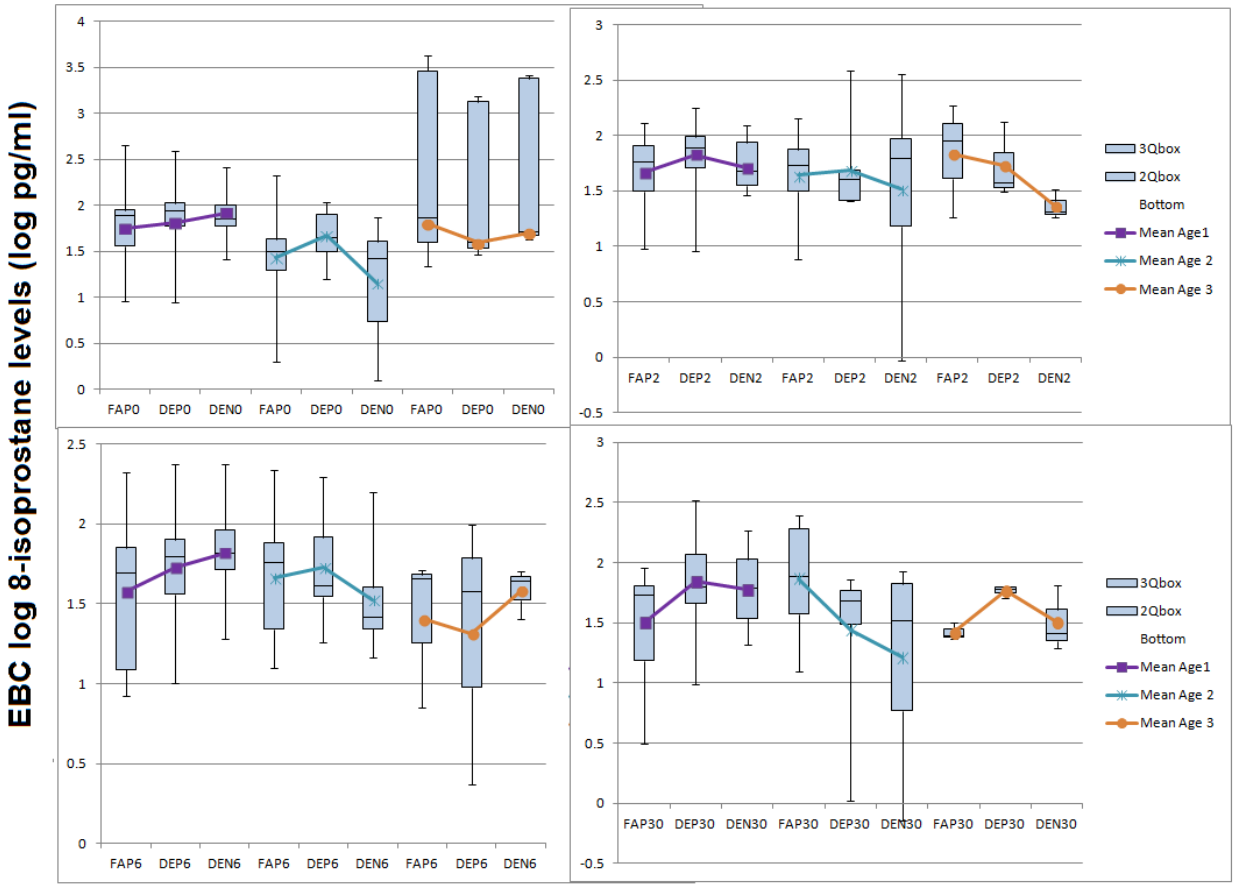

Figure 41. EBC log 8-isoprostane stratified by exposure for age 1(purple), age 2 (blue) and age 3 (orange) subjects for all time points (From top left clockwise baseline, 2, 30 and 6 hours after exposure).

Briefly, Age 1 subjects show EBC 8-isoprostane increasing from FAP to DEP for all time points; while from DEP to DEN 0 and 6 hours increase and 2 and 30 hours decrease in log 8-isoprostane.

Age 2 subjects increase in EBC log 8-isoprostane 0, 2 and 6 hours from FAP to DEP while decrease 30 hours after exposure. EBC log 8-isoprostane levels decrease from DEP to DEN for all time points.

Age 3 subjects show a decrease in log 8-isoprostane for all time points except 30 hours after exposure which increases from FAP to DEP. From DEP to DEN 0 and 6 hours increase in log 8-siprostane while 2 and 30 hours decrease in biomarker levels.

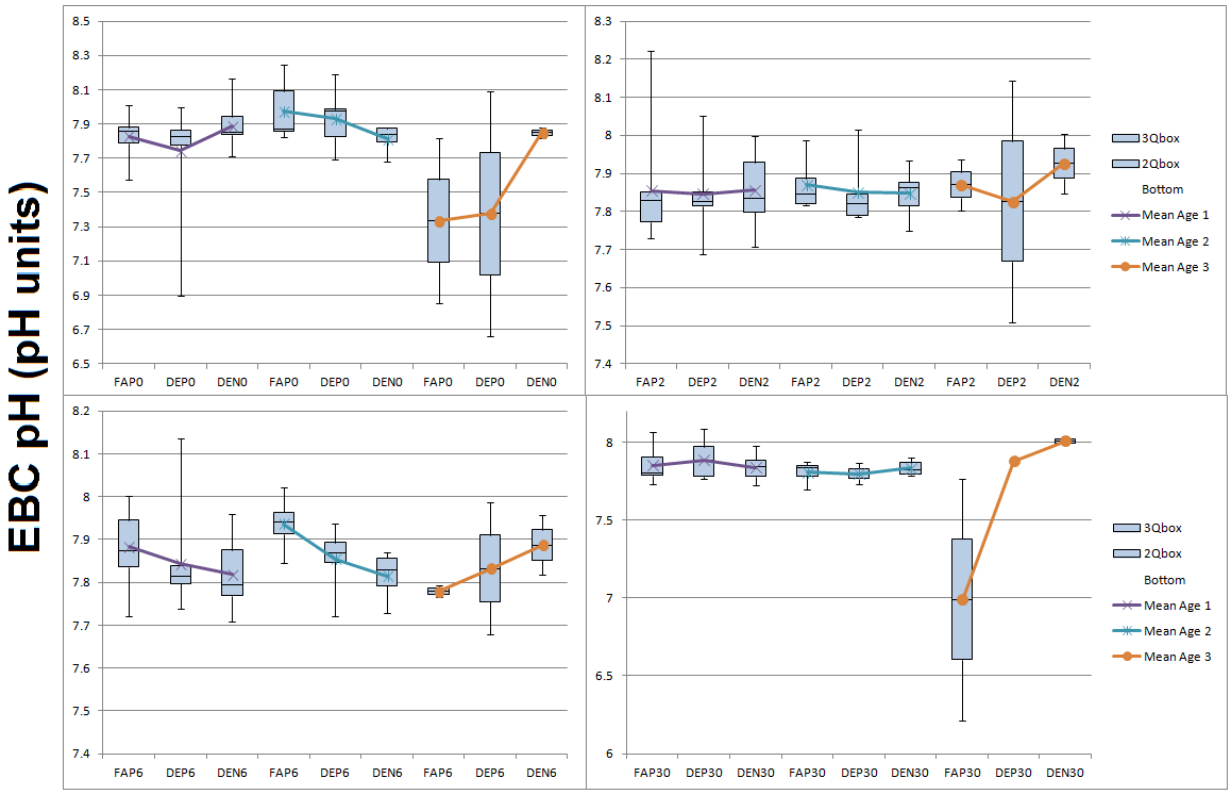

Figure 42. EBC pH stratified by exposure for age 1(purple), age 2 (blue) and age 3 (orange) subjects for all time points (From top left clockwise baseline, 2, 30 and 6 hours after exposure).

Briefly, Age 1 subjects show an acidification 0 and 2 hours after exposure while show s no change after 6 hours and increase in pH 30 hours after exposure from FAP to DEP. From DEP to DEN, Age 1 subjects increase in pH 0 and 2 hours after exposure while acidify 6 and 30 hours after exposure.

Age 2 subjects decrease in EBC pH at all time points from FAP to DEP, acidify from DEP to DEN 0 and 6 hours after exposure, while remain unchanged 2 and 30 hours after exposure.

Age 3 subjects show an increase in pH at all time points except 2 hours after exposure from FAP to DEP which decreases; while pH increases for all time points from DEP to DEN.

Table 46: Univariate analyses for effect modification by age on delta biomarkers

	Delta 8-isoprostane			Delta pH		
	β (pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Delta 2						
Age1	-30.3	-204, 144	0.95	0.03	-0.19, 0.25	0.65
Age2	**62.1**	**9.96, 114**		**-0.08**	**-0.24, 0.08**	
Age3	**46.0**	**-25.2, 117**	**0.06**	**0.32**	**0.10, 0.54**	**0.005**
Delta 6						
Age1	-25.7	-105, 53.7	0.83	0.12	-0.27, 0.51	0.62
Age2	45.6	-4.18, 95.4		-0.17	-0.46, 0.12	
Age3	24.7	-43.3, 92.7	0.20	0.19	-0.22, 0.60	0.28
Delta 30						
Age1	-24.8	-177, 128	0.92	0.15	-0.73, 1.03	0.87
Age2	48.6	1.95, 95.2		-0.25	-0.54, 0.04	
Age3	19.0	-44.9, 82.9	0.13	-0.05	-0.48, 0.38	0.28

Age 2 borderline significantly increased delta 2 8-isoprostane. Age 2 was typically associated with larger levels than Age1 or 3, Age 3 had little change in 8-isoprostane levels, while Age 1 subjects had lower levels after exposure than at baseline. Age 2 subjects were consistently associated with an acidification while age 1 and 3 subjects consistently with an increase in pH.

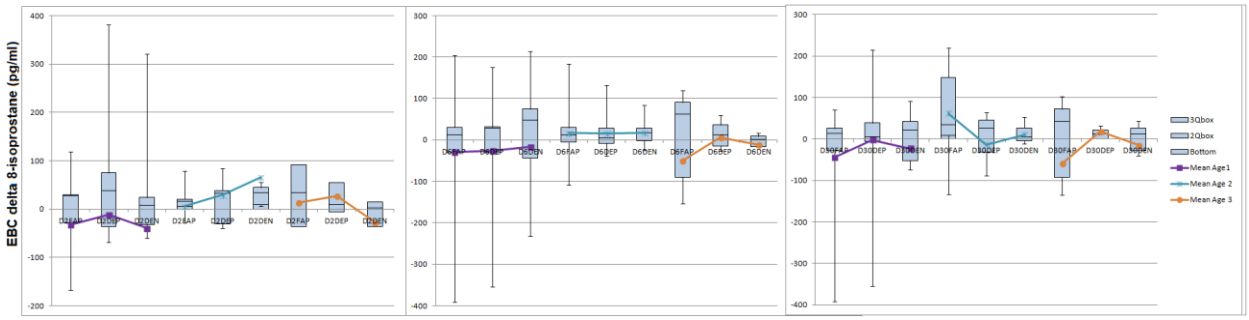

Figure 43. EBC delta 8-isoprostane stratified by exposure for age 1(purple), age 2 (blue) and age 3 (orange) subjects for all time points (left to right 2, 30 and 6 hours after exposure).

Briefly, Age 1 subjects show an increase from FAP to DEP and decrease from DEP to DEN in delta pH levels at all time points. Age 2 subjects show no change from FAP to DEP across all time points while show increase from DEP to DEN across all time points. Age 3 subjects decrease from FAP to DEP 2 hours after exposure; while they remain the same from FAP to DEP 6 hours after exposure. 30 hours after exposure, Age 3 subjects increase in delta pH from FAP to DEP. Age 3 subjects decrease from DEP to DEN across all time points.

Due to the significant effect age had on EBC delta pH 2 hours after exposure, potential effect modification by age was investigated.

Briefly, Age1 subjects show no change in EBC delta 8-isoprostane 6 hours after exposure. From FAP to DEP 2 and 30 hours after exposure, levels increase; while from DEP to DEN, levels decrease.

For Age 2 subjects from FAP to DEP and from DEP to DEN EBC delta 8-isoprostane levels increase 2 hours after exposure. Subjects show no change in EBC delta 8-isoprostane 6 hours after exposure throughout exposures. Age 2 subjects shown an increase from FAP to DEP and decrease from DEP to DEN in delta 8-isoprostane.

Age 3 subjects EBC delta 8-isoprostane levels increase from FAP to DEP for all time points while decrease from DEP to DEN for all time points.

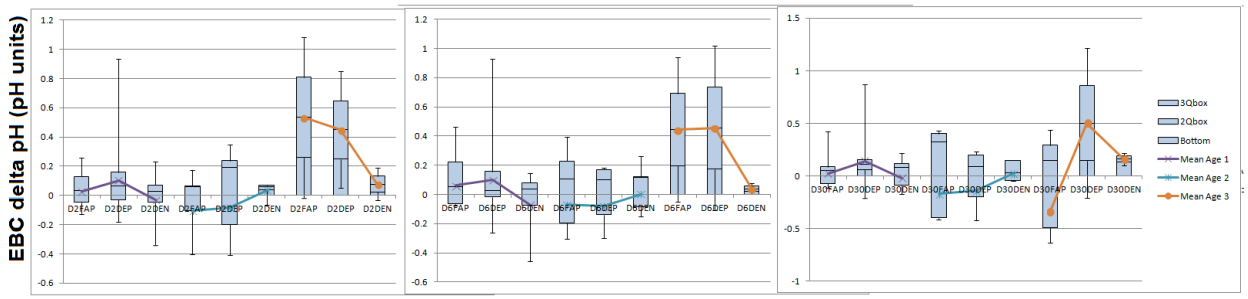

Figure 44. EBC delta pH stratified by exposure for age 1(purple), age 2 (blue) and age 3 (orange) subjects for all time points (left to right 2, 30 and 6 hours after exposure).

6. Secondary analysis: effect modification by body mass index

Table 47: Univariate analyses for effect modification by body mass index on biomarkers

	8-isoprostane			pH		
	β (log pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Baseline						
BMI1	3.55	1.57, 5.53	0.006	7.74	7.07, 8.41	<0.0001
BMI2	0.07	-0.64, 0.78		0.05	-0.52, 0.62	
BMI3	0.01	-1.03, 1.05	0.98	0.17	-0.40, 0.74	0.84
2 hours after exposure						
BMI1	3.38	0.73, 6.03	0.02	7.84	7.76, 7.92	<0.0001
BMI2	-0.13	-0.93, 0.67		**0.12**	**-0.04, 0.28**	
BMI3	0.71	-0.49, 1.91	0.38	**0.14**	**0.003, 0.28**	**0.05**
6 hours after exposure						
BMI1	3.25	1..49, 5.01	0.0003	7.86	7.68, 8.04	<0.0001
BMI2	0.26	-0.41, 0.93		0.03	-0.09, 0.15	
BMI3	0.57	-0.41, 1.55	0.50	0.01	-0.11, 0.13	0.89
30 hours after exposure						
BMI1	3.24	2.22, 4.26	<0.0001	7.81	7.64, 7.99	<0.0001
BMI2	0.15	-0.56, 0.86		0.01	-0.28, 0.30	
BMI3	0.15	-0.89, 1.19	0.91	0.08	-0.21, 0.37	0.86

BMI3 subjects were consistently associated with larger 8-isoprostane levels compared to BMI1 subjects. BMI3 subjects also borderline significantly increased pH compared to other BMI groups.

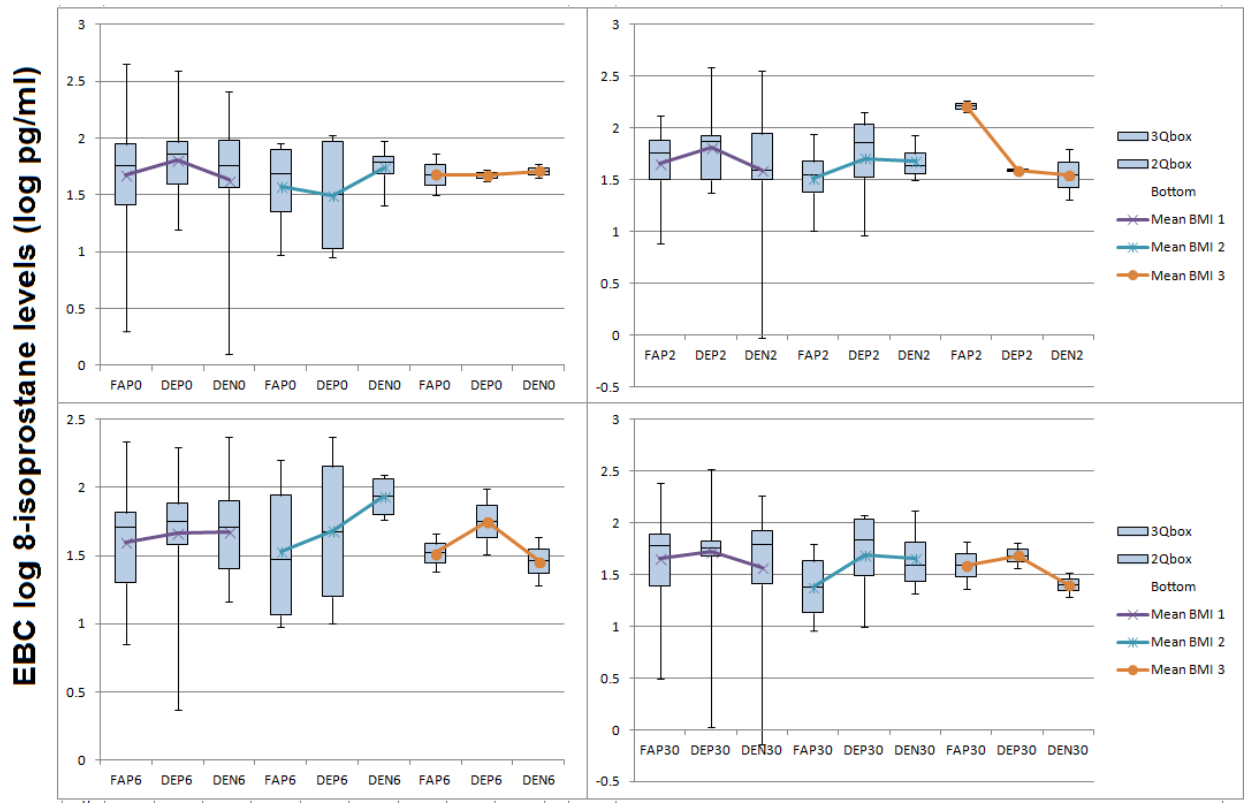

Figure 45. EBC log 8-isoprostane stratified by exposure for BMI 1(purple), BMI 2 (blue) and BMI 3 (orange) subjects for all time points (From top left clockwise baseline, 2, 30 and 6 hours after exposure).

Briefly, BMI1 subjects showed increased EBC log 8-isporstane from FAP to DEP and decreased from DEP to DEN across all time points. BMI2 subjects showed an increase in log 8-isoprostane from FAP to DEP 2, 6 and 30 hours after exposure while levels decreased 0 hours from FAP to DEP. Log 8-isprostane increased 0 and 6 hours after exposure and remained the same 2 and 30 hours after exposure from DEP to DEN for BMI2 subjects. BMI3 subjects saw no change at baseline across all exposures, increased then decreased from FAP to DEP and DEP to DEN for 6 and 30 hours after exposure; while decreased across all exposures for 2 hours after exposure.

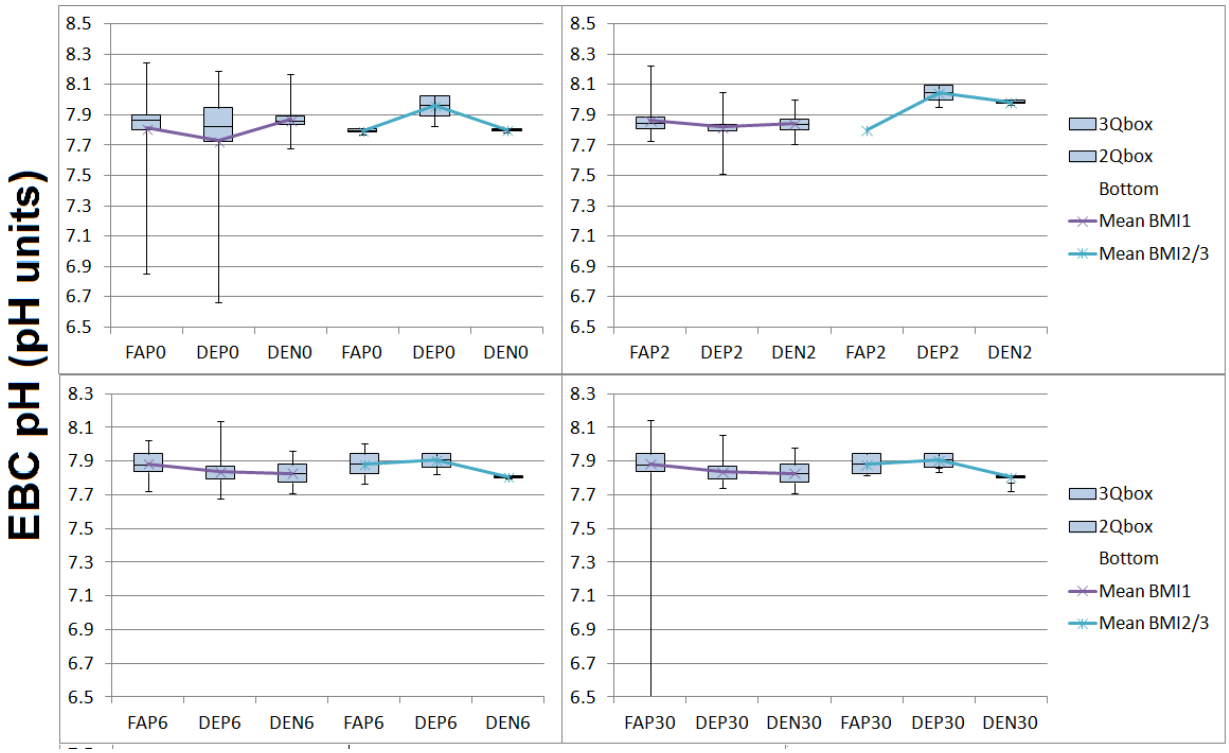

Figure 46 EBC pH stratified by exposure for BMI1 (purple) and BMI2/3 (blue) subjects for all time points (From top left clockwise baseline, 2, 30 and 6 hours after exposure).

Table 48: Univariate analyses for effect modification by genetic GSTP1 status on delta biomarkers

	Delta 8-isoprostane			Delta pH		
	β (pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Delta 2						
BMI1	-13.8	-155, 128	0.97	0.04	-0.14, 0.22	0.59
BMI2	17.6	-34.5, 69.7		0.11	-0.26, 0.48	
BMI3	41.6	-35.2, 118	0.55	0.03	-0.28, 0.34	0.83
Delta 6						
BMI1	-24.5	-91.1, 42.1	0.78	0.11	-0.24, 0.46	0.59
BMI2	24.9	-23.3, 73.1		-0.03	-0.60, 0.54	
BMI3	39.7	-31.2, 111	0.44	-0.16	-0.73, 0.41	0.85
Delta 30						
BMI1	-13.9	-74.7, 46.9	0.79	0.08	-0.28, 0.43	0.69
BMI2	11.8	-34.1, 57.7		-0.05	-0.62, 0.52	
BMI3	9.42	-58.0, 76.8	0.87	-0.09	-0.68, 0.50	0.94

There were no significant differences between BMI groups. However, BMI3 was associated with higher levels of 8-isoprostane while BMI1 subjects typically had lower levels after exposure than before their exposure. BMI2 and 3 had larger acidifications at 6 and 30 hours after exposure compared to 2 hours after exposure.

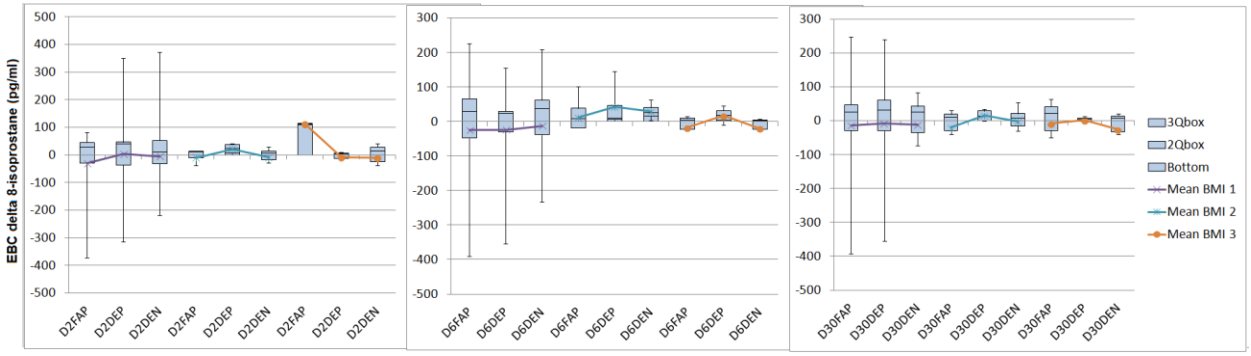

Figure 47 EBC delta 8-isoprostane stratified by exposure for BMI 1(purple), BMI 2 (blue) and BMI 3 (orange) subjects for all time points (left to right 2, 30 and 6 hours after exposure).

Age1 subjects show small negative to no change in EBC 8-isoprostane levels identifying that levels prior to exposure were higher than after exposure. BMI2 subjects show little positive or no changes across exposures, typically higher after DEP exposure. BMI 3 subjects show differences between exposures but it is not clear if there is a trend across exposures.

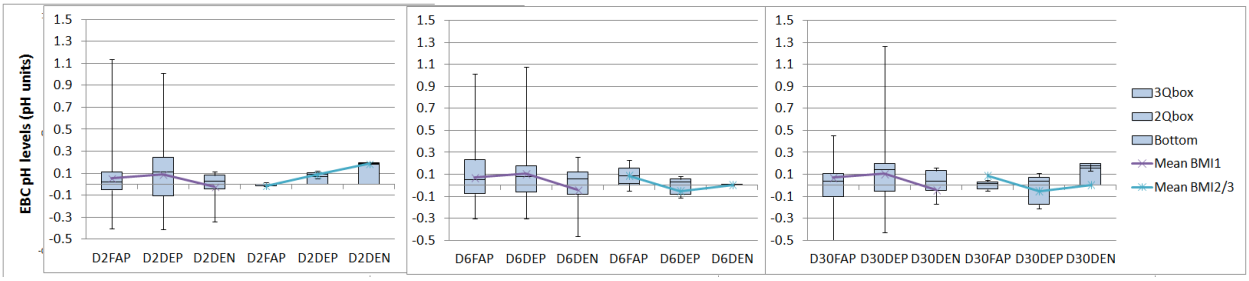

Figure 48 EBC delta pH stratified by exposure for BMI 1(purple), BMI 2/ BMI 3 (blue) subjects for all time points (left to right 2, 30 and 6 hours after exposure).

BMI1 subjects have small positive pH changes after FAP and DEP exposures while show no change in pH after DEN exposure. BMI2/3 subjects show an acidification from FAP to DEP exposures while increase after DEN exposure.

7. Secondary analysis: effect modification by order

Table 49: Univariate analyses for effect modification by order for biomarkers

	8-isoprostane			pH		
	β (log pg/ml)	Confidence Interval	p-value	β (pH units)	Confidence Interval	p-value
Baseline						
Order1	3.46	2.19, 4.73		7.62	7.19, 8.05	
Order2	0.17	-0.61, 0.95		0.16	-0.17, 0.49	
Order3	0.23	-0.55, 1.01	0.84	0.23	-0.10, 0.56	0.37
2 hours after exposure						
Order1	3.35	2.57, 4.13		7.86	7.74, 7.98	
Order2	0.06	-0.88, 1.00		-0.0005	-0.08, 0.08	
Order3	0.12	-0.82, 1.06	0.97	-0.0066	-0.09, 0.08	0.98
6 hours after exposure						
Order1	3.29	2.25, 4.33		7.88	7.80, 7.96	
Order2	-0.26	-1.00, 0.49		-0.037	-0.10, 0.02	
Order3	0.19	-0.56, 0.94	0.50	0.007	-0.06, 0.07	0.47
30 hours after exposure						
Order1	3.27	2.19, 4.35		7.83	7.67, 7.99	
Order2	-0.14	-0.90, 0.62		-0.062	-0.24, 0.11	
Order3	0.14	-0.62, 0.90	0.77	-0.00026	-0.18, 0.18	0.72

Order had no effect on biomarker levels. There is no clear trend in 8-isoprostane levels after any time point. EBC pH seems to acidify for Order 2 and 3 2, 6 and 30 hours after exposure.

Table 50: Univariate analyses for effect modification by order for delta biomarkers

	Delta 8-isoprostane			Delta pH		
	β (pg/ml)	Confidence Interval	p-value	B (pH units)	Confidence Interval	p-value
Delta 2						
Order1	19.6	-327, 367		0.073	-0.16, 0.31	
Order2	-39.8	-98.2, 18.6		0.0017	-0.19, 0.19	
Order3	-35.2	-93.6, 23.2	0.35	-0.078	-0.27, 0.11	0.63
Delta 6						
Order1	1.95	-67.8, 71.7		0.24	-0.62, 1.10	
Order2	-24.6	-79.9, 30.7		-0.193	-0.51, 0.12	
Order3	-7.12	-62.4, 48.2	0.67	-0.24	-0.57, 0.09	0.32
Delta 30						
Order1	-0.32	-54.6, 53.9		0.26	-0.07, 0.59	
Order2	-18.5	-70.8, 33.8		-0.27	-0.60, 0.06	
Order3	-3.09	-55.4, 49.2	0.76	-0.28	-0.61, 0.05	0.19

Order had no significant effect on delta biomarker levels. There is a consistent decrease in 8-isoprostane for deltas 2 and 3 as well as for delta pH levels.

Table 51: Multivariate analyses for effect modification by age on baseline EBC log 8-isoprostane

		EBC log 8-isoprostane			
		β (log pg/ml)	Confidence Interval	p-value	ANOVA p-value
Baseline					
Intercept		3.10	1.85, 4.35	<0.0001	<0.0001
DEP		0.47	-0.53, 1.47	0.36	
DEN		0.74	-0.30, 1.78	0.17	0.34
	Sex	0.27	-0.40, 0.94	0.47	0.12
	MRS	0.57	-0.25, 1.39	0.19	0.75
	Atopy	0.48	-0.28, 1.24	0.24	0.14
	GSTP1	-0.41	-1.14, 0.32	0.28	0.35
	Order 2	0.16	-0.60, 0.92	0.69	
	Order 3	-0.0001	-0.77, 0.76	0.99	0.89
	Age 2	**-0.90**	**-2.11, 0.32**	**0.15**	
	Age 3	**-1.12**	**-2.90, 0.66**	**0.23**	**0.005**
	BMI 2	0.18	-0.51, 0.87	0.61	
	BMI 3	0.65	-0.47, 1.77	0.26	0.52
DEPAge2		0.34	-1.33, 2.00	0.69	
DENAge2		-1.18	-2.90, 0.55	0.19	
DEPAge3		-0.10	-2.39, 2.19	0.93	
DENAge3		0.03	-2.28, 2.34	0.98	0.45

Age remained significant but was independent of exposure. Multivariate models for log 8-isoprostane showed age (p=0.005) to be significant at baseline.

Table 52: Multivariate analyses for effect modification by sex and BMI on EBC log 8-isoprostane 2 hours after exposure

	EBC log 8-isoprostane			
	β (log pg/ml)	Confidence Interval	p-value	ANOVA p-value
2 hours after exposure				
Intercept	2.94	-1.02, 6.90	0.15	0.07
DEP	0.45	-1.24, 2.14	0.60	
DEN	0.19	-1.50, 1.88	0.83	0.96
Sex	**1.30**	**-0.09, 2.69**	**0.08**	**0.09**
MRS	0.74	-0.30, 1.78	0.17	0.63
Atopy	0.29	-0.67, 1.25	0.56	0.24
GSTP1	-0.99	-1.91, -0.07	0.04	0.22
Order 2	0.11	-0.83, 1.05	0.82	
Order 3	0.10	-0.90, 1.10	0.84	0.96
Age 2	-0.36	-1.42, 0.52	0.43	
Age 3	-1.86	-3.37, -0.35	0.02	0.31
BMI 2	0.03	-1.40, 1.46	0.96	
BMI 3	2.73	0.53, 4.93	0.02	0.03
DEPSex	**-0.67**	**-2.55, 1.21**	**0.49**	
DENSex	**-0.55**	**-2.41, 1.31**	**0.56**	**0.82**
DEPBMI2	0.001	-1.98, 1.98	0.99	
DENBMI2	0.24	-1.84, 2.32	0.82	
DEPBMI3	-1.30	-4.22, 1.62	0.39	
DENBMI3	-1.30	-4.24, 1.64	0.39	0.86

Sex and BMI remained borderline significant but was independent of exposure. Multivariate models for log 8-isoprostane showed sex (p=0.07) and BMI (p=0.02) to be significant 2 hours after exposure.

Table 53: Multivariate analyses for effect modification by sex, MRS and atopy on EBC pH 2 hours after exposure

		pH			
		β (pH units)	Confidence Interval	p-value	ANOVA p-value
2 hours after exposure					
Intercept		7.94	7.74, 8.14	<0.0001	<0.0001
DEP		-0.17	-0.35, 0.006	0.09	
DEN		-0.009	-17.6, 17.6	0.92	0.88
	Sex	**0.09**	**-0.05, 0.23**	**0.20**	**0.02**
	MRS	**-0.07**	**-0.23, 0.09**	**0.45**	**0.09**
	Atopy	**-0.02**	**-0.24, 0.20**	**0.85**	**0.03**
	GSTP1	-0.03	-0.11, 0.05	0.41	0.64
	Order 2	-0.03	-0.13, 0.07	0.48	
	Order 3	-0.04	-0.12, 0.04	0.35	0.95
	Age 2	0.05	-0.03, 0.13	0.27	
	Age 3	-0.05	-0.21, 0.11	0.52	0.67
	BMI 2	0.16	-0.06, 0.38	0.16	
	BMI 3	0.11	-0.11, 0.33	0.33	0.30
DEPSex		0.04	-0.14, 0.22	0.67	
DENSex		0.008	-0.17, 0.18	0.93	0.51
DEPMRS		-0.02	-0.22, 0.18	0.86	
DENMRS		0.02	-0.20, 0.24	0.87	0.92
DEPAtopy		0.17	-0.10, 0.44	0.23	
DENAtopy		-0.007	-0.28, 0.27	0.96	0.32

Multivariate models for EBC pH showed sex (p=0.01), MRS (p=0.08) and atopic status (p=0.02) significantly modified EBC pH 2 hours after exposure. Interaction terms were not significant, as such no effect modification was observed for the EBC pH multivariate model 2 hours after exposure.

Table 54: Multivariate analyses for effect modification by MRS on EBC log 8-isoprostane 6 hours after exposure

	EBC log 8-isoprostane			
	β (log pg/ml)	Confidence Interval	p-value	ANOVA p-value
6 hours after exposure				
Intercept	3.94	2.45, 5.43	<0.0001	<0.0001
DEP	0.15	-0.85, 1.15	0.77	
DEN	-0.22	-1.22, 0.78	0.67	0.99
Sex	-0.39	-1.15, 0.37	0.33	0.41
MRS	**-0.39**	**-1.70, 0.92**	**0.56**	**0.09**
Atopy	0.16	-0.66, 0.98	0.71	0.53
GSTP1	-0.31	-1.70, 0.45	0.44	0.71
Order 2	-0.45	-1.31, 0.41	0.32	
Order 3	0.06	-0.76, 0.88	0.88	0.52
Age 2	-0.13	-0.87, 0.61	0.74	
Age 3	-0.005	-1.30, 1.29	0.99	0.81
BMI 2	0.31	-0.42, 1.04	0.40	
BMI 3	0.71	-0.49, 1.91	0.24	0.47
DEPMRS	-0.64	-2.34, 1.07	0.46	
DENMRS	0.34	-1.39, 2.06	0.70	0.56

Multivariate models for log 8-isoprostane showed MRS (p=0.09) to be significant 6 hours after exposure. This was independent of exposure.

Table 55: Multivariate analyses for effect modification by age on EBC delta 8-isoprostane 2 hours after exposure

	Delta 8-isoprostane			
	β (pg/ml)	Confidence Interval	p-value	ANOVA p-value
Delta 2				
Intercept	-10.1	-122, 102	0.86	0.91
DEP	25.1	-53.5, 104	0.53	
DEN	-9.13	-89.9, 71.6	0.83	0.95
Sex	4.93	-54.1, 63.9	0.87	0.86
MRS	-65.8	-134, 2.40	0.06	0.18
Atopy	37.6	-23.9, 99.1	0.24	0.30
GSTP1	24.7	-32.5, 81.9	0.40	0.19
Order 2	-40.4	-100, 19.4	0.19	
Order 3	-30.4	-91.2, 30.4	0.33	0.33
Age 2	**46.2**	**-48.3, 141**	**0.34**	
Age 3	**112**	**-30.9, 255**	**0.13**	**0.04**
BMI 2	20.7	-36.5, 11.9	0.48	
BMI 3	36.0	-60.2, 132	0.47	0.71
DEPAge2	-19.3	-150, 111	0.77	
DENAge2	63.9	-71.7, 199	0.36	
DEPAge3	-75.3	-255, 104	0.42	
DENAge3	-68.9	-249, 112	0.46	0.58

Multivariate models for delta 8-isoprostane showed age to be significant (p=0.04) 2 hours after exposure. This was independent of exposure.

Table 56: Multivariate analyses for effect modification by MRS and age on EBC delta 8-isoprostane 6 hours after exposure

		Delta 8-isoprostane			
		β (pg/ml)	Confidence Interval	p-value	ANOVA p-value
Delta 6					
Intercept		7.69	-95.4, 111	0.88	0.77
DEP		-0.81	-84.9, 83.3	0.98	
DEN		-22.9	-109, 63.1	0.60	0.99
	Sex	-46.1	-101, 8.58	0.10	0.31
	MRS	**-118**	**-221, -15.1**	**0.03**	**0.05**
	Atopy	31.2	-27.8, 90.2	0.30	0.33
	GSTP1	11.4	-43.7, 66.5	0.69	0.65
	Order 2	-39.9	-102, 21.8	0.21	
	Order 3	-14.8	-75.8, 46.2	0.64	0.68
	Age 2	44.7	-46.8, 136	0.34	
	Age 3	128	-20.6, 277	0.09	0.06
	BMI 2	11.5	-40.1, 63.0	0.66	
	BMI 3	-1.8	-86.1, 82.5	0.96	0.88
DEPMRS		10.0	-132, 152	0.89	
DENMRS		105	-38.5, 248	0.16	0.43
DEPAge2		-8.63	-135, 118	0.89	
DENAge2		-7.19	-139, 124	0.92	
DEPAge3		-14.52	-216, 187	0.89	
DENAge3		-80.0	-282, 122	0.44	0.95

Multivariate models for delta 8-isoprostane showed MRS (p=0.04) and Age (p=0.05) to be significant 6 hours after exposure. This was independent of exposure.

Table 57: Multivariate analyses for effect modification by age and BMI on EBC delta pH 2 hours after exposure

		pH			
		β (pH units)	Confidence Interval	p-value	ANOVA p-value
Delta 2					
Intercept		-0.04	-0.39, 0.31	0.83	0.59
DEP		0.07	-0.17, 0.31	0.51	
DEN		-0.03	-0.28, 0.22	0.80	0.57
	Sex	0.08	-0.10, 0.26	0.39	0.91
	MRS	0.03	-0.19, 0.25	0.79	0.19
	Atopy	-0.02	-0.31, 0.27	0.92	0.11
	GSTP1	0.01	-0.15, 0.17	0.87	0.68
	Order 2	-0.005	-0.18, 0.17	0.95	
	Order 3	-0.06	-0.26, 0.14	0.56	0.70
	Age 2	**-0.06**	**-0.35, 0.23**	**0.68**	
	Age 3	**0.87**	**-0.40, 1.34**	**0.0008**	**0.02**
	BMI 2	**0.20**	**-0.23, 0.63**	**0.36**	
	BMI 3	**-0.62**	**-1.09, -0.15**	**0.02**	**0.02**
DEPAge2		-0.08	-0.45, 0.30	0.69	
DENAge2		0.15	-0.26, 0.56	0.48	
DEPAge3		-0.19	-0.74, 0.36	0.49	
DENAge3		-0.42	-0.95, 0.11	0.13	0.29

Multivariate models for delta pH showed age (p=0.02) and BMI (p=0.02) to be significant 2 hours after exposure. This was independent of exposure.