ADIPOKINE RESPONSES IN THE LUNG AND CIRCULATION IN ATOPIC ADULTS UPON EXPOSURE TO ALLERGEN AND DIESEL EXHAUST

by

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B.Sc., The University of Victoria, 2013

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE
in
THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Occupational and Environmental Hygiene)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

July 2015

Abstract

Introduction:

Adipokines are inflammatory mediators released primarily from the adipose tissue. These proteins are now recognized as active elements of systemic and pulmonary inflammatory responses, whose dysregulation can prime the development of allergic lung diseases. The purpose of this study was to measure adipokine responses in an atopic adult study population following exposure to allergen and diesel exhaust. Characterizing adipokine responses in the lung and in the serum, in an atopic but otherwise healthy population, can provide insight into adipokine responses in sensitized to specific allergens, yet without co-morbidities.

Methods:

Lung and blood samples were collected from subjects participating in a randomized, double-blinded controlled human study with crossover to two conditions: inhaled diesel exhaust and inhaled filtered air, each of which were followed by lung-instilled allergen (and contralateral saline control). Serum samples collected at baseline, 4, 24 and 48 hours after allergen instillation, and lung samples collected at 48 hours after allergen were assayed for total adiponectin, leptin and resistin using ELISA. Mixed-effects models were used for statistical analysis to determine exposure effect, and effect modification by sex, BMI status and airway responsiveness.

Results:

Adiponectin and leptin were significantly increased in the lung in response to allergen. Leptin and resistin changed in the serum in a diurnal pattern, but levels were not altered by diesel exhaust. Diesel exhaust and allergen co-exposure significantly increased the adiponectin/leptin ratio in the lung relative to allergen alone, in subjects with normal airway responsiveness.

Conclusion:

Increases in lung adipokines in response to allergen exposure were identified in the context of a controlled human exposure study. Some effect modification by sex, BMI and airway responsiveness occurred. Diesel exhaust along with allergen induced a protective adipokine pattern in the lung in those with normal airway responsiveness. The clinical relevance and generalizability of these findings, herein noted in atopic individuals, warrants further study.

Preface

All work presented in this thesis was conducted at the Vancouver General Hospital in the Air Pollution Exposure Laboratory (APEL) and at the Jack Bell Research Centre. The human controlled study, that provided the human samples used in this study, was designed by Dr. Christopher Carlsten and was approved by the ethical review boards of the University of British Columbia and the Vancouver Coastal Health Research Institute. This study is registered at clinicaltrials.gov (trial no. NCT01792232), and is covered by UBC Ethics Certificate number H11-01831. This thesis is the original intellectual product of the author, M. Kramer, who was responsible for thesis project development, data collection and analysis, as well as thesis composition. C. Carlsten was the supervisory author on this project, and was involved throughout project conception and data analysis, and provided edits and suggestions during thesis composition. The data contained in Chapter 4, Sections 4.4 and 4.5 will be submitted for publication shortly.

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List of Abbreviations

µm	micrometer
ANOVA	analysis of variance
APEL	Air Pollution Exposure Laboratory
BAL	bronchoalveolar lavage
BW	bronchial wash
BMI	body mass index, kg/m^2
cc	cubic centimeter
CO	carbon monoxide
CO_2	carbon dioxide
COPD	chronic obstructive pulmonary disease
DE	diesel exhaust
DEA	diesel exhaust and allergen
DES	diesel exhaust and saline
DPM	diesel particulate matter
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FA	filtered air
FAS	filtered air and saline
FAA	filtered air and allergen
FEF25-75	forced expiratory flow at 25-75% of the pulmonary volume
FEV_1	forced expiratory volume in first second of exhalation
FVC	forced vital capacity
H_0	statistical null hypothesis
IL	interleukin
IQR	interquartile range
kW	kilo Watt
LPS	lipopolysaccharide
ln	natural logarithm (base e)
log	logarithm (base 10)
mg/mL	milligram per milliliter
MS	metabolic syndrome
ng/mL	nanograms per milliliter
NO	nitric oxide
NO_2	nitrogen dioxide
O_2	oxygen
OD	optical density
PAHs	polycyclic aromatic hydrocarbons
PC_{20}	methacholine concentration that causes a 20% reduction in FEV_1
pg/mL	picograms per milliliter
PM	particulate matter
$PM_{2.5}$	particulate matter less than 2.5 microns in aerodynamic diameter
ppb	parts per billion
ppm	parts per million
s	standard deviation

$\mathbf{s_g}$	geometric standard deviation
$\mathbf{SO_2}$	sulfur dioxide
TNF-α	tumor necrosis factor-alpha
VOC	volatile organic compound
$\mathbf{\bar{x}}$	sample mean
$\mathbf{\bar{x}_g}$	geometric mean

Acknowledgements

There are many people who allowed this thesis experience to be the most rewarding and fulfilling challenge of my academic career to date. First and foremost, the completion of this thesis project would not have been possible without the positive, yet realistic, guidance of my supervisor Chris Carlsten. He knew when to set deadlines, when to allow me freedom to explore, when to encourage focus, and was always available when I needed a little extra help. Thank you for supporting me throughout this process, and also for supporting my future goals and aspirations, ones that are now coming true.

To my committee members, Kay Teschke and Akshay Sood, thank you for your focused and timely advice and feedback, and for helping me see my project from different perspectives. This thesis was much improved by your valuable input.

To the other members of the Carlsten lab who assisted me with lab techniques, who asked me questions that I had yet to answer, and who provided me with friendly encouragement and advice, I am truly appreciative. Thanks also to my fellow students in the OEH program – we learned a lot together and survived an onerous first-year course load, while still managing to enjoy our time in Vancouver.

Lastly, thank you to the NSERC CREATE-AAP program for providing me with funding and training.

I will most certainly carry the knowledge, skills and experience I gained from the OEH program into the next chapter of my academic journey.

Dedication

This thesis is dedicated to my family who always knew I could succeed, even when I wasn't so sure.

Chapter 1: Introduction

1.1 Purpose and Objectives of Study

Adipokines are inflammatory mediators first discovered to be released from the adipose tissue. Animal models have shown that these mediators are secreted in response to inflammatory stimuli in the lung, and that the spillover causes enhanced systemic and pulmonary inflammation. Although adipokines have been historically studied in the obese, who have enhanced risk of asthma, adipokine levels are also known to change in response to inhaled inflammatory stimuli. Hypothetically, those with dysregulated adipokine responses, more skewed towards the pro-inflammatory, may be more prone to developing asthma and other inflammatory lung diseases in response to repeated or prolonged allergen exposure, while those with more appropriate adipokine responses may never develop any condition more serious than an allergy. The purpose of this study was to further elucidate the change in adipokine concentrations in response to allergen exposure, among an atopic yet otherwise healthy population. Changes in systemic and airway adipokine concentrations in response to diesel exhaust (DE) and allergen co-exposure, were examined and compared. Although animal models suggest acute changes in adipokines will occur in response to allergen exposure, few clinical human studies have investigated this question, none have included a controlled lung allergen challenge, and none have co-exposed to an adjuvant such as diesel exhaust. Accordingly our study design, involving allergen and diesel exhaust co-exposure, uniquely investigates the potential for augmentation of allergen effects on adipokine responses in the controlled context of a crossover study design.

1.2 Research Methodology

To investigate these aims, blood and lung samples collected within this controlled human exposure study were examined. An *in vivo* experiment of 18 atopic individuals was performed using a randomized, balanced, double-blinded crossover study design in a laboratory setting. Each participant was exposed to diesel exhaust and filtered air in addition to a saline-controlled segmental allergen challenge, allowing each participant to serve as his/her own control. Controlled chamber studies like this one allow researchers to assess the acute health effects of individual pollutants (1), or in the case of this study, the interaction of specific exposures. It was within the context of controlled human exposure studies that diesel exhaust was directly recognized to cause inflammatory changes in the airways (2–8), as well as increased airway resistance (9,10) and responsiveness (9).

The atopic individuals selected for study allow adipokine changes in response to specific allergen exposure to be detected in a relatively young and healthy population, one that is without the complication of comorbid conditions. It will be investigated if exposure to inhaled diesel exhaust and allergen in these individuals alters

adipokine levels within 48 hours of exposure. Data from this randomized double-blinded crossover study was analyzed to complete the following objectives:

1) To investigate the effects of controlled allergen and diesel exhaust co-exposure on adipokine levels in the airways and in the blood.
2) To determine if diesel exhaust augments the response expected from allergen exposure.
3) To examine the time course of adipokine responses.
4) To examine how sex and BMI status affect systemic and lung adipokine concentrations, both at baseline and post-exposure.

These objectives were investigated using 4 hypotheses with specific primary, secondary and tertiary aims that are detailed in the Methods section.

1.3 Significance of Knowledge

The specific role of adipokines in response to allergen and diesel exhaust exposure is not known, nor is there a good understanding of what characterizes adipokine response to inhaled allergen. This controlled human exposure study aims to examine the changes in adipokine levels in an atopic, yet otherwise healthy study population. This information will add understanding to the role that adipokines may play in inflammatory processes in the lung, and will further identify participant factors that may alter adipokine responses. Focus on both pro- and anti-inflammatory adipokine changes in response to allergen exposure provides valuable insight into the biochemical events that may underlie allergic lung disease.

Chapter 2: Background & Literature Review

2.1 Diesel Exhaust

Diesel exhaust exposure is a relevant exposure both in the workplace, as well as in the general population. Diesel engines are used widely in occupational settings in large transportation vehicles and in heavy equipment machinery (11). Therefore, many workers in occupational settings are at risk of high and chronic diesel exhaust exposure, contributing to respiratory irritation and asthma. CAREX (CARcinogen EXposure) Canada estimates that 897,000 workers are exposed in Canada, with transportation industries having the highest prevalence of exposure (12), and those working in the mining industry having the highest levels of exposure (13).

Diesel exhaust is also a key contributor to ambient particulate matter less than 2.5 microns in aerodynamic diameter ($PM_{2.5}$) (14). Therefore, the general population is also widely exposed to diesel particulate matter (DPM). Because of the large number of people exposed, the health implications of this exposure are considerable. Those most vulnerable to the effects of air pollution among the general population include children under 15 years of age, the elderly aged 65 years and older, those with existing respiratory conditions, or those with poor cardiovascular health (11,15,16). Evidence is also emerging that pregnant women must also be considered amongst this vulnerable population, as ambient levels of DPM and other diesel exhaust components may have an adverse impact on fetal growth and development (17).

2.1.1 Components of Diesel Exhaust

Diesel exhaust is released due to the incomplete combustion that occurs when diesel is used as a fuel source (16) which results in a complex and variable emission mixture (18). The emission mixture is highly dependent on various factors including the type and quality of fuel used, as well as the type, age and technology of the engine (18,19). When compared with gasoline exhaust emissions, diesel exhaust contains smaller amounts of carbon monoxide, hydrocarbons and carbon dioxide, but contains larger amounts of nitrogen oxides and aldehydes, and most importantly high amounts of particulate matter (16,20,21). The wide range of uses of diesel fuel combined with a diesel engine's propensity to release large amounts of particulate matter as well as black carbon is a clear indication of why diesel exhaust contributes largely to the ambient concentration of particulate matter.

Diesel exhaust is a complex mixture of gases and solid particulates (22,15) containing hundreds of constituents (15) all of which contribute separately to the overall toxicity of the exposure. These constituents may also interact to further increase the health risks of this complex mixture (23). The most common gaseous

components of diesel exhaust include nitric oxide (NO), nitrogen dioxide (NO_2), sulfur dioxide (SO_2), carbon dioxide (CO_2), carbon monoxide (CO), water vapour, oxygen (O_2) and various low molecular hydrocarbons: including formaldehyde, acetaldehyde, acrolein, benzene, methane, 1,3-butadiene and polycyclic aromatic hydrocarbons (15,16,24–26). The solid portion of the mixture is the DPM (22,15,24,25,27) that was mentioned previously. The DPM fraction forms when diesel exhaust is cooled upon mixing with ambient air (11), and is responsible for the black smoke that is associated with the use of diesel engines (26). The composition of DPM, also referred to as diesel soot (16,27) consists of an aggregated solid elemental carbon core, along with as many as 400 different adsorbed substances (28) including soluble organic carbon compounds, sulfates, nitrates, metals and trace elements present on the rough surface of the particles (22,15), shown below in Figure 2.1 published by Ristovski et al. 2012 (29). The adsorbed compounds similarly contribute to the overall toxicity of the poorly defined chemical species that is diesel exhaust. The quantity and types of compounds present on the surface of DPM is considerably affected by the type of engine and its emission-cleansing technologies (30).

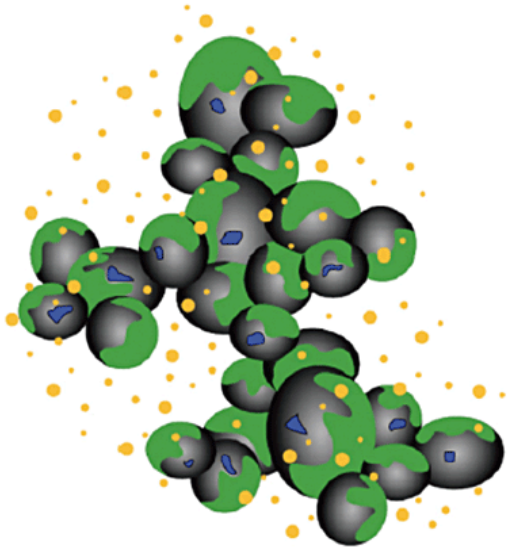

Figure 2.1. The chemical composition and structure of DPM, from Ristovski et al., who adapted the figure from Mariq [31] with permission. Soot (black), condense hydrocarbons/SO_4 (green), nucleation mode/particles in the gas phase mode (yellow) and adsorbed metals (blue) are depicted in this diagram (accessed from: http://www.ncbi.nlm.nih.gov/pubmed/22126432/).

Of the adsorbed compounds that may be present on DPM, the most harmful include the polycyclic aromatic hydrocarbons (PAHs) and nitro-PAHs which have been determined to exhibit carcinogenic and mutagenic activities (11,31). The risk of adsorbed chemicals has an impact on human health due not only to the large surface area of particulate matter (11,15) that enables a large concentration and mixture of compounds to be present, but also on the size of diesel particulates that allows them to penetrate into the alveoli, bringing adsorbed components into the deep lung (11).

Diesel particulate matter is defined by its size or aerodynamic diameter as fine (<2.5 μm, $PM_{2.5}$), ultrafine (<1.0 μm) or as nanoparticles (<50 nm) (32). The size of the PM is a characteristic that affects particle penetration and deposition in the respiratory tract, and therefore affects its toxicity. The majority of diesel particulate matter is defined as ultrafine (15), with 50-90% of the particles present in diesel exhaust falling in this size range (11). Previous experiments using radioactive particles have shown that 83% of the particles with an aerodynamic diameter under 2.5 μm are deposited in the lung, a rate that increases for smaller particles (33). Respirable particles in the size range between 0.01 to 1 μm have the greatest effect in the alveoli of the lungs (34), while larger inhalable particles between 10 and 50 μm have the greatest effect in the upper respiratory tract (34). Ultrafine DPM is not only able to penetrate deeply into the respiratory tract, but it may also enter pulmonary circulation, causing not only a pulmonary but also a systemic inflammatory response (22). DPM is the fraction most consistently associated with adverse health effects (11,35), with modest increases being related to significant increases in respiratory and cardiovascular morbidity and mortality (36–38). It is also the portion most commonly regulated by air quality standards, in the form of $PM_{2.5}$.

2.1.2 Relevance of Exposure

Diesel exhaust is a more energy dense and efficient fuel than gasoline and is therefore used to power large transportation vehicles such as trains, boats, tankers, buses and semi-trucks, as well as many types of essential equipment in a variety of industries including "transportation, mining, construction, agriculture, as well as many manufacturing operations" (39) and are the chosen engine type for these applications due to their efficiency and durability (11). A report from 2007 indicated that in British Columbia, diesel exhaust is the second most common workplace carcinogen, with 107,959 workers exposed (40).

Because of its widespread use in occupational settings, many workers are at risk of elevated DPM exposures. Mechanics in North America have reported exposures to respirable $PM_{2.5}$ of 150 $\mu g/m^3$ and 240 $\mu g/m^3$ (41), while exposure for miners ranges up to approximately 1,280 $\mu g/m^3$, with lower ranges occurring for railroad workers (39-191 $\mu g/m^3$), public transit personnel (7-98 $\mu g/m^3$), and mechanics and dockworkers (5-65 $\mu g/m^3$) (15). Chronically exposed workers are at risk of developing many occupational lung diseases, the most common one being work related asthma (42). It is estimated that exposure to fuel exhaust causes 20% of occupational asthma cases (43,44), which in turn accounted for 52% of occupational lung disease in British Columbia (45). Exposures to diesel exhaust can be reduced through the use of engineering controls including routine engine maintenance, the use of exhaust filters and low sulfur fuels and restricting the amount of diesel-powered equipment in confined areas (46). The development of these diseases over the working lifetime however, depends on individual susceptibility.

Exposures to diesel exhaust among the general population are generally lower than the values quoted above. The areas of potential greatest diesel exhaust exposure for those in urban areas include the areas nearest highways and busy roads, construction sites, transportation depots including ports and bus and truck depots, major bridges and traffic tunnels (47). As can be seen in Figure 2.2 below, the closer people are living to highways and freeways, the higher their exposures are to diesel exhaust and more generally, particulate matter (47). Epidemiology has found that living within 200 to 500 meters of a 4-lane highway causes increased prevalence of asthma or wheezing (48–50) and impaired lung function in children (51), and greater risk of cardiovascular events in susceptible adults (52). These health effects are mostly attributed to long-term exposures.

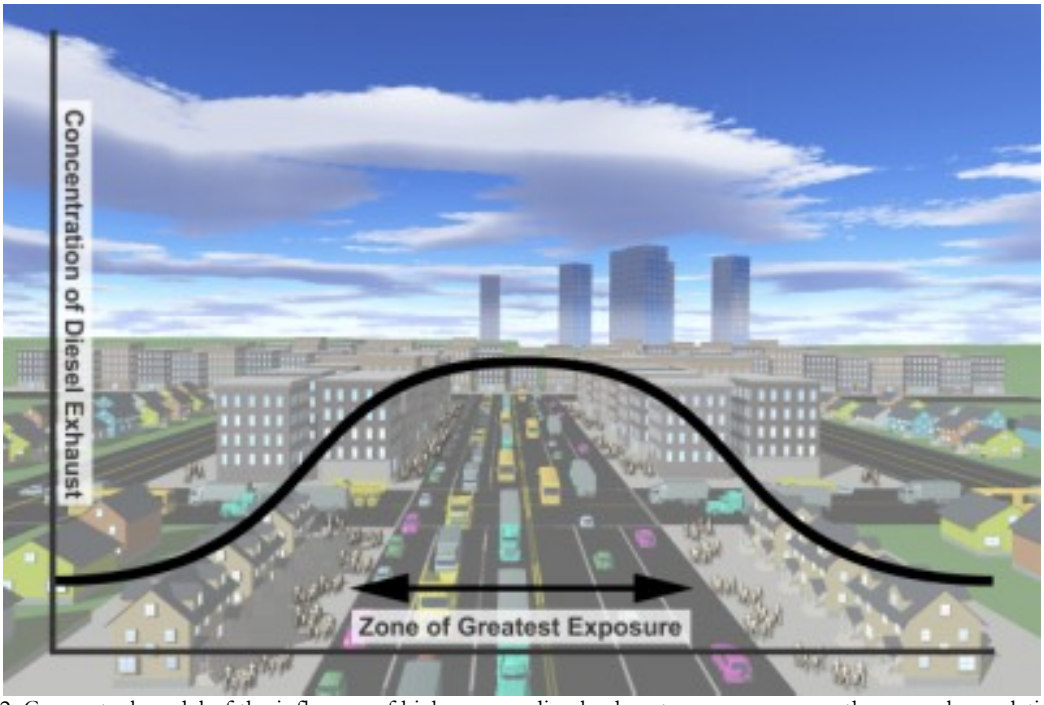

Figure 2.2. Conceptual model of the influence of highways on diesel exhaust exposure among the general population (accessed from: http://www.catf.us/methane/black_carbon/diesel/dieselhealth/faq.php?site=0).

2.2 Diesel Exhaust Health Effects

2.2.1 Acute Health Effects

In healthy subjects and working populations, relatively short-term exposures have been observed to cause respiratory irritation (10,53–55) and inflammatory symptoms (20–24), as well as transient decrements in lung function (57–59). In those with compromised respiratory function, exposure to DE has been associated with the exacerbation of asthmatic symptoms and existing allergies (6,38,60,61). DPM as well as having an

inflammatory effect in the lungs can cause secondary, low-grade systemic inflammation that has negative impacts on cardiovascular function (5,18,62–64). Epidemiological studies have associated episodes of heavy PM air pollution events with increased cardiovascular disease mortalities (65,66). Short-term exposure to DPM has also been associated with ventricular arrhythmia, stroke and mortality caused by ischemic heart disease (67). Additionally, large cohort studies consistently demonstrate that those exposed to higher ambient particulate matter exposure ($PM_{2.5}$) are at higher risk of mortality, with the strongest associations found with cardiopulmonary deaths (68)(69).

2.2.2 Long-term Health Effects

As of June, 2012 diesel exhaust was classified as a Group 1 carcinogen or a "known human carcinogen" (70). Evidence from long-term chronically exposed occupational groups, support a causal association between diesel exhaust and lung cancer (71). Long-term exposure also elevates the risk of respiratory infections (72,73), asthma and bronchitis exacerbation, and increased cardiopulmonary mortality in susceptible individuals (22,74). These adverse health effects occur as a result of exposure over a period of years. *Short-term exposures in controlled human exposures do not directly contribute to the chronic responses associated with long-term air pollution exposure but offer a window into those long-term effects which are, effectively, the accumulation of innumerable such short-term exposures over years.*

2.2.3 Diesel Exhaust Biochemistry, Mode of Action

The adverse health effects caused by diesel exhaust, and in particular diesel particulate matter, are mediated mainly through inflammation (74–77), although understanding of the toxicological mechanisms of such effects remains incomplete (77). Activated immunological cells including macrophages and neutrophils release cytokines, lipid mediators and reactive oxygen species that act to further propagate particulate-induced damage (18). The epithelium itself may also release pro-inflammatory mediators further contributing to inflammation. There is also evidence that lung inflammation can lead to systemic inflammatory responses as well (78–80). Upon deposition, DPM is phagocytized by macrophages and neutrophils that are attracted to the particle by chemokines (81). DPM also causes the release of inflammatory cytokines including interleukin (IL)-8, IL-6, tumor necrosis factor-alpha (TNF-α) and granulocyte-macrophage colony-stimulating factor (82). These mediators can be released by immune cells as well as from airway cells such as bronchial epithelial cells (83).

2.3 Allergen Health Effects

As has been noted, this study is designed to study the health effects not only of diesel exhaust, but also of the combined exposure between allergen and diesel exhaust. There exists lots of data indicating that diesel exhaust can augment the effects of allergen exposure.

2.3.1 Allergen Mode of Action

Allergens are defined as proteins with the ability to cause an allergic or hypersensitive reaction. An atopic individual is someone who develops immunoglobulin E (IgE) antibodies following exposure to a specific allergen (84), or more generally, those who are immunologically sensitized towards one or many, specific allergens. Skin prick tests with common allergens (84) is one way to assess an individual's atopic status, and the one used in this study. Sensitization to one or more allergens is one of the most relevant risk factors for asthma development (85). Upon exposure to specific allergen, a complex series of cellular responses occur resulting in allergic symptoms or responses (86). The acute-phase of allergen response is largely driven by mast cell degranulation (release of histamine and other mediators) followed by the expression of pro-inflammatory molecules (86). Mast cells are also known to produce chemoattractants that cause eosinophils, neutrophils, basophils and lymphocytes to populate the airway tissue. The accumulation of these cells, predominantly eosinophils, and their release of pro-inflammatory cytokines (IL-1α, TNF-α, IL-3, IL-5, IL-8) constitute the late-phase allergen response that may last several hours after allergen exposure (86).

2.3.2 Allergen-Diesel Exhaust Synergy

Data indicate that air pollution components, including diesel exhaust particulate, have the potential to augment allergen effects in the nose, and likely in the airways. It appears that combustion particles can act as carriers of allergens, increasing their ability to penetrate into the airways (87). Air pollutants are also hypothesized to act indirectly to alter and augment allergen health effects. Data from *in vitro* studies and rodent models have confirmed that diesel exhaust particulate augments allergen effects on TH2 cytokines, eosinophil activity, and eotaxin levels (88–90). Diesel exhaust augmentation of allergenic effects has been confirmed in a human nasal exposure model (91–94), and with related particulate exposures including PM_{10} (95) and cigarette smoke (96). Therefore, the present study was designed specifically to investigate and demonstrate diesel exhaust augmentation of allergenic effects *in vivo,* in the human lung.

2.4 Adipokines

The adipose tissue is now recognized as an active endocrine organ and an active participant in the regulation of pathologic and physiologic processes (97). The products produced primarily in the adipocytes, aptly named

adipokines, are active participants in the regulation of pathologic processes, including inflammation (98,99). Adipose tissue products and mediators can enhance local and systemic inflammatory responses in response to several exposures, and under several pathogenic conditions (100). In lean individuals, adipose tissue secretes limited amounts of pro-inflammatory cytokines (TNF-α, IL-6, IL-8) and pro-inflammatory adipokines (leptin), but in an obese state, the tissue becomes infiltrated with macrophages that secrete large amounts pro-inflammatory cytokines influencing the release of pro-inflammatory adipokines (101). This observation led to the idea that the adipose tissue perpetuating "metabolic inflammation" could have an impact on systemic inflammation, and inflammation in other sites, such as the airways (102). This potential connection between adipokines and airway inflammation, was first identified in epidemiologic data that indicated there was an increased prevalence of asthma in obese individuals (103). Now, several connections between adipokines, allergen exposure and skewed pro-inflammatory reactions have been realized. Although the term "adipokine" is given to any protein that is produced and released from adipocytes (104), the three most widely studied include adiponectin, leptin and resistin. These will also be the focus of this study.

2.4.1 Characteristics of Key Adipokines – Adiponectin, Leptin, Resistin

2.4.1.1 General Characteristics: Adiponectin

Adiponectin, also referred to as adipocyte-complement-related protein, is a 30 kDa insulin sensitizing protein (99,105). It is an abundant serum protein that constitutes 0.01% of all circulating proteins, and was first recognized as the most abundant transcript expressed by adipocytes (106,107). Although abundantly secreted from visceral adipocytes (108), systemic adiponectin levels are inversely correlated with body mass index (BMI) (107,109), although this relationship is not always consistent (110). The basic adiponectin monomer multimerizes and circulates in the blood in low (trimers), medium (hexamers) and high molecular weight (higher order multimers) forms (111). The isoforms may vary in efficacy; however, differential airway effects of the various isoforms remain largely unknown (99). Sex differences exist in circulating adiponectin concentrations with women having higher total serum adiponectin and higher absolute levels of all isomers than men (112). In addition to adipocyte production, adiponectin is also produced in the lung and has been detected in bronchoavleolar lavage (BAL) fluid and in airway epithelial cells (102,113,114). Overall, total and isoform measures in the blood and airway are poorly correlated (114,115). This likely indicates that adiponectin, does not pass freely through pulmonary vasculature (99).

Unlike leptin, serum adiponectin levels are not altered by meals or food intake, or during the menstrual cycle (116). Circulating adiponectin levels in nonobese subjects also exhibit diurnal variation with a clear ultradian pulsatility (117) characterized by a nocturnal decline starting during the late evening and declining to reach the lowest levels in the early morning (95). Daytime levels of adiponectin are generally higher with a peak in the

late morning, and minimal variation during the day, and finally with a slight decrease in the early afternoon and a plateau until evening (118). These variations are absent in obese subjects (119,120). Normal physiologic adiponectin concentrations are generally within the range of 5 to 30 mg/L in lean individuals (121,104).

2.4.1.2 General Characteristics: Leptin

Leptin is a 16-kDa protein encoded from the obese (*ob*) gene (122) that has a structure similar to cytokines (123), in particular IL-6 (124). Leptin is primarily a satiety hormone that regulates appetite and plays a large role in energy balance and body weight (105). The almost exclusive source of leptin is from mature adipocytes (125), causing systemic leptin concentrations to correlate with adipose tissue mass and BMI (126). As expected from the BMI correlation, plasma leptin levels are generally elevated in obese individuals when compared with lean individuals, however, absolute concentrations at a given weight (and fat content) are found to be somewhat variable (127). A more consistent relationship is that women and post pubertal girls have 40-200% higher circulating leptin concentrations than their male equivalents following adjustment for body fat composition (128–131). Various pulmonary cell types also express leptin such as bronchial epithelial cells and immune cells including alveolar type II pneumocytes and macrophages (132–134). As a consequence of this production, leptin has been measured in sputum and in BAL fluid. These airway leptin concentrations are highly correlated (r^2 of 0.61 and 0.55 respectively) with serum leptin levels, indicating that leptin is transported from the lung to the blood (and perhaps vice versa) easily (99,114,115). The clinical significance of pulmonary production of leptin when compared to systemic levels is not known (99,100).

Additional clinical considerations affect systemic leptin concentrations. Serum leptin concentrations increase following food intake, and decrease following short-term fasting (135). It is of note also, that leptin varies slightly during the menstrual cycle, with lower mean concentrations during the follicular phase than during the midcycle and luteal phases (116). Serum (and plasma) leptin levels have been found to have both ultradian and circadian rhythms characterized by a nocturnal increase with peak values occurring from midnight to about 2:00 a.m., and a decrease to the lowest values occurring between noon and midafternoon (136–138). This pattern is also reflected in protein expression profiles (117,139). Despite these fluctuations, under normal eating cycles and standardized conditions, such as those present in a clinical trial setting, a single leptin measurement in the morning or early afternoon is considered informative and clinically relevant (140). Normal physiologic leptin concentrations are expected within the range of 2 to 8 μg/L in the serum (135).

2.4.1.3 General Characteristics: Resistin

Resistin ("resistance to insulin") is a 12.5 kDa protein, was named for its ability to reduce insulin sensitivity in mice (141). Resistin is part of the "found in inflammatory zone" FIZZ protein family, and has been studied

extensively for its functional role in the pathogenesis of type II diabetes (141,142). This adipokine and its family member "Resistin-like molecule-β have been observed, in animal models, to stimulate vascular smooth muscle proliferation and angiogenesis (143,144), however, its functional role in human disease is still somewhat unknown. Resistin is secreted in large part by monocytes and circulating macrophages (144,145), and therefore has no correlation with BMI. Although generally referred to as an "adipokine" there exists conflicting evidence as to whether adipocytes express resistin (104). Expression of resistin is highest in the bone marrow: it is 10-fold higher in the bone marrow over the next highest expression levels in the lung (144,146), and resistin has been observed to be produced particularly by monocytes during inflammatory responses (147). Significantly increased levels of resistin have been observed in obese patients, so it is likely that some relationship may exist between this protein and metabolic disorder (144). Resistin, like adiponectin, circulates as homodimers or higher order multimers, however these forms may not be required for biologic activity, and have not yet been related to disease or disorder (144).

Like leptin, resistin levels are found to vary with the menstrual cycle, with slight increases observed during the luteal phase, when compared with the follicular and midcycle phases (116). No circadian or diurnal variations in systemic resistin concentrations have been studied in human subjects. However, the resistin mRNA profile in mice exhibits rhythmic expression and follows the profile of adiponectin (148). Additionally, resistin mRNA expression in mice appears to be related, like leptin, to nutritional status as expression increases with feeding and decreases with fasting (149). These data have not been confirmed in human subjects. Normal serum resistin concentrations fall within the range of 7-22 ng/mL (144).

2.4.2 Measurement of Adipokines

Adipokines are routinely measured in the serum and plasma in clinical and research settings alike. Adipokine levels in blood samples are generally quantified using various commercial ELISA kits. Measurement in lung samples is less routine, however, adiponectin and leptin have both been measured in BAL samples (102,110,114) and sputum samples (150–152). To our knowledge, no studies have quantified adipokine levels in bronchial wash (BW) samples using ELISA or any other methods. Immunohistochemistry analysis of adiponectin and leptin protein levels is routinely carried out in lung tissue samples with several commercial antibodies available against both adipokines, as well as their receptors.

2.4.2.1 Adipokine Stability

Adiponectin has a reported half life of between 5 and 6 hours in the serum (112). Leptin concentrations were observed to remain stable over 6 freeze-thaw cycles, nor were they altered when blood was allowed to stand 1, 2, 4 or 6 h prior to or after separation before freezing at -70°C (153).

2.4.3 Historical Study of Adipokines: Obesity

Adipokines have historically been studied because of their dysregulation in obese individuals. Because of adipokine roles in metabolism and feelings of satiation and hunger, their role in energy balance has long been hypothesized. Serum concentrations of leptin and resistin have been found to be elevated in obese individuals, while adiponectin serum levels are suppressed as compared to their lean counterparts (99). These alterations in adipokine levels are greater than what BMI and fat mass correlations would indicate. Serum concentrations of adipokines have been found to not only associated with BMI and obesity, but also with a variety of inflammatory conditions (97) and obesity complications such as type II diabetes, hypertension and atherosclerotic cardiovascular disease (99). The pathogenic role of adipokines is not entirely known. An emerging hypothesis adipokines have the ability to contribute to the "low grade inflammatory state" that characterizes obesity (145).

Furthermore, and relevant to the study question, is the hypothesis that adipokines represent one of several mechanistic links between obesity and the increased risk of allergic asthma, and more generally, the increased prevalence of allergic disorders (99,154). The contribution of these inflammatory mediators to the pathogenesis of pulmonary disease, and more generally, on individual susceptibility to air pollution exposure in an ongoing area of study (100).

2.4.4 Inflammation, Air Pollution and Metabolic Syndrome

Exposure to particulate air pollution is known to cause pulmonary and systemic inflammatory responses as was described above (22). It is conceivable therefore, that the health effects of PM air pollution will be enhanced in people with existing inflammatory conditions (155). This could include conditions such as obesity, diabetes or hypertension, all of which are linked to chronic inflammation (156). These conditions fall under the umbrella term metabolic syndrome (MS) that describes a group of metabolic abnormalities that can lead to insulin resistance, central adiposity, hyperglycemia, and hypertension: all of which are risk factors for cardiovascular disease (157–159). Emerging epidemiologic evidence suggests that individuals with certain components of metabolic syndrome including hypertension (160,161), obesity (155,162,163) and diabetes mellitus (161,164,165), may be more susceptible to the cardiovascular effects of PM exposure. These individuals exhibit poorer clinical outcomes and elevated inflammatory responses. Additionally, the degree of metabolic syndrome also appears to alter individual susceptibility. One study investigating the long-term impacts of ambient PM exposure found that there was a graded positive response between PM_{10} and systemic markers of inflammation across subpopulations with 3, 4, or 5 metabolic syndrome components (166).

There are currently investigations seeking to discover the underlying mechanisms that cause this increased susceptibility. Increased airway responses, as measured by histological assessment of inflammatory cell infiltration in the lung, in response to diesel exhaust particulate exposure was measured in obese rats as compared to their normal weight counterparts. (167) Toxicological studies in animals with metabolic abnormalities have investigated these mechanisms, but several questions remain. It is likely that a preclinical pathophysiological process underlies MS-susceptibility to PM pollution. As stated above, various adipokines are dysregulated in not only obesity, but also in other inflammatory MS components. In fact serum measures of leptin, adiponectin, and their ratio have been investigated for their potential role in the clinical diagnosis of MS among several ethnic populations (168–172). Adipokines may represent one mechanism by which MS increases susceptibility to particulate pollution. Therefore, investigating acute changes in adipokines following exposure to diesel exhaust, a significant component of ambient air pollution is highly relevant.

2.5 Adipokine Data: Animal Studies

Adipokines have been observed to mediate and respond to pro- and anti-inflammatory cytokines, such as those involved in responses to allergen and diesel exhaust exposure. The cell targets of such effects are unknown. In animal models, it has been found that adipose tissue can respond to inflammatory stimuli in the lung. As is depicted in Figure 2.3 published by Mancuso (2010), adipokines and other inflammatory mediators produced and released from the adipose tissue contribute to systemic inflammation, and act to enhance pulmonary inflammation (100). These responses are known to be elevated in the context of obesity, but still occur in non-obese contexts. The exact relationship between pulmonary and systemic inflammatory mediators is unclear, however, it is likely that a positive feedback-like response takes place.

Adiponectin appears to be an anti-inflammatory mediator in most situations. It has been demonstrated to inhibit IL-6 expression and macrophage TNF-α secretion (173), while promoting anti-inflammatory cytokine (IL-10 and IL-1 receptor agonist) expression (174,175). However, under certain conditions, pro-inflammatory effects of adiponectin manifest. In animal models, adiponectin levels decrease in response to inflammation (176), and treatment with adiponectin attenuates airway inflammation in response to chronic (177) and acute allergen challenge (178), while conversely, adiponectin deficient mice exhibit pulmonary inflammation and lung remodeling (179). Similar to responses to pro-inflammatory stimuli, long-term $PM_{2.5}$ exposure in mice fed normal chow was found to significantly decrease adiponectin levels (180). Concentrated ambient particulate exposure (441 $\mu g/m^3$) in rats fed a high-fructose diet decreased adiponectin gene expression (181).

Leptin is generally considered to be a pro-inflammatory mediator. In animal models and clinical studies, leptin is acutely elevated in response to TNF-α, IL-1β, IL-6 and LPS administration, following cytokine-like

response kinetics (182–186). It has also been observed that leptin enhances TH1 cytokine production (Interleukin, IL-2, interferon-ϒ, TNF-α), while suppressing TH2 cytokine production (IL-4, IL-5, IL-10) (187)(188). Increases in leptin have also been observed in infectious and inflammatory states in mice (189,190). In terms of particulate exposures, long-term exposure to $PM_{2.5}$ in mice was found to significant reduce leptin serum concentrations (191). Data in humans, however, is less consistent, and does not always match what is observed in rodent models (186).

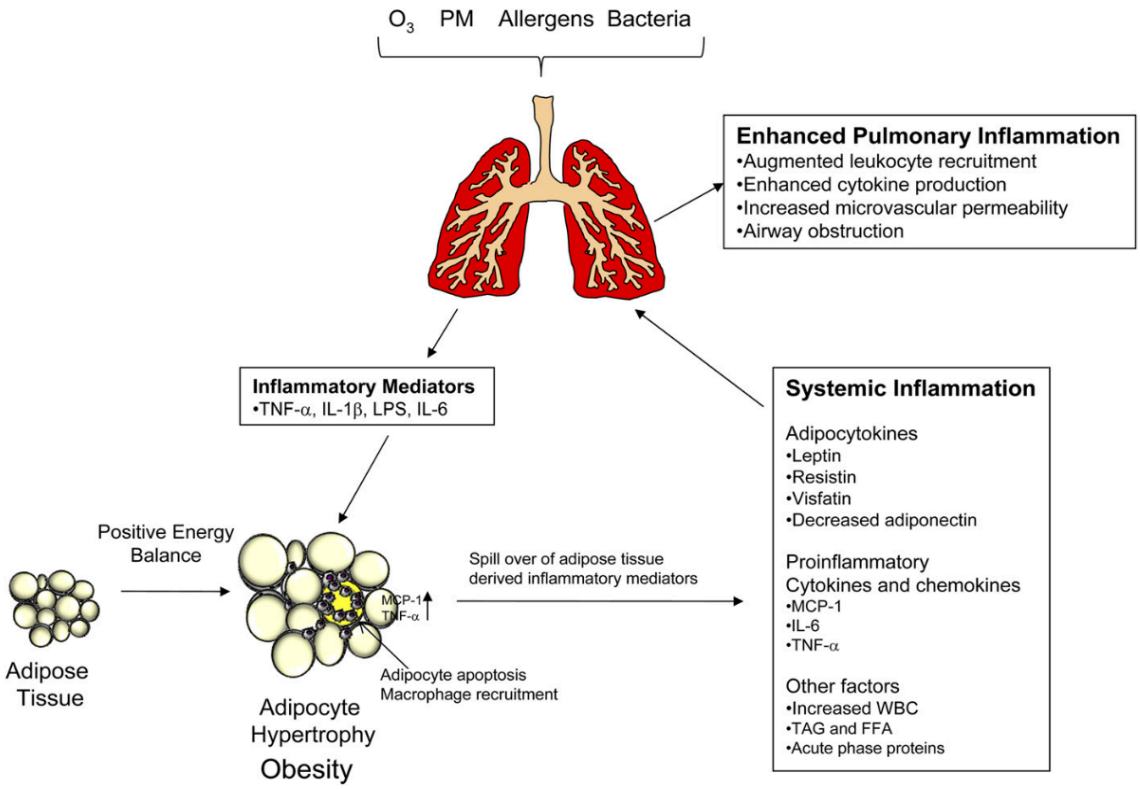

Figure 2.3. The influence of the adipose tissue on inflammation in the lung when challenged with exposures like ozone and particulate matter. Increased fat tissue mass acts to increase production of pro-inflammatory adipokines (leptin, resistin) and reduces anti-inflammatory mediators like adiponectin (100).

Like leptin, resistin is also considered to be a pro-inflammatory mediator (192). Resistin appears to both induce and be induced by IL-6 and TNF-α in human peripheral blood mononuclear cells (146,193). Resistin gene expression has been reported to increase in human and mouse macrophages in response to LPS administration (192,193). Additionally, in a mouse model of diet-induced obesity, five-month $PM_{2.5}$ exposure was found to significantly increase resistin concentrations in the serum, over filtered air exposure (194).

2.6 Adipokine Data: Human Studies

There exists a large body of epidemiological evidence relating adipokines levels to asthma and other allergic diseases, however data from clinical human studies is much more limited.

2.6.1 Epidemiological Studies: Asthma Prevalence and Severity

Population-based studies have associated serum adipokine levels to asthma prevalence and severity. Although no causality can be determined from these studies, the underlying assumption is that there is a mechanistic link between adipokines and asthma, (99) and that these correlations will help inform and guide future mechanistic studies and controlled human studies, such as this one. Conflicting results have been obtained for adiponectin. In premenopausal women adiponectin was found to have a beneficial effect on asthma prevalence, with lower adiponectin levels predicting future incident asthma (102,115,195). No prevalence associations were found in males. However, in men, high serum adiponectin levels are associated with increased asthma severity, while no such associations were found in women (196). However, one study found that during acute asthma exacerbations female patients exhibited lower serum adiponectin levels when compared to 8-weeks post-exacerbation (197). Significant differences in BAL adiponectin levels between asthmatics and controls have not been confirmed upon adjustment for BMI (114), or in small case-control studies (198).

In premenopausal women (199) and postmenopausal women (197), higher levels of serum leptin are associated with asthma prevalence, independent of weight status. Leptin has been negatively correlated with baseline pulmonary function measures (FEV_1 and FEF25-75) (98), and is generally found to have harmful effect on clinical pulmonary measures in postmenopausal women (197). Pulmonary leptin levels in the sputum have been found to be greater in asthmatics than in controls (200), however, this result has not been replicated using BAL samples (102,114).

Resistin has also been linked to asthma development (201), however, much less data exist as to its relationship with asthma. Asthmatic patients with moderate to severe asthma were found to have elevated plasma resistin levels over controls (202). Further studies are needed to clearly define the role of resistin in asthma prevalence and severity, and to confirm any sex or BMI modifications of these associations.

2.6.2 Clinical Studies: Allergen and other Pro-Inflammatory Exposures

Clinical studies, whether interventional or observational (203), provide valuable information regarding adipokine responses to inflammatory exposures. Specific allergen inhalation found no differences in adiponectin or leptin responses between asthmatic and control patients, when they were exposed to specific

allergen, irrelevant allergen, or methacholine. However, acute adipokine changes in response to any inhalational challenge were observed. Adiponectin serum levels were immediately increased, and remained elevated above baseline for about an hour. Conversely, an immediate decline in serum leptin was observed that lasted many hours, and returned to baseline in the morning (204). In patients with suspected cedar asthma, inhalational challenge with plicatic acid increased adiponectin significantly in the sputum over levels obtained after methacholine challenge, as measured at six hours post-exposure (152). Neither of these exposures appeared to affect serum adiponectin levels (152). Leptin has been found to be elevated in allergic rhinitis patients during symptomatic periods (205). The role of leptin in inflammation among allergic rhinitis patients may be due to its role as a survival factor for human eosinophils (154). A study investigating effects of ambient $PM_{2.5}$ found that diabetic patients with low circulating adiponectin concentrations were more susceptible to PM-associated decrements in endothelial function (206). In studies involving cancer patients, administration of IL-1β or TNF-α increased serum leptin levels, as would be predicted from animal studies (184,185). Conversely, LPS injection in healthy volunteers did not increase leptin levels (207), nor did it change adiponectin levels in healthy adults as measured in two separate studies (147,208), but it was seen to dramatically increase circulating resistin levels (193).

2.6.2.1 Lung Versus Serum Measures

Serum measures of adipokines provide a relatively non-invasive method for the quantification of adipokine responses often at several time points in a study. However, to thoroughly investigate adipokines' role in the pathogenesis of lung diseases, the measurement of these mediators in the lung itself is crucial. It has often been suggested that measurement of serum adipokine levels in isolation is a limitation, and that lung measures provide a more accurate picture of mediators reaching the target cells in the lung (99). Because bronchoscopy is a relatively invasive procedure, few clinical studies have measured adipokine levels in the lung. Some small clinical studies have measured lung adipokine levels, and have confirmed some of the trends seen in the serum data. For example, within a clinical cohort of women undergoing bariatric surgery it was found that BAL leptin was higher, and BAL adiponectin lower in asthmatics when compared to controls by Sildelva et al., (102). No associations between asthma or BMI status were found for BAL adiponectin measures (114). Another study by Holguin et al., found that BAL leptin levels increased according to BMI category, and that obese asthmatics had higher BAL leptin levels than lean asthmatics. A few studies have measured adipokine levels in induced sputum, and this is a somewhat less invasive procedure (209) that can be used as a surrogate for BAL (210). However, the measurement of adipokines by ELISA in sputum samples is less established. Sputum adiponectin has been found to be lower in asthmatics than controls (115), while another study found that adiponectin sputum increases in response to plicatic acid challenge in patients with suspected cedar asthma (152). Finally, sputum leptin levels have found to be positively correlated with both BMI, and serum

leptin measurements (200). Although airway adipokine levels have begun to be investigated, the relationship between systemic and airway inflammation within the context of adipokine study however, is unclear.

2.6.3 Lung Physiology

Physiological relevance of adipokines in lung pathology and disease requires that receptors specific to these mediators be expressed in the lung. Recent studies have found the lung to be a leptin producing and leptin responsive organ (211). Both the long (LepRb) and short (LepRa) leptin receptor isoforms are expressed alveolar and bronchial epithelial cells, as well as by alveolar macrophages (133,212). Additionally, human eosinophils are known to express the leptin receptor (LepRb) (213). All of the three known adiponectin receptors, AdipoR1, R2 and T-cadherin, are expressed in the lungs (178). T-cadherin, in particular, appears (in mouse models) to be important for adiponectin transport across pulmonary vasculature, and may act as an adiponectin repository in the lungs (214)(215). A newly identified human resistin receptor is adenylyl cyclase-associated protein (CAP1). This receptor has the ability to mediate the pro-inflammatory actions of resistin. CAP1 exhibits similar expression to resistin, and more importantly, is detectable in the lung tissue (216).

Additionally, the site of action is important. The collection of BW samples and BAL samples allow different portions of the airways to be explored. The BW is a sample of the central airways, or the so-called conducting airways, while the BAL sample is from the alveolar spaces (217). Although physiologically related, these spaces are not identical and have different cellular profiles (218,219).

2.6.3.1 Immunohistochemistry Analysis

Further evidence of adipokine and adipokine receptor expression has been analyzed at the protein level. Expression of adipokines and their receptors have been investigated in the lung, and commercial antibodies are available for immunohistochemistry analysis. Immunohistochemistry for leptin and its receptor (Ob-R) has been performed on bronchial epithelium of patients with asthma of various severity, and controls (133). Asthmatic patients showed a decreased expression of leptin and the receptor as compared to controls. Leptin was also stained in lung sections of patients with chronic obstructive pulmonary disorder (COPD). Conversely, leptin expression was elevated in patients with COPD verses current, never, and ex-smokers who showed similar staining patterns (132). Similarly, leptin and Ob-R receptor was more readily detectable in non-small-cell lung cancer patient samples over control patient samples (220).

Adiponectin and its receptors, AdipoR1 and AdipoR2, have been detected in non-neoplastic lung tissues (221), as well as lung samples of COPD-E patients, predominantly in airway epithelial cells (113). Expression of adiponectin and AdipoR1 were found to be elevated in COPD-E patients over controls, but not AdipoR2

(113). No inhalational challenge experiments involving leptin or adiponectin immunohistochemistry were identified.

No immunohistochemistry against resistin has been performed in human lung tissue samples to our knowledge. However, immunohistochemistry against resistin-like molecule-β (RELM-β) has been performed in mild asthmatic airway biopsies. Interestingly, four days after repeated allergen challenge in these subjects, increases in RELM-β expression were detected in airway epithelium (222). It is conceivable that a similar increase in resistin, and perhaps in other adipokines or their receptors could be detected following allergen and diesel exhaust co-exposure.

2.7 Study Rationale

In summary, the literature presented above suggests that:

- Adipokine levels, both in the blood and in the lungs, will likely change in response to allergen challenge in the lung; it is also plausible that diesel exhaust will augment allergen effects.
- Adipokines are expressed in the lung and have physiological relevance there.
- Detailing pulmonary adipokine levels will add another dimension of understanding the response to common inhalants.
- Changes in the BAL (distal) versus the BW (proximal) segments of the lung may be different.
- Pro-inflammatory (leptin and resistin) and anti-inflammatory (adiponectin) may respond to exposures differently.

There exists a strong base of animal data and epidemiological study, along with limited clinical studies, that supports the use of precious clinical human samples to investigate the change in adipokines in response to allergen and diesel exhaust co-exposure. Our study helps to elucidate the temporal sequence of the adipokine relationship with allergen exposure, and leads to the weight of causality that adipokines contribute to allergic lung disease progression.

Chapter 3: Methods and Study Design

3.1 Hypotheses Overview

The overall, primary hypotheses to be assessed in this thesis are listed below:

1) **Adipokine differences will exist between males and females, and between overweight and normal BMI individuals.**
2) **Allergen will alter adipokine concentrations in the lung; diesel exhaust will augment these effects.**
3) **Allergen will alter adipokine concentrations in the serum acutely; diesel exhaust will augment these effects.**
4) **Correlation will exist between leptin lung and serum adipokine measures.**

These will be described in further detail in Section 3.7 below, with their individual methodology.

3.2 Study Overview

The ethical review boards at the Vancouver Coastal Health Institute and at the University of British Columbia approved the study. This clinical trial (also called an interventional study) was registered at clinicaltrials.gov (trial no. NCT01792232). Written informed consent was obtained from each study participant. To study the impact of diesel exhaust and allergen co-exposure on adipokine concentration, the Air Pollution Exposure Laboratory (APEL) headed by Dr. Christopher Carlsten, was used for controlled human exposure to diesel exhaust (223,224). The experiment was designed as a randomized, balanced, double-blinded crossover study, meaning each subject is exposed to all conditions, and can serve as his or her own control, thus eliminating all individual potential confounders (225–227). An overview diagram describing the crossover study design is described below in Figure 3.1. The two exposure triads include: 2-hour inhaled exposure to DE and filtered air (FA) followed by segmental allergen challenge, with specific allergen and saline instilled into right and left lung lobes. The two triads were separated by a 4-week washout period to minimize carryover effects.

Each triad depicted in grey and blue are comprised of three days of study. In Day 1, the baseline and 4 hour post-exposure serum samples are collected following DE or FA and allergen exposure. In Day 2, 24-hour post-exposure serum samples are collected. And in Day 3, the 48-hour post-exposure lung and serum samples are collected. Study procedures will be described in further detail below.

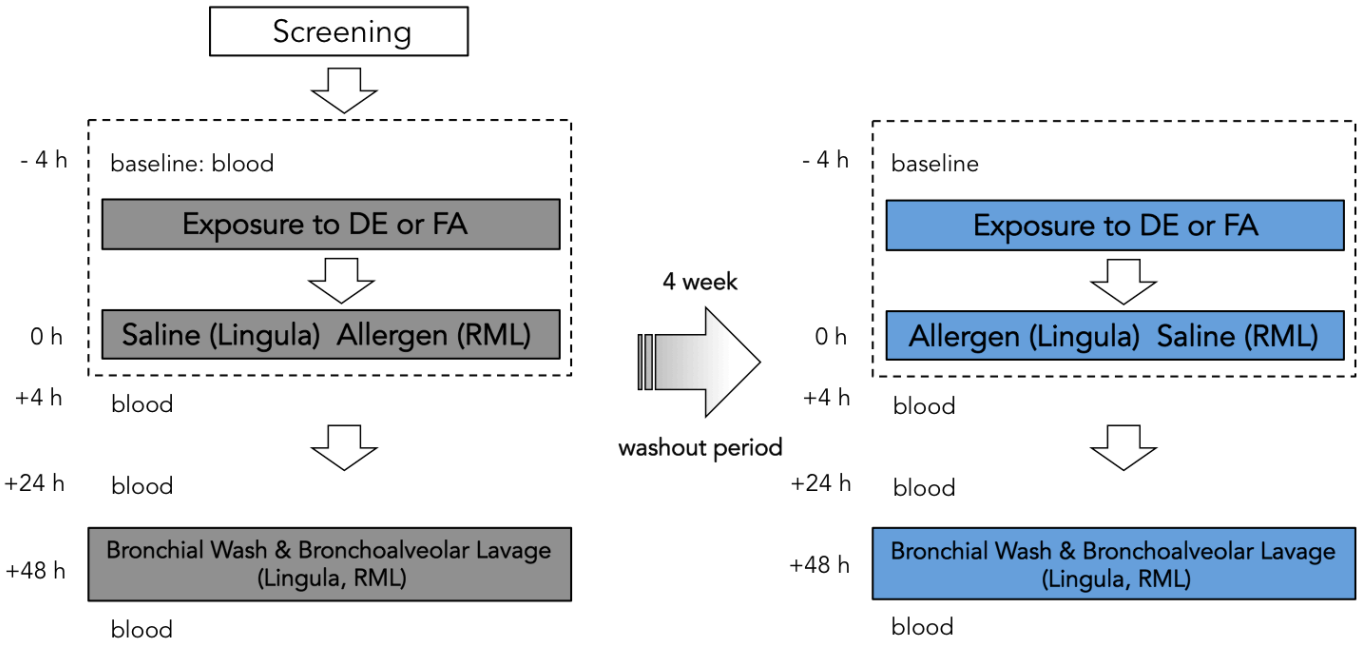

Figure 3.1. Human crossover study design performed in the Air Pollution Exposure Laboratory (APEL). 18 consented, atopic adults were exposed to inhaled diesel exhaust (DE) or filtered air (FA) for 2 hours, followed by segmental allergen challenge via bronchoscopy. Distinct middle lobe lung sub-segments are used in post-washout phase (eg. Sub-segments from pre-washout are avoided). The alternate exposure was performed after a minimum 4-week washout period. RML: right middle lobe.

3.3 Selection of Study Participants

Study participants between the age of 19 and 49 were recruited through local advertising and referral of clinic patients, following University of British Columbia Research Ethics Board considerations. Primary screening excluded participants with any of the following: (1) pregnancy/breastfeeding, (2) use of inhaled corticosteroids, (3) regular use of bronchodilator medication, (4) unstable asthma symptoms, (5) any use of vitamins A, C, E or other antioxidant supplements, (6) co-morbid conditions judged by the investigators to increase risk of dropout, or (7) work in an industrial setting or other setting of significant inhaled exposures.

Secondary screening involved blood collection and genotyping, baseline lung function (FEV_1), and airway responsiveness to methacholine (PC_{20}) measurements. Sensitization to birch, Pacific grasses and house dust mite (*Dermatophagoides pteronyssinus* group 1) allergen was determined by skin prick with standard extracts (medical grade; Hollister-Stier, Spokane, WA) in a solution of 50% vol/vol glycerine diluent solution. Inclusion in the study required a wheal > 3-mm to one of the allergens listed above. A series of 10-fold dilutions were used to determine the lowest dose necessary to elicit a 3-mm wheal. A total of 18 subjects were recruited and participated in both exposure triads of the crossover study.

3.4 Study Procedures

All study procedures were carried out in APEL at Vancouver General Hospital between October 8, 2010 and February 20, 2013.

3.4.1 Diesel Exhaust and Filtered Air Exposure

Consented subjects underwent a double-blinded crossover experiment (225–227) using two exposure conditions of two-hour duration, diesel exhaust (DE) 300 $PM_{2.5}/m^3$ of filtered air (FA) 8 $PM_{2.5}/m^3$. The treatments were randomly assigned to the two exposure periods for each participant (228). DE exposure was generated by a EPA Tier-3 compliant diesel generator that has been previously described (223), however, a 2.5kW constant load was used. Briefly, ultra-low sulfur diesel fuel (sulfur < 15 ppm) was used to produce exhaust, a portion of which was first diluted 9:1 with compressed air, diluted 25:1 with high efficiency particulate air (HEPA)-filtered air, and finally aged for four minutes prior to release into the 4 x 6 x 7-foot exposure booth (223) to achieve the exposure conditions listed below in Table 3.1.

Table 3.1 Exposure components used in the crossover experiment design of the two exposure conditions: DE and FA.

Condition	$PM_{2.5}$ ($\mu g/m^3$)	Particle Number ($\#/cm^3$)	CO (ppm)	NO (ppb)	NO_x (ppb)	NO_2 (ppb)	NO_2/PM# ($\mu g/\#$)
Filtered Air	8.2 ± 6.9	1750.4 ± 235.1	2.8 ± 0.1	25.3 ± 5.0	71.1 ± 9.8	45.9 ± 7.7	4.9×10^{-9}
Diesel Exhaust	302.0 ± 30.5	5.4×10^5 ± 6.4×10^4	14.1 ± 2.0	8665.5 ± 1287.1	9185.3 ± 1366.1	519.7 ± 118.6	1.8×10^{-9}

Values are reported as mean ± SD. $PM_{2.5}$: particulate matter less than 2.5 microns in diameter; CO: carbon dioxide; NO: nitrogen oxide; NO_x: oxides of nitrogen; NO_2: nitrogen dioxide.

DE exposure components are monitored to characterize exposure, and well as to ensure participant safety. A nephelometer measures particle concentration, a Tapered Element Oscillating Microbalance (TEOM) measures $PM_{2.5}$ mass concentration, a Scanning Mobility Particle Sizer (SMPS) measures total particle number concentration, two Thermo Model analyzes measure CO and NO_x concentrations, while a GrayWolf probe measures total VOC, CO_2, temperature and relative humidity in the booth (223). The level of DE obtained is a safe yet realistic exposure representative of some occupational exposures occurring during mining operations, at highway toll booths and railways. These concentrations are also representative of environmental exposures including busy urban locations (near busy roads, bus terminals) as was mentioned before, and occur commonly in large cities in the less developed world, and occasionally in cities of the developed world (223).

3.4.2 Allergen Exposure

One hour following the DE or FA exposure condition, segmental allergen challenge was performed. Positive skin prick tests to birch pollen (n=3), grass pollen (n=7), or house dust mite (*D. pteronyssinus*) allergen (n=8) were obtained among the study population. Bronchoscopy was used to deliver a diluent-controlled solution of the positive skin prick allergen extract, at a concentration 10-fold lower than the dose producing a positive wheal, into the right middle lobe (RML) sub-segment. A 5 mL diluent control was delivered into a lingular sub-segment. 48 hours post-allergen challenge, bronchial wash (the collection from the first 40 cc [20 + 20] of instilled saline) and bronchoalveolar lavage (the collection from the next 100 cc [50 + 50] instilled saline) samples were obtained from the same sub- segments exposed to allergen or saline.

Following a washout period of a minimum of four weeks, exposure and segmental treatments were reversed. In sum, four exposure conditions at one time point per subject were obtained regarding lung samples: FAS (filtered air and saline) and FAA (filtered air and allergen), then DES (diesel exhaust and saline) and DEA (diesel exhaust and allergen, or vise versa. In the serum, only two exposure conditions can be compared at four different time points: FAA and DEA.

3.4.3 Lung Function Testing and Methacholine Challenge

Lung function parameters including FEV_1 and PC_{20} measurements were collected according to standard procedures. Participants are told to sit up straight with their feet flat on the ground, and with the nose clip pinching their nose, exhale as forcefully as they can after a full inspiration. Baseline measures as well as several spirometry measurements were taken at several points during the exposure triads. Forced vital capacity (FVC) and forced expiratory volume (FEV_1) are both commonly measured components of lung function measured using spirometric tests (209). FVC is the measured volume of air exhaled during a forceful expiration, and FEV_1 is the volume exhaled in the first second (209). Although these measures were not evaluated in this study, the baseline measures are reported to assess the general respiratory health of the study population (229).

Methacholine challenge was performed using the two-minute tidal breathing dosing protocol (230), that is similar to the protocol recommended by the Canadian Thoracic Society to assess airway responsiveness (231,232). Briefly, baseline spiromtery values are collected, and the FEV_1 target value indicating a 20% drop is calculated (FEV_1 x 0.8). Two-fold diluted concentrations of methacholine were prepared from 0.03 mg/mL up to the maximum dose of 16 mg/mL. The lowest concentration of methacholine was nebulized and inhaled by participants for two minutes while breathing quietly. FEV_1 values were obtained 30 and 90

seconds after inhalation. If FEV_1 does not drop 20% the next highest concentration was inhaled. The concentration that caused a 20% drop in FEV_1 (PC_{20}) was the reported PC_{20} value (233).

3.4.4 Control of Diet, Fasting Status

Subjects were not specifically required to fast in this study, but were asked to continue their normal eating habits. However, on the study days in which participants underwent bronchoscopy, a procedure requiring mild sedation, participants were fasted. Therefore, participants fasted in the morning prior to the Day 1 and the Day 3 bronchoscopy procedures, but did not fast in the afternoon of these days, nor during Day 2. Only samples collected at baseline and 48 hours post-exposure were under a fasting state.

3.5 Laboratory Methods

All sample processing and ELISA procedures were performed at the Jack Bell Research Centre at Vancouver General Hospital between September 2014 and March 2015. All sample processing was carried out under containment level two requirements. Freeze-thaw cycles were minimized.

3.5.1 Blood Collection and Processing

Venous blood was drawn into sterile blood tubes into serum separating tubes and EDTA-containing tubes. Blood was collected at baseline prior to each exposure triad denoted -4 hours, and 4, 24 and 48 hours after segmental allergen exposure. Complete blood collection sets (8 samples/subject) were obtained from 17 participants. Blood samples were stored at 4°C, and were processed within 24 hours. Aliquots were stored at -80°C until use.

3.5.2 BW and BAL Collection and Processing

BAL and BW samples were collected at 48 hours after allergen exposure. Complete lung collection sets (4 BAL and 4 BW samples/subject) were obtained from 18 subjects originally, however sample volume was limited for several subjects causing reduced sample number for several adipokine endpoints. Following collection by bronchoscopy, the BW and BAL samples were processed. Briefly, BW and BAL samples were poured through a mesh filter to filter out mucus and centrifuged. Aliquots of supernatant were stored at -80°C.

3.5.3 ELISA procedures

Serum, BAL and BW samples were assayed in singlicate for total adiponectin, leptin, and resistin. All serum adipokine levels and resistin in BAL and BW samples was measured with commercial ELISA kits (R&D Systems Inc., Catalog #DRP300, #DLP200 and #DRSN00). The minimum (mean) detection limits of the

R&D adiponectin, leptin and resistin kits are 0.246 ng/mL, 7.8 pg/mL and 0.026 ng/mL respectively. Adiponectin and leptin in BAL and BW samples were measured with more sensitive commercial ELISA kits (Invitrogen Corp., Catalog #KHP0041 and #KAC2281) respectively. The minimum detection limits of the adiponectin and leptin kits were 0.1 ng/mL and 3.5 pg/mL respectively. Additionally, a freeze-thaw experiment was performed for leptin determining the effect of a thaw at room temperature over 24 hours. The results of this experiment are included in Appendix A.3.

3.5.3.1 Sample Preparation

All samples were thawed and used at room temperature. BAL samples were centrifuged at 3000 revolutions per minute (rpm) for 10 minutes prior to use, and were not vortexed prior to loading. All other samples were vortexed thoroughly prior to loading onto the ELISA plate.

3.5.3.2 Commercial ELISA Kit Procedures

All serum adipokine measurements using R&D ELISA kits, and adiponectin and leptin measuring using Invitrogen ELISA kits in the BAL and BW were done according to the manufacturer's instructions. Invitrogen kits were used to measure adiponectin and leptin in the lung samples because they have lower sensitivities than the corresponding R&D ELISA kits. Some changes were made to the Invitrogen protocol used to measure leptin in the BAL and BW because the majority of samples were below the limit of detection. These included increasing the initial incubation with Biotin Conjugate solution to three hours and increasing the final incubation with Stabilized Chromogen to one hour. Additionally, further dilution of the leptin standard to include two additional points below the lowest standard curve point (15.6 pg/ml) was used to detect low leptin levels in the BAL and BW samples. A microplate reader was used to obtain the optical density (OD) results at wavelengths of 450 and 540 nanometers.

Microsoft Excel was used to calculate ELISA results. Either linear (of log OD vs. log adipokine concentration) or polynomial curves were fit to the data as recommended by the protocols, with the best fit being determined by the R^2 value closest to one. A sample calculation for serum adiponectin is shown in Appendix A.4. Samples with concentrations beyond the upper limit of the standard curve were diluted and run again. Additionally, samples falling between the zero standard and the first standard point were considered to be under the limit of detection, and were not used or interpreted.

3.5.4 Adiponectin/Leptin Ratio Calculation

The adiponectin/leptin ratio was calculated using the following formula:

$$\frac{\text{Adiponectin (ng/mL)}}{\text{Leptin (ng/mL)}}$$

This unit-less value was calculated for each exposure condition and time point in BAL and serum for each subject that had reliable measures of both adiponectin and leptin.

3.6 Data Overview

3.6.1 Dependent and Independent Variables

In total, nine dependent continuous adipokine variables were measured. These are the biological outcomes of interest in this thesis. An overview of these variables can be seen below in Table 3.2. Three additional dependent variables were created when the ratio of adiponectin to leptin was examined. Analyses examined whether each dependent variable was associated with the following independent variables: exposure (DES, DEA, FAS and FAA), sex (M, F), and BMI (above and below 25 kg/m^2). In the serum, an additional independent variable, time (at baseline, 4, 24 and 48 hours post-exposure), was examined under two exposure conditions (DEA, FAA).

Two additional dependent variables are listed as "other" independent variables because they were tested to ensure certain assumptions were correct. The first assumption was that order did not have an effect on the results of exposure. The second assumption was that the presence of baseline airway hyperresponsiveness ($PC_{20} \leq 8$ mg/mL) did not significantly affect baseline adipokine levels or adipokine responses.

Table 3.2 Overview of the dependent and independent variables examined in the statistical analysis of lung and serum data. The "other" independent variables were tested statistically to confirm certain study design assumptions.

Variable Type	Sample Type	
	BAL/BW	Serum
Dependent	Adiponectin	Adiponectin
	Leptin	Leptin
	Resistin	Resistin
	Adiponectin/Leptin Ratio	Adiponectin/Leptin Ratio
Independent	Exposure DEA DES FAA FAS	Exposure DEA FAA
	Time 48 hours	Time Baseline 4 hours 24 hours 48 hours
	Sex 0: Male 1: Female	Sex 0: Male 1: Female
	BMI 0: < 25 kg/m^2 1: ≥ 25 kg/m^2	BMI 0: < 25 kg/m^2 1: ≥ 25 kg/m^2
"Other" Independent	Order 0: FA first 1: DE first	Order 0: FA first 1: DE first
	Airway Responsiveness 0: PC_{20} > 8 mg/mL 1: PC_{20} ≤ 8 mg/mL	Airway Responsiveness 0: PC_{20} > 8 mg/mL 1: PC_{20} ≤ 8 mg/mL

BAL: bronchoalveolar lavage; BW: bronchial wash; DES: diesel exhaust + saline; DEA: diesel exhaust + allergen; FAS: filtered air + saline; FAA: filtered air + allergen.

3.6.2 Distribution of Dependent Adipokine Variables

Histograms of the dependent adipokine variables were produced using JMP® software to examine the normality or the lognormality of the distribution. Descriptive statistics including the arithmetic and geometric mean and standard deviation values, and the median values were also calculated using this software. Goodness of fit tests including the Kolmogorov-Smirnov test for lognormality and the Shapiro-Wilk test for normality were performed. Visualization of the histogram, comparison of the descriptive statistics and goodness of fit tests were all used to decide if the variables should be transformed.

3.6.3 Outliers

Adipokine measures that were beyond mean ± two standard deviations were reviewed. First, the biological plausibility of a specific value was looked up in the literature, and the value was compared to other measures from the subject to see how it compared. Additionally, the study notes from that participant and ELISA data

were checked for potential sources of error. If no conclusion could be made from these investigations, and the value was considered biologically plausible, then it was included in the analysis. After these checks all "outliers" existing in the dataset were included in the analysis, as adipokine concentrations are known to vary over a large range.

The exception to the above statement was when data points fell above the standard curve of the ELISA kit, and due to cost and supply constraints could not be diluted and run again. This was true for two BAL leptin data points, and two BW resistin data points.

3.7 Hypotheses and Statistical Methodology

Statistical analyses were performed using JMP® software (version 11.0, SAS Institute Inc., 2014) (234) and R software version 3.1.0 (235). A value of $p < 0.05$ was considered significant. All in-text values are reported as mean ± standard deviation.

The specific scientific hypotheses for this study are shown below in Tables 3.3-3.6. The directional hypothesis and sample type is listed in the first two columns with the support from clinical and epidemiological studies listed in the third column. The strength of evidence is considered high if there is evidence from human/related epidemiologic studies, and medium or low depending on the number of relevant studies, and their overall agreement. The studies that support the opposite directional hypothesis are shown in red.

3.7.1 Hypothesis 1: Baseline Sex and BMI Status Comparison

A set of hypotheses will determine differences in baseline adipokine concentrations (Table 3.3). These differences will be assessed between males and females, and between overweight (BMI ≥ 25 kg/m^2) and normal (BMI < 25 kg/m^2) BMI individuals. These are parameters chosen for their relevance to adipokine concentrations as assessed by literature.

3.7.1.1 Statistical Analysis

To evaluate differences in baseline adipokine measures according to sex and BMI status, the FAS lung exposure condition and the baseline serum data were examined. In the lung, the FAS values could be compared directly, and in the blood, the two baseline (-4 hour) values obtained at the beginning of each triad were averaged for each participant. These values were compared between males and females, and according to BMI status (above or below 25 kg/m^2) using an unpaired t-test.

Table 3.3. Primary hypotheses to be tested using baseline serum, BAL and BW adipokine data

Type	Hypothesis	Support	Strength of Evidence	Statistical Analysis IV: Independent Variable DV: Dependent Variable
Primary	At baseline, men and normal BMI individuals will have lower serum leptin than women and overweight individuals respectively.	Epidemiologic evidence for sex (129–131) and BMI (126,127).	High	Unpaired, two-tailed *t*-test **IV:** Sex; BMI status **DV:** Adipokine concentration
	As well as in BAL/BW.	As above.		
	At baseline, men and overweight individuals will have lower serum adiponectin than women and normal BMI individuals respectively.	Epidemiologic evidence for sex (112) and BMI (107,109,110).	Medium-High	
	As well as in BAL/BW.	As above.		
	At baseline, men and normal BMI individuals will have lower serum leptin than women and overweight individuals respectively.	Epidemiologic evidence for sex (236)0) and BMI (107,237)1).	N/A	
	As well as in BAL/BW.	As above.		

BAL: bronchoalveolar lavage; BW: bronchial wash; BMI: body mass index.

3.7.2 Hypothesis 2: Lung Adipokine Responses

A set of hypotheses will be explored using available BAL and BW lung samples collected 48 hours after exposure (Table 3.4). The overall hypothesis in the lung is that specific allergen challenge will alter adipokine concentrations to a greater extent than saline exposure. The secondary hypothesis aims to determine if diesel exhaust further augments the effect of allergen. The tertiary hypothesis will explore if sex, BMI status or airway responsiveness act to modify the effect of exposure. The direction of response is hypothesized from epidemiologic studies, and the limited clinical data that was presented in the introduction. Studies considered most relevant are those measuring adipokine changes using lung samples. There is very little evidence to suggest that adipokine responses will be sustained over 48 hours in the lung, excepting a paper that found differences in immunohistological staining of resistin like molecule-β 4 days after allergen challenges (222). Sex is a known factor regulating adipokine regulation, and it was been observed that obese individuals have altered adipokine circadian rhythms (120), and adipokine dysregulation as was noted in the introduction.

Table 3.4. Primary, secondary and tertiary hypotheses to be tested using BAL and BW adipokine data.

Type	Hypothesis	Support	Strength of Evidence	Statistical Analysis IV: Independent Variable DV: Dependent Variable
Primary	Lung leptin levels following specific allergen exposure will be greater than those following saline exposure.	Epidemiologic evidence (205) and limited clinical evidence (197,238)2). Allergen inhalational study (204).	Medium-High	i) Mixed Effects Model **Response Variable (DV):** Adipokine concentration **Fixed Effects (IV):** Exposure (4: DES, DEA, FAS, FAA) and Sex; BMI status; Airway responsiveness; Order **Random Effect:** Subject ID ii) Post-hoc analysis: ANOVA least-squares mean comparisons with tukey multiple comparisons correction
	Lung adiponectin levels following specific allergen exposure will be greater than those following saline exposure.	Allergen inhalational study (152,204). Limited clinical evidence (197).	Medium-High	
	Lung resistin levels following specific allergen exposure will be greater than those following saline exposure.	Clinical study (147) and epidemiologic evidence (202).	Low	
	Lung adiponectin/leptin ratio levels following specific allergen exposure will be greater than those following saline exposure.	Epidemiologic evidence (124) and limited clinical evidence (197).	Low	
Secondary	DE will augment the effect of allergen.	Allergen synergy evidence (91–94).	Low	
Tertiary	BMI and sex are effect modifiers of the above hypotheses.	Sex-dependent regulation has been identified for adiponectin (239)3) and leptin (240,241)5), as well as BMI modification (120)	Medium	

ANOVA: Analysis of Variance; DES: diesel exhaust + saline; DEA: diesel exhaust + allergen; FAS: filtered air + saline; FAA: filtered air + allergen.

3.7.2.1 Statistical Analysis

Prior to analysis, data was formatted to the correct 'long' structure, and all dependent variables and the subject ID variable were changed to factor variables (242). The R packages 'lmerTest' and 'lsmeans' was used to carry out the mixed effects analysis, and relevant post hoc tests as required. The advantage of using this particular R package is that p-values are calculated from the analysis using the restricted maximum likelihood (REML) method (243). Mixed-models were used to determine if the main fixed effect of interest, exposure, had an effect on the response variable, and also to see if additional demographic variables including sex (M/F), BMI status (above or below 25 kg/m^2) and airway responsiveness (normal or hyperresponsive).

had an effect on the response variable, and also to see if additional demographic variables including sex (M/F), BMI status (above or below 25 kg/m^2) and airway responsiveness (normal or hyperresponsive).

First, the interaction between exposure and order was assessed to see if a carryover effect was present. If a statistical interaction was observed, no further analysis was performed. Second, effect modification by sex, BMI and airway responsiveness was investigated by testing if any of these variables interacted significantly with exposure. If significant interaction terms were found, they were included in the final model and main effects of exposure were not interpreted (244). If no significant interactions were identified, all interaction terms were removed from the model. Third, assuming there were no statistical interactions with exposure identified, an initial mixed-effects model, including the fixed effects of exposure as well as sex, BMI status, airway responsiveness and order was constructed to assess what variables were significant in predicting adipokine responses. Subject ID was added into the model as a random effect. Fourth, the initial model was refined to produce a final mixed-effects model containing exposure as well as any variables with a statistically significant effect ($p < 0.05$). Because exposure was the main effect of interest, it was included in all models regardless of significance. From this final model, the effect of each exposure condition, and the effect of other independent variables on adipokine responses was assessed using pairwise least-squares means *post hoc* comparisons, with a Tukey correction for multiple comparisons. For exposure effects, only four comparisons were considered relevant: DEA-DES, FAA-FAS, DEA-FAA and DES-FAS to assess the effect of allergen versus saline and diesel exhaust verses filtered air. Effect estimates of log-transformed response variables were reported directly from the model to preserve the directionality of the estimate. The effect estimates were exponentiated in the text to facilitate interpretation of the effect size.

3.7.3 Hypothesis 3: Serum Adipokine Responses

A set of hypotheses will be explored using available serum samples at baseline, 4, 24 and 48 hours after allergen exposure (Table 3.5). The primary hypothesis will be to see if adipokine concentrations change in response to allergen. Because serum data from several time points was available, time is a component of these primary hypotheses. The overall effect of time (the time course of adipokine change) will be investigated. It should be noted that the directional hypotheses for adiponectin and leptin, although contrary to the trends observed in epidemiological research, are in line with the results from a controlled allergen exposure study (204). Because of its similarity to this study, it was given the most weight in determining our hypotheses. The hypothesized direction of change in the serum is also not necessarily the same as it is hypothesized to be in the lung. The secondary and tertiary hypotheses will examine if diesel exhaust augments the allergen effect, and if sex, BMI status or airway responsiveness act to modify the effect of exposure.

Table 3.5. Primary, secondary and tertiary hypotheses to be tested using serum adipokine data.

Type	Hypothesis	Support	Strength of Evidence	Statistical Analysis IV: Independent Variable DV: Dependent Variable
Primary	Leptin will decrease acutely from baseline after allergen exposure, and will return to baseline within 24 hours.	Allergen inhalational study (204). Epidemiologic evidence (205) and limited clinical evidence (184,185).	Medium-Low	i) Mixed Effects Model **Response Variable (DV):** Adipokine concentration **Fixed Effects (IV):** Condition (4: DES, DEA, FAS, FAA); Time (4: -4, 4, 24, 48); Sex; BMI status; Airway responsiveness; Order **Random Effect**: Subject ID ii) Post-hoc analysis: ANOVA least-squares mean comparisons with tukey multiple comparisons correction
	Adiponectin will increase acutely from baseline after allergen exposure, and will return to baseline within 24 hours.	Allergen inhalational study (152,204). Limited clinical evidence (197).	Medium	
	Resistin will be acutely increased in the serum following allergen exposure, and will return to baseline within 24 hours.	Clinical evidence (147) and epidemiologic evidence (202).	Medium	
	The adiponectin/leptin ratio will increase following allergen exposure.	Epidemiologic evidence (124) and limited clinical evidence (197).	Medium	
Secondary	DE will augment the effect of allergen.	Allergen synergy evidence (91–94).	Low	
Tertiary	BMI and sex are effect modifiers of the above hypotheses.	As above.	Medium	

ANOVA: Analysis of Variance; DES: diesel exhaust + saline; DEA: diesel exhaust + allergen; FAS: filtered air + saline; FAA: filtered air + allergen.

3.7.3.1 Statistical Analysis

The same procedure described for lung adipokine responses was followed for serum adipokine responses. The only difference is that time (baseline, 4, 24 and 48 hours) was an additional variable tested for effect modification, and for main effect on adipokine response.

3.7.4 Hypothesis 4: Lung and Serum Comparisons

A set of hypotheses will investigate the correlation of lung and systemic adipokine measures after FAA exposure (Table 3.6). The comparison of change after FAA exposure was based on the need to compare at 48 hours after allergen exposure, the time point at which both of these sample types was collected. It was deemed inappropriate to compare changes in adipokine levels from baseline, as there is no baseline lung adipokine measure to compare to.

Table 3.6. Primary hypotheses to be tested using BAL, BW and serum adipokine data.

Type	Hypothesis	Support	Strength of Evidence	Statistical Analysis IV: Independent Variable DV: Dependent Variable
Primary	Systemic and lung concentrations of leptin will be positively correlated.	Clinical study (114) and epidemiologic evidence (99).	High	i) Pearson Correlation **IV:** Serum adipokine concentration **DV:** Lung (BAL or BW) adipokine concentration
	Systemic and lung concentrations of total adiponectin will not be correlated.	Clinical study (114) and epidemiologic evidence (99,115).	High	
	Systemic and lung concentrations of resistin will not be correlated.	Protein structure information (144).	Low	

BAL: bronchoalveolar lavage; BW: bronchial wash.

3.7.4.1 Statistical Analysis

Pearson correlation was used to determine if lung and systemic adipokine concentrations were related at 48 hours post-FAA exposure. A Pearson correlation was performed on transformed data in all cases except BAL resistin, to ensure that data approximated a linear distribution. The direction and strength as indicated by the value of the Pearson product-moment correlation coefficient, and the statistical significance, p-value, of the correlations will be reported. These values are not adjusted for demographic variables.

3.8 Figure Design

The majority of figures in this thesis were created using the R package entitled 'ggplot2' (245). The remaining figures were created using GraphPad Prism Version 6.0e for Mac OS X (246). Untransformed data is shown in the figures, regardless if log-transformed data was used in the statistical analysis.

3.9 Power Calculation

If a result of physiologic interest was identified that was not statistically significant a power calculation was carried out to determine the sample size necessary to show statistical significance. This value was calculated using G*Power version 3.1. The appropriate statistical test was chosen according to the particular scenario, and the effect size was calculated from group parameters, with an alpha level of 0.05 and power of 80% based on data used and assumptions made by Sood et al. (204).

Chapter 4: Results

Overall, 18 subjects completed the DE2 crossover experiment. A total of ten patients were exposed to DE first, and FA second, while the remaining eight participants were exposed in the reverse order. The number of subjects with samples from all exposure conditions was 11 to 15 subjects for BAL and BW lung samples, and 17 subjects for serum samples.

4.1 Study Participants

Participant characteristics for the eighteen consented subjects are shown below in Table 4.1. A more detailed list of individual participant characteristics can be found in Appendix A, Table A.1.

Table 4.1. Baseline (serum -4 hours, lung FAS exposure) clinical and biochemical characteristics of the study population.

Characteristic	n	Mean ± SD	Median	Lower and Upper Quartiles
Women : Men	11:7	–	–	–
Age, yr	18	28.0 ± 7.6	26	23, 31
BMI, kg/m^2	18	25.8 ± 4.7	24.4	23.4, 27.6
FEV_1, L	18	3.66 ± 1.1	3.32	3.05, 4.12
FEV_1, % predicted	18	103.6 ±18.6	104.7	100.5, 112.8
Skin test atopy, no. positive tests	18	–	–	–
Birch	3			
Pacific grasses	7			
D. pterinyssinus (house dust mite)	8			
Serum adiponectin, ng/mL	17	8294 ± 6034	7,906	3,884, 11,050
Serum leptin, ng/mL	17	14.5 ± 11.2	11.9	5.69, 19.0
Serum resistin, ng/mL	17	9.95 ± 2.14	9.72	8.27, 11.1
BAL adiponectin, ng/mL	15	4.29 ± 6.79	1.55	1.32, 3.44
BAL leptin, pg/mL	14	7.80 ± 6.46	5.45	3.13, 8.98
BAL adiponectin/leptin ratio	13	570 ± 487	558	185, 749
BAL resistin, ng/mL	12	5.44 ± 4.11	3.06	2.27, 8.67
BW adiponectin, ng/mL	14	1.21 ± 0.54	1.31	1.16, 1.39
BW resistin, ng/mL	11	0.91 ± 1.07	0.61	0.39, 1.00

BMI: body mass index; FEV_1: maximal forced expiratory volume in one second. BAL: bronchoalveolar lavage; BW: bronchial wash.

The complete study population consisted of 11 premenopausal women and 7 men between 19 and 42 years of age, and a BMI range of 19.1 to 40.5 kg/m^2. The average BMI was slightly overweight but typical of Canadians currently. Obese/overweight (BMI ≥ 25 kg/m^2) subjects made up 33% of the study population, with only two participants had BMI values falling within the obese category (BMI ≥ 30 kg/m^2). Ethnicity of the study population was predominantly Caucasian (61.1%), with the remaining participants being of Asian (33.3%), and other descent (5.6%). All 18 participants are allergic to one or more of the allergens or allergen groups listed in the table above as confirmed by positive skin tests. 11 study participants had PC_{20} values >16 mg/mL, while the remaining seven participants had PC_{20} values ranging from 0.2 to 13.9. Six of these participants were defined as having baseline hyperresponsiveness to methacholine (PC_{20} ≤ 8 mg/mL), but

only three had doctor-diagnosed asthma. This is because methacholine hyperresponsiveness is only one feature that contributes to the diagnosis of asthma, not a defining feature of asthma alone (233).

Baseline adipokine concentrations of the study population were within expected physiological ranges (Table 4.1). At baseline (-4 hours), adiponectin was highest in the serum, followed by leptin, and finally by resistin. Adipokine levels in the lung were generally much lower (by several magnitudes for leptin and adiponectin, and by about 50% for resistin). BW levels were approximately the same as BAL adiponectin and a magnitude lower than BAL resistin.

4.2 Descriptive Statistics

Descriptive statistics including the arithmetic mean ($\bar{x}$), the geometric mean ($\bar{x}_g$), the standard deviation (s) and the geometric standard deviation (s_g) are shown for each dependent variable (Table 4.2). These data were used together with visualization of the histograms and goodness of fit tests to determine if log-transformation was necessary for each variable. This process is detailed further in Appendix A3. All dependent variables except BAL resistin were determined to have a lognormal distribution with geometric standard deviations ranging from 1.35 to 4.35. Transforming the variables by taking the natural logarithm of each value in the data sets was found to better approximate a normal distribution, although not perfectly in most cases. Inferential analyses were performed using the transformed data sets for all variables except BW resistin, while the untransformed data is presented in the figures and tables.

The degree of skewness of each variable was used to determine the value that should be substituted for data points falling under the limit of detection (LOD). The LOD of each ELISA kit was defined as the mean minimum detectable dose as reported by the kit suppliers. LOD values in distributions that were found to be highly skewed and/or those that had a s_g near to or above 3.0 were substituted with LOD/2, while the LOD/$\sqrt{2}$ substitution method was used if the distributions were less skewed or had a s_g between 1.5 and 2.5 (247). The LOD/2 substitution method was used for all variables with values falling under the LOD, with the exception of BAL leptin and resistin in which the LOD/$\sqrt{2}$ method was used. These substitution methods were only utilized if less than 50% of the values in the dataset fell under the LOD. The use of LOD substitution in the BW leptin data set, in which 64% of values were under the LOD, was not appropriate as the geometric mean and standard deviation estimates would likely be biased or inaccurate (247). Therefore, BW leptin data was not assessed further.

Table 4.2 Descriptive statistics and LOD substitution decisions for each measured dependent variable.

Measurement	n	$\bar{x}$ (ng/mL)	$\bar{x}_g$ (ng/mL)	Median (ng/mL)	S (ng/mL)	s_g	LOD (ng/mL)	% < LOD	LOD Substitution (ng/mL)
BAL adiponectin	15	15.8	5.99	4.76	21.1	4.35	0.1	1.67 %	L/2 = 0.05
BW adiponectin	14	6.04	2.52	1.48	12.4	2.97	0.1	8.93 %	L/2 = 0.05
Serum adiponectin	17	7851	5378	6104	5901	2.69	0.246	0%	—
BAL leptin	14	0.019	0.013	0.010	0.022	2.31	0.0035	20.4%	L/√2 = 0.0025
BW leptin	11	—	—	—	—	—	0.0035	63.6%	—
Serum leptin	17	11.7	8.02	9.06	0.92	2.5	0.0078	0%	—
BAL adiponectin/ leptin ratio	13	963	679	680	1.17	3.22	n/a	0%	n/a
Serum adiponectin/ leptin ratio	17	1271	671	839	1311	3.74	n/a	0%	n/a
BAL resistin	12	6.23	4.43	6.28	4.21	2.75	0.026	4.17%	L/√2 = 0.018

Measurement	n	$\bar{x}$ (ng/mL)	$\bar{x}_g$ (ng/mL)	Median (ng/mL)	S (ng/mL)	s_g	LOD (ng/mL)	% < LOD	LOD Substitution (ng/mL)
BW resistin	11	1.16	0.647	0.607	1.43	3.11	0.026	2.27%	L/2 = 0.013
Serum resistin	17	11.4	10.8	11.0	3.75	1.35	0.026	0%	—

n: number of subjects in data set; $\bar{x}$: arithmetic mean; $\bar{x}_g$: geometric mean; s: standard deviation; s_g: geometric standard deviation; LOD: limit of detection (sensitivity or minimum mean detection limit of ELISA kit).

4.3 Hypothesis 1: Baseline Sex and BMI Status Comparison

Overall Hypothesis: Adipokine differences will exist between males and females, and between overweight and normal BMI individuals.

Baseline adipokine data compared by sex and BMI status can be seen below in Table 4.3 and Table 4.4, and depicted visually in Figure 4.1-4.3. There was no significant difference in BMI between males (mean = 27.9 kg/m^2) and females (mean = 24.1 kg/m^2) as assessed by an unpaired t-test. After stratification into overweight and normal BMI groups, there was also no difference in BMI between males and females in each group as assessed by t-test ($p = 0.692$ and $p = 0.789$ respectively). There were an unequal number of participants in the overweight and normal groups in the complete study population ($n = 6$ and $n = 12$ respectively). There were more males in the overweight BMI group and more females in the normal BMI group. Baseline adipokine values did not differ by airway responsiveness as assessed by unpaired t-test (p-values ranged from $p = 0.086$ to $p = 0.887$).

Table 4.3. Baseline lung (BAL and BW) adipokine levels compared by sex and by BMI status (normal vs. overweight/obese) using an unpaired t-test. Values are presented as mean ± standard error. Number of males and females, and normal and overweight individuals compared is different for each adipokine measured.

Outcome	Sex		Unpaired t-test	BMI		Unpaired t-test
	Male (n = 7)	Female (n = 10)		Normal (< 25 kg/m^2) (n = 12)	Overweight (≥ 25 kg/m^2) (n = 6)	
BAL adiponectin (ng/mL)	1.60 ± 0.98	6.09 ± 8.43	0.150	5.63 ± 8.63	2.27 ± 1.41	0.283
BAL leptin (pg/mL)	4.01 ± 2.63	10.3 ± 7.60	0.058	7.27 ± 4.79	8.03 ± 9.08	0.845
BAL resistin (ng/mL)	5.32 ± 4.09	5.53 ± 4.44	0.935	7.56 ± 4.51	3.32 ± 2.43	0.078
BW adiponectin (ng/mL)	0.85 ± 0.63	1.47 ± 0.32	0.064	1.31 ± 0.56	1.02 ± 0.55	0.370
BW resistin (ng/mL)	0.73 ± 0.40	2.57 ± 3.89	0.299	2.47 ± 3.52	0.45 ± 0.49	0.184

BMI: body mass index, kg/m^2; BAL: bronchoalveolar lavage; BW: bronchial wash

No significant differences were found as assessed by unpaired t-test. Females appear to have higher mean BAL leptin and BW adiponectin levels than males, and those with normal BMI status appear to have higher mean BAL resistin levels than those with higher BMI values. Although only an observed trend, females and those in the normal BMI category generally had higher mean adipokine levels measured in the BAL and BW.

Table 4.4. Baseline serum adipokine levels compared by sex and by BMI status (normal vs. overweight/obese) using an unpaired t-test (n = number of subjects in data set). Values are presented as mean ± standard error.

Outcome	Sex		Unpaired t-test	BMI		Unpaired t-test
	Male (n = 7)	Female (n = 10)		Normal (< 25 kg/m²) (n = 11)	Overweight (≥ 25 kg/m²) (n = 6)	
Serum adiponectin (ng/mL)	9015 ± 7524	7789 ± 5128	0.716	6687 ± 4523	11241 ± 7713	0.230
Serum leptin (ng/mL)	6.63 ± 5.48	20.1 ± 10.9	**0.0048**	15.0 ± 11.0	13.6 ± 12.5	0.696
Serum resistin (ng/mL)	9.42 ± 2.40	10.3 ± 1.99	0.432	10.2 ± 2.17	9.48 ± 2.20	0.542

Values are presented as mean ± standard error.

In the serum, the only statistically significant difference at baseline was that females had higher mean leptin levels than males did. No additional trends in the serum data were observed.

Overall Result: At baseline, very few statistically significant differences were observed based on sex or BMI status differences. Females were found to have higher serum leptin levels than males; this was the apparent trend in most cases, however, these differences were not statistical. There were no patterns for BMI status.

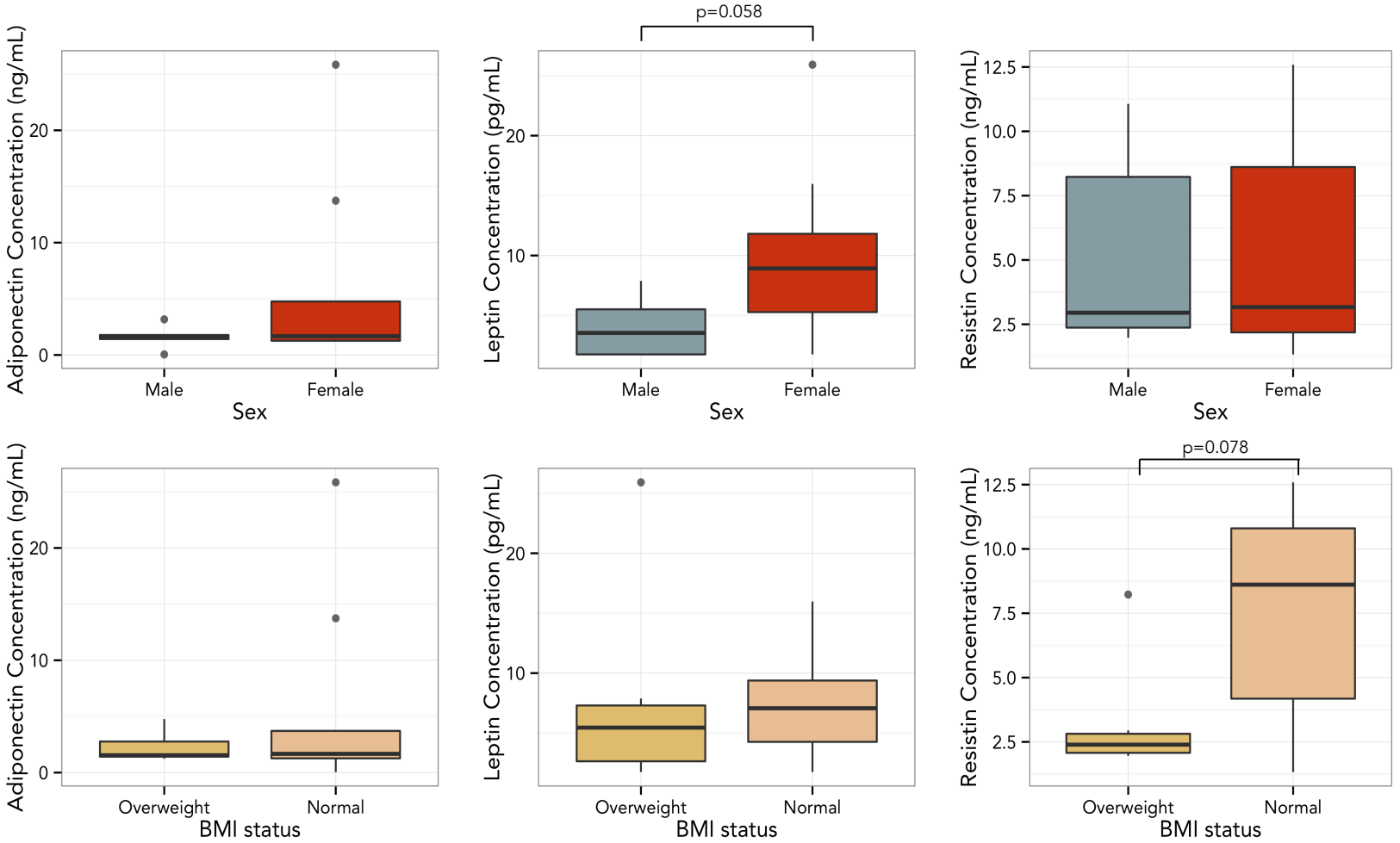

Figure 4.1. Baseline BAL adipokine levels compared in females and males, and in normal (<25 kg/m^2) and overweight (≥25 kg/m^2) BMI individuals. Number of males and females, and normal and overweight individuals compared is different for each adipokine measured. Significant unpaired t-test p-values are indicated above the pairs found to be statistically different. The median, 1st and 3rd quartiles are represented by the middle, lower and upper lines of the box, while the upper whisker is Q3 + 1.5*interquartile range and the lower whisker is Q1 – 1.5*IQR.

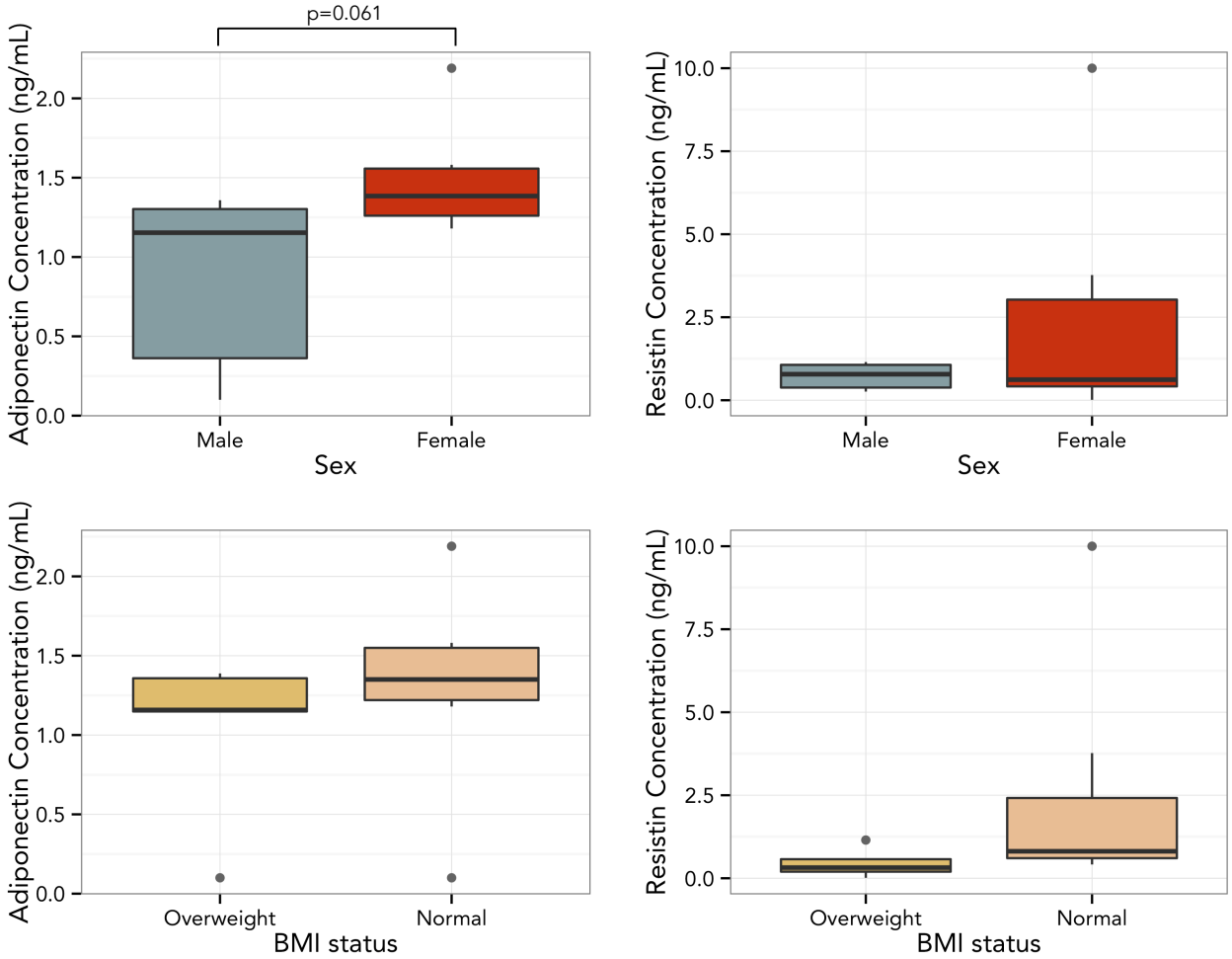

Figure 4.2. Baseline BW adiponectin and resistin levels compared in females and males, and in normal (<25 kg/m^2) and overweight (≥25 kg/m^2) BMI individuals. Number of males and females, and normal and overweight individuals compared is different for each adipokine measured. Significant unpaired t-test p-values are indicated above the pairs found to be statistically different. The median, 1st and 3rd quartiles are represented by the middle, lower and upper lines of the box, while the upper whisker is Q3 + 1.5*interquartile range and the lower whisker is Q1 – 1.5*IQR.

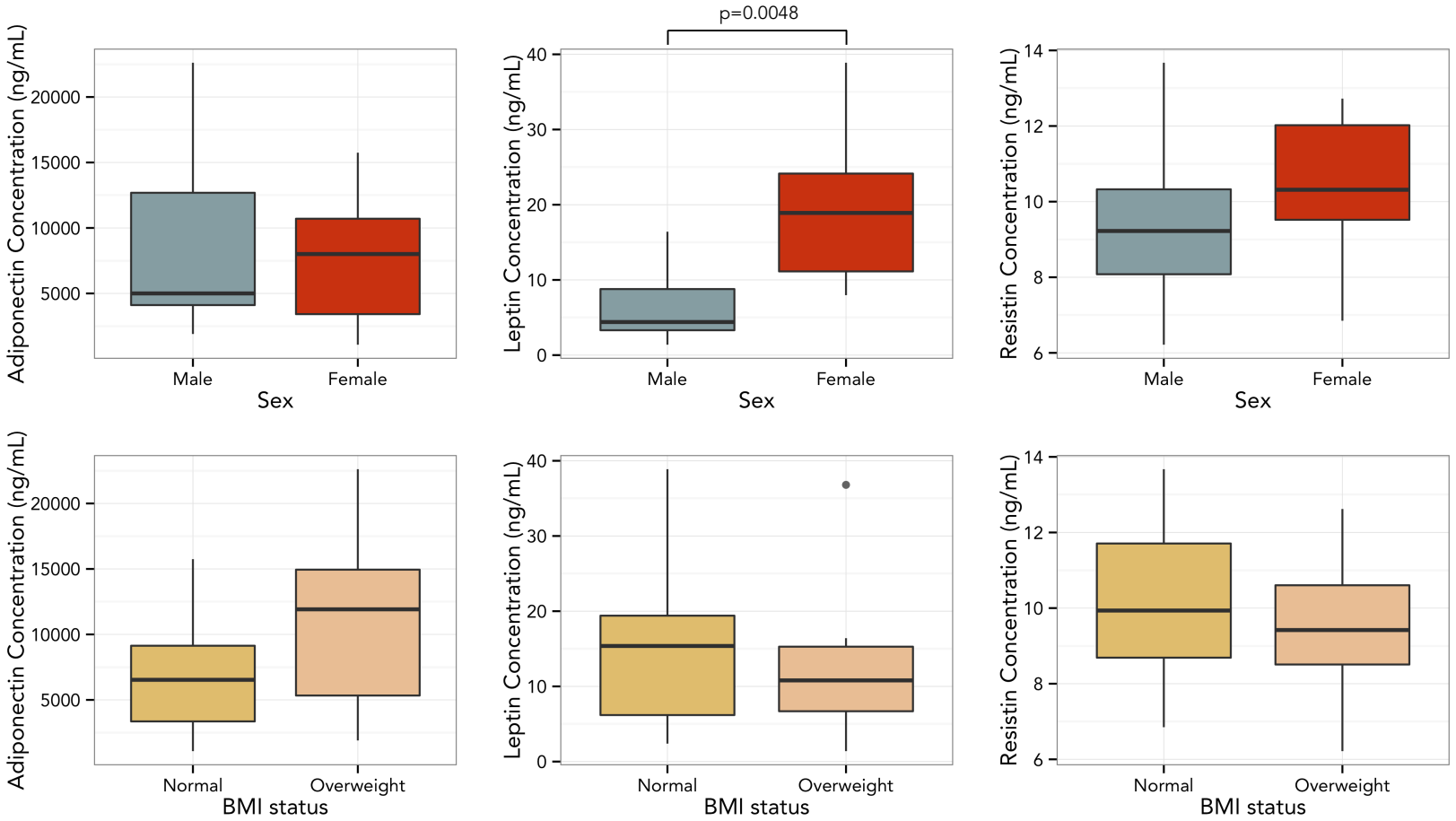

Figure 4.3. Baseline serum adipokine levels compared in females (n = 10) and males (n = 7), and in normal (<25 kg/m^2) (n = 11) and overweight (≥25 kg/m^2) (n = 6) BMI individuals. Significant unpaired t-test p-values are indicated above the pairs found to be statistically different. The median, 1st and 3rd quartiles are represented by the middle, lower and upper lines of the box, while the upper whisker is Q3 + 1.5*interquartile range and the lower whisker is Q1 – 1.5*IQR.

4.4 Hypothesis 2: Lung Adipokine Responses

Overall Hypothesis: Allergen will alter adipokine concentrations in the lung; diesel exhaust will augment these effects.

4.4.1 Test for Carryover Effect

There was no significant carryover effect detected in the lung adipokine response variables (interaction p-values ranged from 0.069 – 0.796). We are not worried about the p-value of 0.07, a value somewhat close to 0.05, due to the fact of multiple comparisons. All variables were assessed further.

4.4.2 Effect Modification by Sex, BMI status, Airway Responsiveness

The term "responsiveness" listed in subsequent tables refers to airway hyperresponsiveness ($PC_{20} \leq 8$ mg/mL) status or normal airway responsiveness ($PC_{20} > 8$mg/mL) at baseline. The interaction between exposure and sex, BMI status and airway responsiveness was assessed, and results are shown below in Table 4.5. There was no evidence of effect modification by any of these variables, and therefore, interaction terms were not included in the subsequent models.

Table 4.5 Mixed-effects model ANOVA test for sex, BMI status, order and airway responsiveness interaction with exposure condition in each dependent adipokine variable (n = number of subjects in data set). Significant p-values ($p < 0.05$) are shown in **bold**.

Interaction	BAL Adiponectin (n = 15)	BW Adiponectin (n = 14)	BAL Leptin (n = 14)	BAL Resistin (n = 12)	BW Resistin (n = 11)	BAL Adiponectin/ Leptin Ratio (n = 13)
	ANOVA p-value (Type III)					
Exposure*Sex	0.819	0.956	0.598	0.730	0.296	0.066
Exposure*BMI	0.999	0.770	0.962	0.241	0.118	0.964
Exposure*Airway Responsiveness	0.151	0.377	0.317	0.724	0.212	0.059

The * indicates an interaction term being tested.

There was suggestion of a potential interaction between exposure and airway responsiveness in the BAL adiponectin/leptin data ($p = 0.059$). This indicates that ratio responses to exposure may have depended on an individual's airway responsiveness status. Because there is biological relevance and support in literature that can explain this finding, the result was investigated further in Section 4.4.5 following the regular mixed-effects analysis.

4.4.3 Significance of Sex, BMI Status and Airway Responsiveness

Data from the initial mixed-effect model that included the fixed effects exposure, sex, BMI status, airway responsiveness and order, and the random effect subject ID are shown in Table 4.6. P-values are shown to assess inclusion of variables in the final mixed-effects models.

Table 4.6. Initial mixed-effects model ANOVA test for statistical significance of sex, BMI status, airway responsiveness and order in predicting each adipokine response variable. Significant p-values ($p < 0.5$) are shown in **bold**.

Fixed Overall Effect	BAL Adiponectin (n = 15)	BW Adiponectin (n = 14)	BAL Leptin (n = 14)	BAL Resistin (n = 12)	BW Resistin (n = 11)	BAL Adiponectin /Leptin Ratio (n = 13)
	ANOVA p-value (Type III)					
Exposure Condition	**4.36×10^{-6}**	**1.25×10^{-6}**	**0.0002**	0.0595	**0.05**	0.070
Sex	**0.046**	0.178	**0.007**	0.226	0.279	0.797
BMI status	0.287	0.887	0.215	0.091	0.171	0.985
Airway Responsiveness	0.285	0.303	0.465	**0.016**	0.270	0.985
Order	0.515	0.752	0.241	0.885	0.981	0.106

Sex was found to have a significant fixed effect in predicting BAL adiponectin and BAL leptin responses. Airway responsiveness was found to be significant in predicting overall BAL resistin responses. These variables were included in the final mixed-effects models. BMI status and order were not significant in predicting adipokine responses and were not included in final mixed-effects models.

4.4.4 Effect of Exposure, Significant Demographic Variables

The effect estimates from the final mixed-effects models can be seen in Table 4.7 below. The effect of exposure on adipokine levels, averaged over significant demographic variables, is depicted visually in Figure 4.4 for the BAL data, and Figure 4.5 for the BW data. Allergen significantly increased BAL adiponectin, BW adiponectin and BAL leptin concentrations, and modestly increased BW resistin, although no significant pairwise effect estimates were observed.

Post hoc least-squares mean analysis revealed that allergen increased BAL adiponectin after DE and FA exposure over the corresponding saline exposures with effect estimate of 5.40 ng/mL and 5.62 ng/mL, respectively. Similarly, allergen increased BAL leptin after DE and FA exposure with effect estimates of 2.13 pg/mL and 2.51 pg/mL respectively. No significant effect of exposure was found for BAL resistin or the BAL adiponectin/leptin ratio but it appears that allergen may also be acting to increase these measures. Although only an observed trend, the mean adiponectin/leptin ratio appears to be highest following DEA exposure, than after any other exposure condition, as seen in panel D of Figure 4.4. This was the only potential evidence in these data for diesel exhaust augmentation of allergen effect.

BW adiponectin was also increased by allergen after DE and FA exposure as compared to the corresponding saline exposures with effect estimates of 3.11 ng/mL and 5.62 ng/mL respectively. Pairwise comparisons of exposure are not shown for BAL resistin as exposure did not have a significant main effect. Although allergen appears to increase BW resistin, no significant pairwise exposure comparisons were found by *post hoc* analysis. As in the BAL samples, there was no evidence indicating diesel exhaust augmentation of allergen effect.

Table 4.7. Least-squares means pairwise comparison for all significant fixed effects in the final mixed-effects model, with Tukey corrected p-values. Only statistically significant effect estimates (standard error) from the log-transformed data are listed in the table. Significant p-values ($p < 0.5$) are shown in **bold**.

Contrast	**BAL Adiponectin (n = 15)**		**BW Adiponectin (n = 14)**		**BAL Leptin (n = 14)**		**BAL Resistin (n = 12)**		**BW Resistin (n = 11)**	
	Effect, ng/mL	p-value	Effect, ng/mL	p-value	Effect, pg/mL	p-value	Effect, ng/mL	p-value	Effect, ng/mL	p-value
DEA – DES	1.69 (0.40)	**0.0006**	1.47 (0.32)	**0.0003**	0.75 (0.23)	**0.0134**	–	–	–	0.108
DEA – FAA	–	0.851	–	0.853	–	0.967	–	–	–	0.999
FAA – FAS	1.72 (0.40)	**0.0005**	1.58 (0.32)	**0.0001**	0.92 (0.23)	**0.0019**	–	–	–	0.338
DES – FAS	–	0.803	–	0.971	–	0.993	–	–	–	0.933
M – F	-0.93 (0.47)	0.067	–	–	-1.07 (0.32)	**0.0056**	–	–	–	–
Normal – Hyper	–	–	–	–	–	–	4.27 (1.38)	**0.013**	–	–

F: female; M: male; Normal: normal airway responsiveness; Hyper: airway hyperresponsiveness.

It should also be noted that sensitivity analysis was performed for the BAL leptin and BW adiponectin data in which outliers can be observed. Without the two BAL leptin FAA outliers, and the single BAL leptin DEA outlier, the significance of exposure ($p= 0.002$) and the significance of the pairwise DEA – DES ($p = 0.020$) and FAA – FAS ($p = 0.024$) comparisons were maintained. Similarly, without the single BW adiponectin FAA outlier, and the two DEA outliers, the significance of exposure ($p = 1.33 \times 10^{-5}$) and the pairwise comparisons between DEA – DES ($p = 0.004$) and FAA – FAS ($p = < 0.001$) remained significant.

Females had a higher predicted value of BAL adiponectin than males as indicated by the negative effect estimate (effect estimate 0.394 ng/mL). Similarly, females had a higher predicted value of BAL leptin than males (effect estimate 0.343 pg/mL). Normal airway responsiveness individuals were predicted to have higher BAL resistin levels than hyperresponsive individuals (effect estimate 4.27 ng/mL).

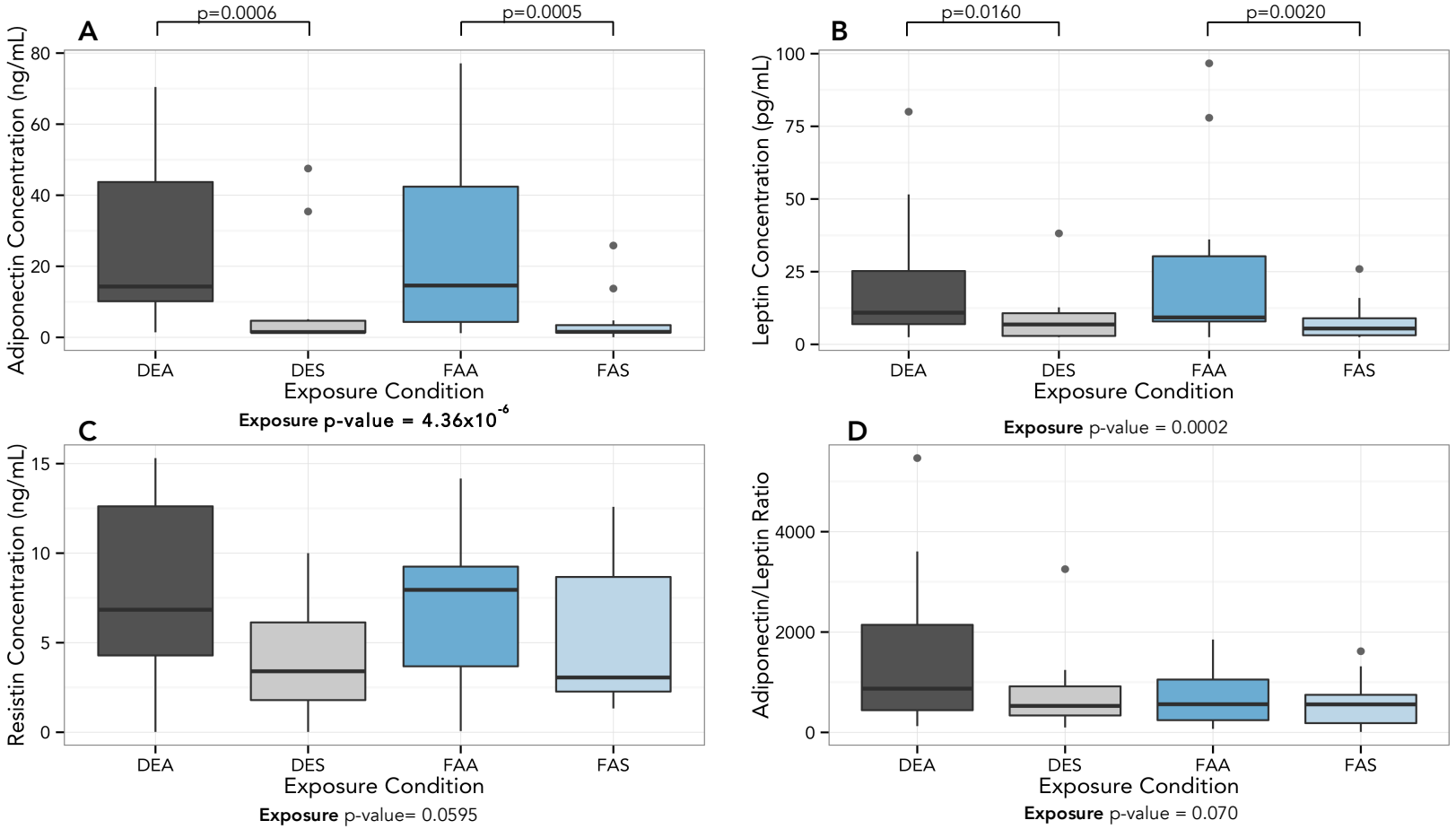

Figure 4.4 BAL adiponectin (n = 15), leptin (n = 14), resistin (n = 12) and adiponectin/leptin ratio (n = 13) following DEA, DES, FAA and FAS exposure, with significant mixed-effects main exposure effect p-values shown below, while significant *post hoc* p-values are shown above bracketing the significant comparison. The median, 1st and 3rd quartiles are represented by the middle, lower and upper lines of the box, while the upper whisker is Q3 + 1.5*interquartile range and the lower whisker is Q1 – 1.5*IQR. Outliers are shown as single points.

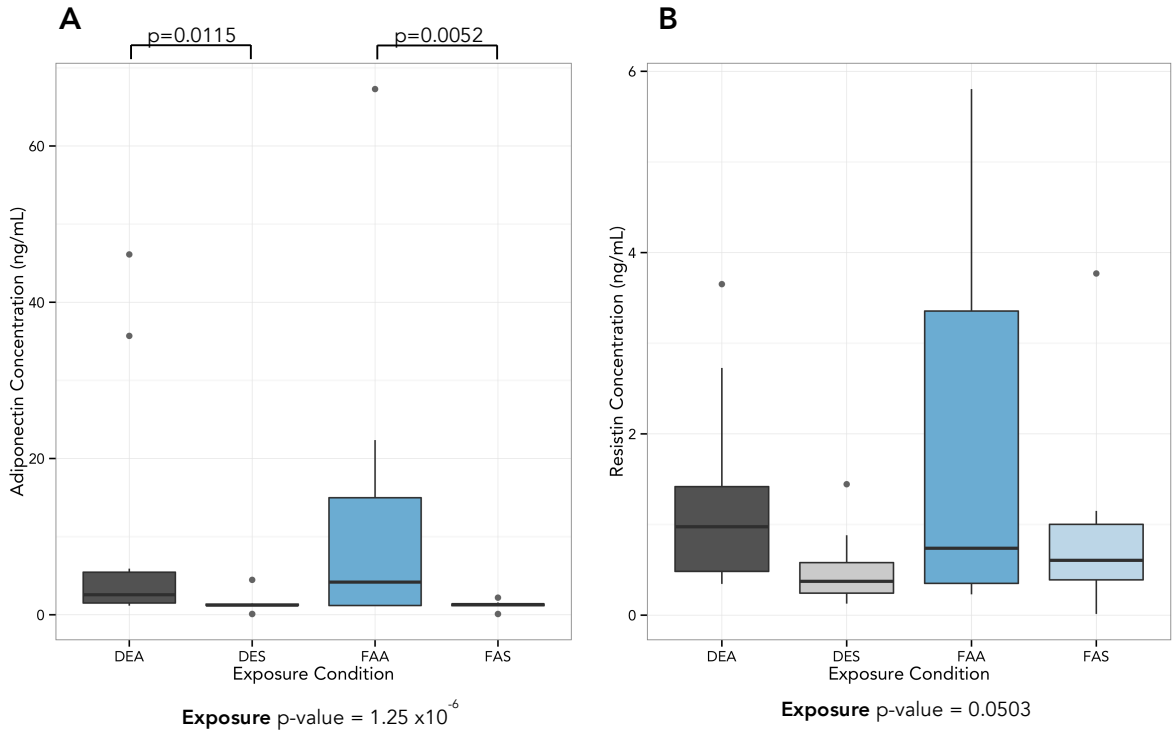

Figure 4.5. BW adiponectin (n = 14) and resistin (n = 11) following DEA, DES, FAA and FAS exposure, with significant mixed-effects main exposure effect p-values shown below, while significant *post hoc* p-values are shown above bracketing the significant comparison. The median, 1st and 3rd quartiles are represented by the middle, lower and upper lines of the box, while the upper whisker is Q3 + 1.5*interquartile range and the lower whisker is Q1 – 1.5*IQR. Outliers are shown as single points.

4.4.5 Effect Modification in the Adiponectin/Leptin Ratio

To further investigate the potential effect modification by airway responsiveness on exposure in the BAL adiponectin/leptin dataset, a final mixed-effects model including the interaction term, fixed effects of exposure and airway responsiveness, and the random effect of subject ID was constructed.

In individuals with normal airway responsiveness, the adiponectin/leptin ratio was found to be modestly higher after DEA exposure than after DES exposure (effect estimate of 2.71 ratio units) and modestly higher after DEA exposure than after FAA exposure (effect estimate of 2.71 and 3.18 ratio units respectively). No increases in the adiponectin/leptin ratio by DEA are observed in hyperresponsive individuals. This result is depicted visually in Figure 4.6 below.

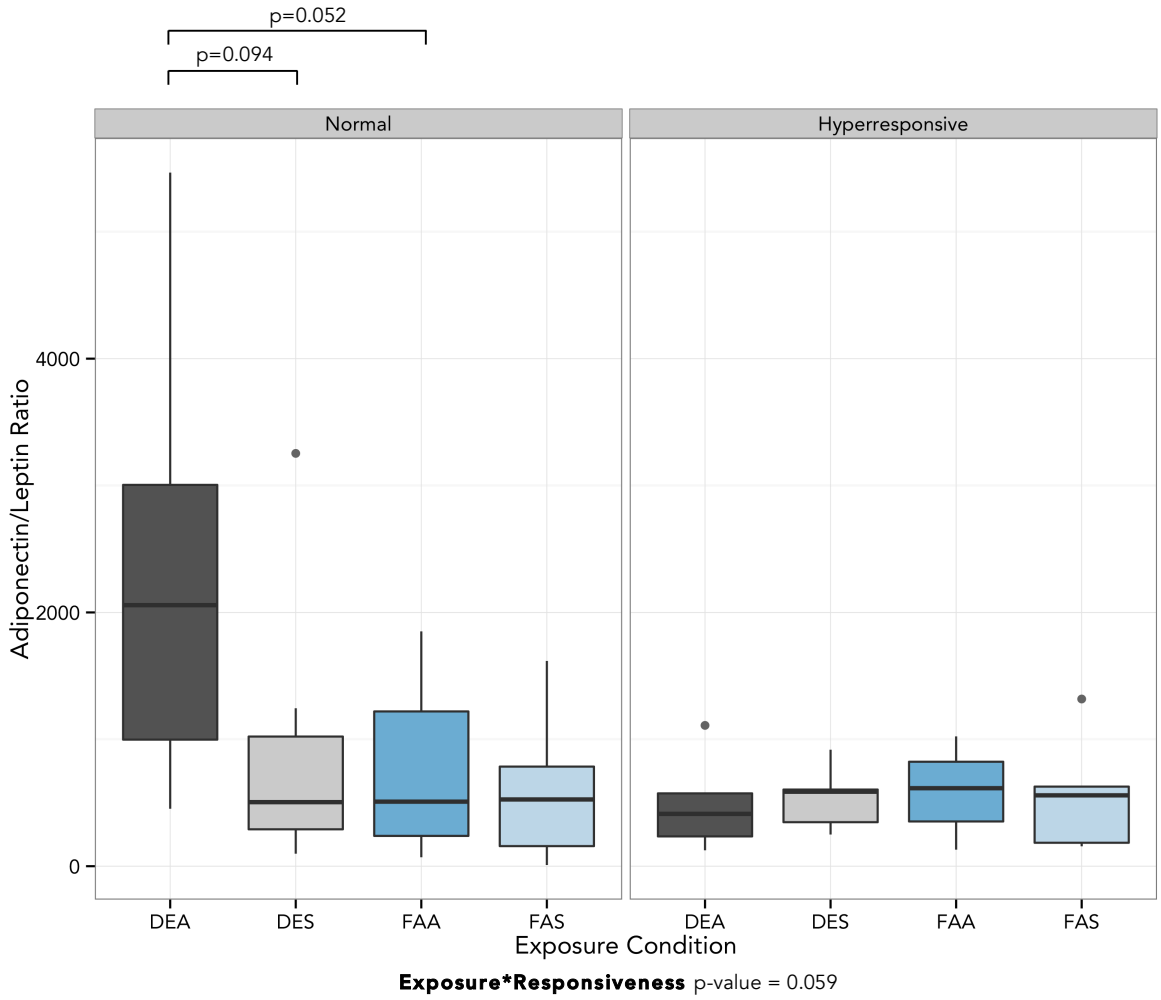

Figure 4.6. Adiponectin/leptin ratio following DEA, DES, FAA and FAS exposure, in normal (n = 8) and hyperresponsive (n = 5) individuals, with the significant mixed-effects interaction p-value shown below, while significant *post hoc* p-values are shown above bracketing the significant comparison. The median, 1st and 3rd quartiles are represented by the middle, lower and upper lines of the box, while the upper whisker is Q3 + 1.5*interquartile range and the lower whisker is Q1 – 1.5*IQR. Outliers are shown as single points.

Overall Result: Allergen exposure (DEA and FAA) was associated with greater concentrations of adiponectin and leptin in the BAL, and greater adiponectin concentrations at 48 hours post-exposure. Diesel exhaust only appeared to augment the effect of allergen in the adiponectin/leptin ratio data, in those with normal airway responsiveness. There was no statistically significant effect modification by sex, BMI status or airway responsiveness.

4.5 Hypothesis 3: Systemic Adipokine Responses

Overall Hypothesis: Allergen will alter adipokine concentrations in the serum acutely; diesel exhaust will augment these effects.

4.5.1 Test for Carryover Effect

Prior to any analysis of lung adipokine responses, the interaction between exposure and order was tested. In both the adiponectin and the adiponectin/leptin ratio data a statistically significant interaction was observed, indicating the presence of carryover effect. Therefore, these variables in the serum could not be analyzed further because an unbiased treatment effect could not be obtained (227). No additional carryover effect in serum leptin or resistin was detected (p-value 0.856 and 0.202 respectively).

4.5.2 Effect Modification by Time, Sex, BMI status, Airway Responsiveness

The interaction between exposure and time, sex, BMI status and airway responsiveness was assessed, and results are shown below in Table 4.9. There was no evidence of effect modification by any of these variables, and therefore, interaction terms were not included in the subsequent models.

Table 4.8. Mixed-effects model ANOVA test for time, sex, BMI status and airway responsiveness interactions with exposure condition in each independent adipokine variable. Significant p-values ($p < 0.5$) are shown in **bold**.

Interaction	Serum Leptin (n = 17)	Serum Resistin (n = 17)
	ANOVA p-value (Type III)	
Exposure*Time	0.940	0.972
Exposure*Sex	0.263	0.626
Exposure*BMI	0.201	0.376
Exposure*Airway Responsiveness	0.749	0.531

The * indicates an interaction term being tested.

4.5.3 Significance of Time, Sex, BMI status and Airway Responsiveness

Data from the initial mixed-effect model that included the fixed effects exposure, time, sex, BMI status, airway responsiveness and order, and the random effect subject ID are shown in Table 4.6. P-values are shown to assess inclusion of variables in the final mixed-effects models. Time was significant in predicting both serum leptin and resistin responses. Sex was also significant in predicting serum leptin response, while airway responsiveness was found to be significant in predicting serum resistin response. These variables were including in the final mixed-effects models.

Table 4.9. Initial mixed-effects model ANOVA test for statistical significance of time, sex, BMI status, airway responsiveness and order in predicting each adipokine response variable. Significant p-values ($p < 0.5$) are shown in **bold**.

Fixed Effect	Leptin (n = 17)	Resistin (n = 17)
	ANOVA p-value (Type III)	
Exposure Condition	0.168	0.556
Time	**< 0.001**	**< 0.001**
Sex	**0.008**	0.249
BMI status	0.244	0.450
Airway Responsiveness	0.736	**0.010**
Order	0.267	0.138

4.5.4 Effect of Exposure, Significant Demographic Variables

The effect estimates from the final mixed-effects models can be seen in Table 4.10 below. The effect of exposure on serum leptin and resistin levels in the 48 hours following exposure, averaged over significant demographic variables, is depicted visually in Figure 4.7. As it was stated before, the effect of exposure is of interest in the serum at several time points, and a time component is included in serum hypotheses, so the fixed effects of both exposure and time are both shown under each panel in Figure 4.7. Serum adipokine concentrations changed over time however, exposure (FAA vs. DEA), had no statistically significant effect on either leptin or resistin. Adipokine concentration changes followed a similar trend over time after FAA and DEA exposure.

Table 4.10. Least-squares means pairwise comparison of all significant fixed effects in the final mixed-effects model, with Tukey corrected p-values. Only statistically significant effect estimates (standard error) from the log-transformed data are listed in the table.

Contrast	Serum Leptin (n = 17)		Serum Resistin (n = 17)	
	Effect, pg/mL	p-value	Effect, ng/mL	p-value
-4 hours – 4 hours	0.42 (0.07)	**< 0.001**	-0.21 (0.05)	**< 0.001**
-4 hours – 24 hours	0.24 (0.07)	**0.004**	-0.23 (0.05)	**< 0.001**
-4 hours – 48 hours	0.27 (0.07)	**< 0.001**	–	0.897
4 hours – 24 hours	-0.18 (0.07)	0.0516	–	0.985
4 hours – 48 hours	–	0.150	0.18 (0.05)	**0.003**
24 hours – 48 hours	–	0.967	0.20 (0.05)	**< 0.001**
M – F	-1.07 (0.36)	**0.009**	–	–
Normal – Hyper	–	–	0.265 (0.096)	**0.014**

The -4 signifies the baseline time-point; F: female; M: male; Hyper: hyperresponsive

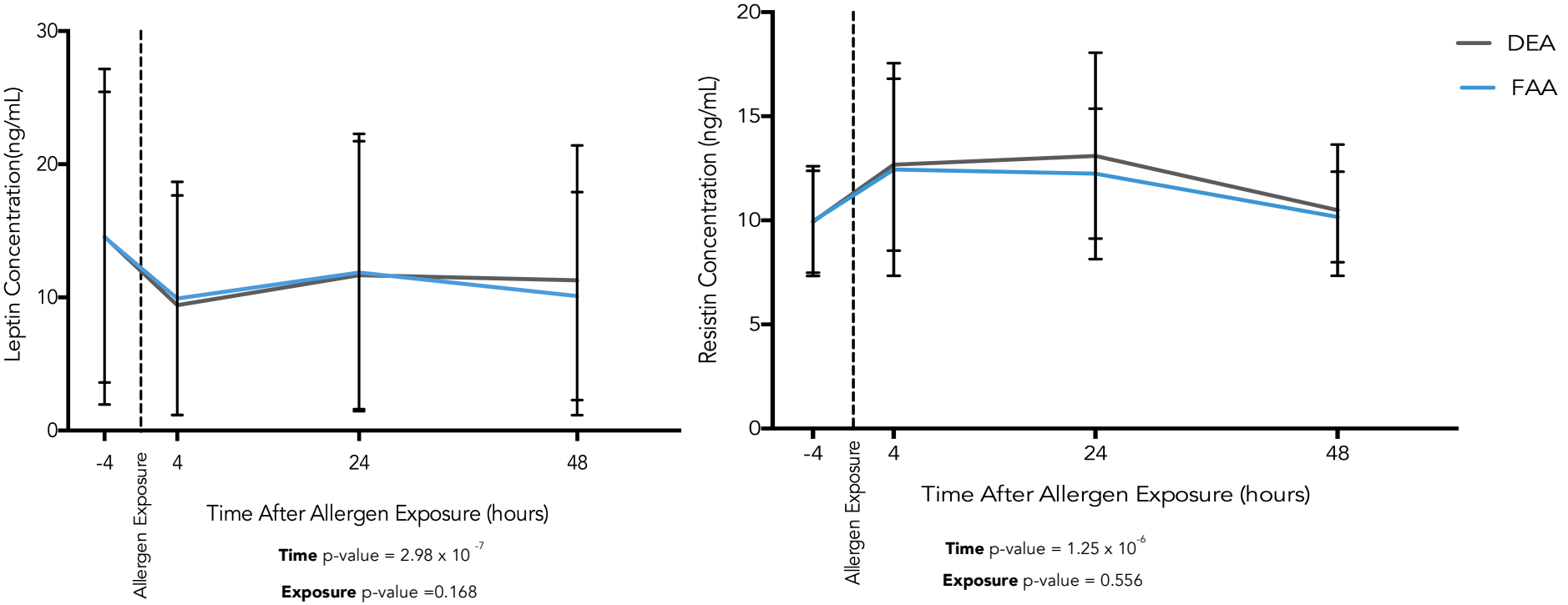

Figure 4.7. Time course of serum leptin and resistin change from baseline (-4 hours) to 4, 24 and 48 hours after allergen exposure indicated by the dashed vertical line. Significant mixed-effects exposure and time p-values are shown below. Points represent mean concentrations, and error bars indicate standard deviation.

Sex significantly predicted serum leptin responses to inhaled allergen with women having higher serum leptin levels overall than men. Airway responsiveness was found to significantly predict serum resistin responses with normal individuals having higher resistin levels than hyperresponsive individuals (effect estimate = 1.30 ng/mL). Serum leptin concentrations decreased significantly from the baseline within 4 hours post-exposure (effect estimate = 1.52 pg/mL). Leptin concentrations then increased slightly at 24 hours post-exposure (effect estimate = 1.27 pg/mL) and remained similar at 48 hours post-exposure, but still significantly lower than baseline concentrations (effect estimate = 1.32 pg/mL). Conversely, resistin concentrations increased significantly from the baseline at 4-hours post exposure (effect estimate = 0.81 ng/mL). Levels remained significantly elevated from baseline at 24 hours post-exposure (effect estimate = 0.79 ng/mL), but returned to baseline levels after 48 hours.

Overall Result: Serum concentrations of leptin and resistin were found to change over time following inhaled allergen exposure. There was no exposure effect, indicating that there is no diesel exhaust augmentation of allergen effects, as was concluded in the lung. Sex was found to be important in predicting leptin responses. BMI status had no relevance in predicting serum adipokine responses. Airway responsiveness was found to be important in predicting serum resistin responses.

4.6 Hypothesis 4: Lung and Serum Comparisons

Overall Hypothesis: Correlation will exist between leptin lung and serum adipokine measures.

The correlation data between log-transformed lung and systemic measures (excepting BAL resistin which was not transformed) at 48 hours following FAA exposure is shown below in Table 4.11. There was a statistically significant positive correlation between BAL leptin and serum leptin, r = 0.775, p = 0.002 with a large strength of association. This association, however, has not been adjusted for covariates like sex or BMI that likely impact this association.

Table 4.11. Pearson's correlation coefficients between measured log-transformed lung and systemic adipokine concentrations at 48 hours post-FAA exposure.

Post-FAA Exposure	BAL Adiponectin (log ng/mL)	BW Adiponectin (log ng/mL)	BAL Leptin (log pg/mL)	BAL Resistin (ng/mL)	BW Resistin (log ng/mL)	BAL Adiponectin /Leptin ratio
Serum Adiponectin (log ng/mL)	0.099 (0.736)	0.198 (0.516)	–	–	–	–
Serum Leptin (log ng/mL)	–	–	0.775 (**0.002**)	–	–	–
Serum Resistin (log ng/mL	–	–	–	0.278 (0.408)	0.439 (0.205)	–
Adiponectin/Leptin ratio	–	–	–	–	–	-0.004 (0.990)

Data are presented with Pearson correlation coefficient (p-value).

Overall Result: Leptin serum and BAL concentrations were found to correlate strongly 48 hours after FAA exposure. No additional correlations between lung and serum adipokine measures were observed.

Chapter 5: Discussion

5.1 Overview

The participation of adipokines in inflammatory responses in systemic and lung environments, and their ability to perpetuate and worsen inflammation causing lung pathogenesis is under great debate and requires directed study to answer. The evaluation of adipokine responses within the context of clinical studies is limited, and the study of adipokine responses in the context of a human controlled crossover study is previously nonexistent.

In this study, we were able to observe adiponectin, leptin and resistin responses after allergen and diesel exhaust co-exposure, to identify potential diesel exhaust augmentation of allergen effect, and were able to observe these responses both in the serum, and in the lung using two different sample types. The time course of serum responses at 4, 24 and 48 hours post-exposure were identified, and sustained effects in the lung at 48 post-exposure were measured. Although this study was not specifically designed or powered for this investigation, several interesting allergen-specific responses were observed. Furthermore, sex, BMI status and methacholine responsiveness interactions and effects were identified, and a diesel exhaust-driven effect was detected in the adiponectin/leptin ratio data.

As was discussed in the methods section, this study aimed to address 4 primary hypotheses, some of which also had secondary, and tertiary aims. The four main hypotheses that will serve as the basis of this discussion are as follows:

1) **Adipokine differences will exist between males and females, and between overweight and normal BMI individuals.**
2) **Allergen will alter adipokine concentrations in the lung; diesel exhaust will augment allergen effect.**
3) **Allergen will alter adipokine concentrations acutely in the serum; diesel exhaust will augment allergen effect.**
4) **Correlation will exist between leptin lung and serum adipokine measures.**

The following is a brief summary. Adipokine differences due to sex and overweight status at baseline were difficult to detect, likely due to insufficient power, and large standard deviations in the data. In the BAL lung samples, allergen exposure (DEA or FAA) increased adiponectin and leptin concentrations over their corresponding saline exposures (DES or FAS). The same allergen-induced increase was observed for BW adiponectin. No diesel exhaust augmentation of these effects was observed. The only potential effect modification and diesel exhaust augmentation of allergen effect was observed in the BAL adiponectin/leptin ratio. In those with normal airway responsiveness only, DEA exposure was associated with higher ratio levels than FAA exposure, indicating an additional effect of diesel exhaust over allergen alone. Leptin and resistin

were found to increase and decrease acutely at four hours post-allergen exposure. Leptin remained depressed below baseline at 24 and 48 hours post-exposure, while resistin returned to baseline within 48 hours. No diesel exhaust augmentation of allergen effect was observed. Finally, there was a strong, significant correlation between BAL and serum measures of leptin at 48 hours post-FAA exposure.

This study affords the ability to detect adipokine changes in a relatively healthy but atopic population, and to establish a benchmark of normal adipokine responses important for later comparisons. The details of the adipokine response results and the independent variables that affect these responses, as well as the biological relevance and overall significance of these results, will now be discussed in more detail.

5.2 Hypothesis 1: Baseline Sex and BMI Status Comparison

It has long been recognized that both sex and BMI status influence the circulating levels of adipokines in the body. There are physiological reasons for these differences, and may even be clinical consequences of such differences. To assess baseline and fasting adipokine differences that can be attributed to sex and BMI status, two baseline values of serum adipokine measures obtained at the start of each exposure triad were averaged, and lung adipokine levels obtained after FAS exposure were used. We hypothesized that females would have higher adipokine concentrations than males, and that overweight BMI individuals would have increased leptin and resistin, and decreased adiponectin levels. These comparisons were hypothesized to be true in both serum and lung.

In the literature, the clearest and most consistent differences have been observed for adiponectin. It was first shown in mice (112) and then confirmed in several human studies (112,248) that females exhibit higher circulating levels of total adiponectin, and particularly the high molecular weight isoform of the protein, than males do. This is due to the action of testosterone, which inhibits HMW adiponectin production during puberty (249). There are also fairly consistent findings that adiponectin is inversely correlated with BMI and fat mass (106), and that obese individuals (BMI > 30kg/m^2) exhibit depressed adiponectin concentrations than their age- and gender-matched normal BMI counterparts (248,250,251). Although the gender and BMI relationships are well established in the literature, baseline measures of this study's participants did not show these clear trends. In the serum, males appeared to have higher adiponectin concentrations than females and those with higher BMI values appeared to have higher adiponectin values, although these trends were not statistically significant. Patterns of adiponectin concentrations in the lung are not well established, as many fewer studies have included lung adipokine measurements and comparisons by sex and BMI (rather than by disease status (102,113,114). It might be suspected that if physiological differences exist that affect serum adipokine levels, the same patterns would occur in the lung. However, correlations between lung and serum adipokine measures have been notably absent (99,114,115). In this dataset, BAL and BW adiponectin follow

the established adiponectin patterns. Females exhibit higher levels of adiponectin, and lower BMI individuals have higher adiponectin levels. However, these differences were not statistically significant. The patterns in the lung at baseline did not seem to correspond to the levels observed in the serum. This poor association may be due to the T-cadherin-based transport, which appears to regulate adiponectin in lungs, and likely explains the poor correlations observed in other studies (214,252).

Similar to adiponectin, women have 40-200% higher serum leptin concentrations than do males, following adjustment for body fat levels (128,129,137,138,248). Severely obese individuals are also know to have 400-600% higher concentrations of serum leptin (127), and more generally, leptin is observed to correlate with adipose tissue mass (126). It has also been shown in a small clinical study that overweight and obese individuals have higher levels of BAL leptin than normal weight individuals (114). Although measurements of leptin in lung samples is again limited, it has been confirmed in several studies that correlations exist between leptin serum and lung measures, indicating that leptin cross into the lung with relative ease (99,114). Therefore, similar trends would be expected in the lung samples. The differences according to sex are as expected, with females having ~300% higher leptin values in the serum. BMI status, as measured in this study, does not seem to have any effect on leptin measures, with both serum and BAL measures statistically indistinguishable between these two groups.

The expression patterns of resistin are much less established than adiponectin or leptin. Resistin levels have been correlated with adiposity measures (107), and obese individuals have exhibited elevated serum resistin levels (237) but these associations are not consistent (248,237). This is unsurprising as the production of resistin by adipocytes is still debated. Gender differences have been detected in epidemiological studies, with females found to have higher resistin levels than males (236), but the evidence of a relationship remains unclear. There is no indication in the literature as to whether correlation should be expected between serum and lung concentrations, however, resistin is known to circulate in part as high molecular weight multimers that would be unlikely to pass into the lungs unaided (144). In this study no significant differences were observed between males and females or overweight or normal BMI individuals. Females also appear to have higher BW resistin level than males, however, this trend is not observed in the serum or the BAL. Overall, it is interesting to note that gender and BMI patterns were similar in BAL and BW samples for all adipokines measured. The potential reasons for this pattern will be explored further in discussion of the second hypothesis.

There are several explanations as to why significant patterns in line with literature and specific study hypotheses with regards to sex and BMI differences were not observed among this cohort. First and foremost is the small sample size of comparison that may not provide adequate power to significantly detect a

difference. There is also large variation in the data (as measured by standard deviations) reflecting the wide physiological range of normal adipokine values, making overall differences difficult to detect by unpaired *t*-test. Additionally, very few individuals in this study are clinically obese, and therefore differences in adipokine concentrations at the cut-off of 25 kg/m^2 may not be dramatic enough to be detected. There is also the limitation in the measure of BMI itself that can be a misrepresentation of actual body fat content, and therefore, may actually correlate poorly with adipokine measures (253).

It is interesting nonetheless to confirm that measured adipokine values are within the expected physiological range, and to see if accepted adipokine patterns are true in this study population. Overall, the differences according to sex seem to be somewhat more consistent and easier to detect statistically. This is likely due to the several limitations discussed above regarding BMI status determination.

5.3 Hypothesis 2: Lung Adipokine Responses

Overall, the literature regarding adipokine response following allergen exposure is complex. The responses that are observed to be consistent in animal models are not always so in humans. Additionally, the responses observed in each study may be relevant only to that particular study population, or are often measured only in the serum and not in the lung, the space that may be most relevant to measuring the response (195). Gender and BMI status may also have a role in altering the responses, making the study of adipokine responses even more complicated. However, this crossover exposure study provides a well-controlled and unique situation in which to assess pulmonary adipokine changes in response to allergen exposure, and potential diesel exhaust augmentation of sex and BMI modification of these responses. We hypothesized that allergen would change adipokine levels in the lung at 48 hours, and specifically that there would be an increase in adiponectin, leptin and resistin levels, and that diesel exhaust would further augment these effects. It was also hypothesized that there would be sex and BMI modification of these responses, although no directional hypotheses were possible. Adipokine responses 48 hours after the four exposure conditions were compared using mixed-effects models.

5.3.1 Exposure Effect

Allergen increased adiponectin concentrations in the lung, a change that lasted at least 48 hours post-exposure. In the BW and BAL samples adiponectin was increased after DEA and FAA exposure. An increase in lung adiponectin is aligned with our hypothesis, and what was expected from review of the relevant literature. A recent inhalational challenge study in a cohort of subjects with suspected Red Cedar Asthma, observed an increase in adiponectin measured in the lung 6 hours post-plicatic acid exposure (152). It is interesting to note however, that Biagioni et al. measured much larger changes in adiponectin from 36.4 ± 9.0

ng/mL after methacholine challenge up to 119.8 ± 40.3 ng/mL after plicatic acid challenge (152). A larger increase in lung adiponectin levels may have been observed if a 6-hour post-exposure lung sample was available for analysis in this study. However, it is also possible that the smaller adiponectin increases observed in this study are merely a consequence of allergen exposure, or due to other factors of study design. This is the only inhalational study in humans measuring adipokine changes in the lung that could be identified for comparison.

It has been suggested in mice models that adiponectin may have a protective role, acting to decrease allergen-induced responses (178), and that in humans, high serum adiponectin may serve as a protective factor against asthma development or prevalence (195,199). While these trends have been observed, there is very limited evidence as to the potential role or source of increased airway adiponectin levels. However, one interpretation is that among healthy individuals who are exposed to allergen, adiponectin levels are increased to limit local inflammation and prevent potential lung damage (254). It has been shown in mice models and *in vivo* and *in vitro* models that adiponectin may have this effect at a cellular by reducing alveolar activation (254), and by inhibition of pro-inflammatory mediators (173,174) and promotion of anti-inflammatory mediators (175). ADN deficient mice have been observed to exhibit greater levels of allergic inflammation and increased eosinophil and monocyte accumulation in the lung following ovalbumin challenge (179,254). Mice without adiponectin are also at greater risk of developing inflammatory diseases including diabetes and atherosclerosis (255–257). The increase in adiponectin levels observed in this study may be reflective of the responses that are expected to occur, and additionally are the responses that can become dysregulated in unhealthy individuals. For example, the result found that adiponectin is decreased in asthmatics during acute exacerbations may be a reflection of the disease state acting to perpetuate pro-inflammatory effects systemically and in the lung (197). Additionally, it has been found that adiponectin and its receptors are down-regulated in obesity, type 2 diabetes and cardiovascular disease indicating a predisposition towards pro-inflammatory responses (258). This idea that adiponectin may play a protective role against the harmful effects of allergy, and the progress of the "allergic march" will be discussed in more detail in the next section.

Allergen also increased leptin concentrations in the lung at least over the 48 hours following exposure. In the lung, leptin was hypothesized to increase after allergen challenge based on several epidemiologic and clinical studies cited in the methods section. Leptin was also observed to increase after FAA and DEA exposure in the BAL. This change could not be observed in the BW because the majority of data fell below the limit of detection. The increase in leptin is aligned with data from three studies chosen for the time frame of adipokine change, and also for the relevance of exposure, however, these changes were all measured in the serum. No relevant studies were found measuring leptin in the lung. Leptin is significantly increased during asthma exacerbation (197), and during allergic rhinitis symptomatic periods (205). These studies have time

frames that are suggestive of longer lasting leptin responses. Additionally, another study found a positive association between leptin and allergic sensitization in a healthy study population, a parameter within a population relevant to my study (238). It is not surprising that leptin, a pro-inflammatory mediator whose levels are increased in response to pro-inflammatory cytokines and whose levels measured in the lung have found to be correlated with airway reactivity (102), would be increased during allergen challenge even in healthy individuals. It may appear from the above adiponectin discussion, that leptin would not be expected to increase in healthy individuals. However, as will be discussed later on, it may be the relative levels of pro- and anti-inflammatory mediators that makes the most difference determining appropriate or dysregulated adipokine responses. This ratio is especially interesting in this study considering that both pro- and anti-inflammatory adipokines appeared to increase following allergen exposure, and it may instead be their ratio rather than their absolute concentrations that are of greater importance (259).

Exposure does not have a statistical effect on the last two parameters measured in the lung, resistin and the adiponectin/leptin ratio. Resistin does appear to be elevated by allergen especially in the BW, as would be expected from its role as a pro-inflammatory mediator. LPS has been observed to increase resistin gene expression in human macrophages, and like leptin, induces and is induced by pro-inflammatory cytokines (192,193). The adiponectin/leptin ratio seems to be elevated after DEA exposure relative to any other exposure. This observation was explored further when a potential effect modification by airway responsiveness was detected. This is a concept has physiological relevance and is supported by literature and for these reasons it was explored further, though significance was not achieved ($p = 0.06$), likely due to limited power. The investigation of lung responses was limited by leptin not being able to be measured in the BW samples. This limitation prevents further insight into the similarity in responses between the two different but related lung samples, and also limits the investigation of the adiponectin/leptin ratio that may respond differently to the combined DEA exposure versus any other exposures investigated.

5.3.2 Effect of Demographic Variables

We evaluated the effect of sex, BMI status and airway responsiveness on the impact of exposure on lung adipokines. No effect modification was observed, however, these variables had main effects on adipokine responses. Sex was significant in predicting overall BAL adiponectin, BW adiponectin and BAL leptin responses. This is expected because not only do baseline differences in these adipokines exist as was previously discussed often due to gender-related adipokine production (112,239–241,249), but also because many associations of adipokines and lung pathogenesis or respiratory disease are often found to be gender specific, especially in regards to asthma prevalence and severity (102,115,195–197). Airway responsiveness was significant in predicting BAL resistin responses ($p = 0.010$), with hyperresponsive individuals predicted

to have lower BAL resistin levels. This is an unexpected result as resistin has been found to be elevated in the serum of individuals diagnosed with moderate or severe asthma, many of whom likely have airway hyperresponsiveness (202). However, this result has not been confirmed in any additional studies, and may therefore not be representative of a consistent relationship. Additionally, serum measures may not align with what is occurring in the lung, and classification by airway responsiveness does not align exactly with asthma diagnosis. BMI status as a dichotomized variable with a cut-off of 25 kg/m^2 does not appear to have any effect on adipokine responses in this study.

Interestingly, the potential modification of exposure effect was found in the BAL ratio in which the effect of exposure was not statistically significant. By analyzing the responses stratified by other independent variables, additional exposure effects were revealed. A modest gender interaction with exposure on the adiponectin/leptin ratio was observed (data not shown). Upon stratification and data visualization, males appear to be driving the observed trend in the data. Something about the combined exposure of diesel exhaust and allergen in males uniquely increased this ratio over the effect of diesel exhaust alone. It could be that this effect is more noticeable in males because their baseline levels of adiponectin are much lower than females. It also could be that there is a physiological basis for this difference, but there is no literature support for this speculation. There has not been a lot of investigation into sex and BMI interactions within adipokine literature, and when they are investigated, no significant interactions have been found (147). Therefore, this finding is still relatively hard to explain in the context of what is currently known. Due to it's lack of significance and relatively unsupported physiological relevance this result could not investigated further in this study.

The investigation of sex and BMI status effects were limited by sample size. In some cases as few as six females and five males and six normal and five overweight BMI status individuals were being compared (in the BW resistin data set). It is valid to question whether these individuals are representative of what normal, healthy adipokine responses should be. Additionally, it would have been very valuable to confirm in the BW samples that male adiponectin/leptin ratio changes in response to DEA exhibit a similar pattern as in the BAL. This would help verify that this result reflects a physiological difference in males. This potential effect modification warrants further investigation in a larger study population.

5.3.2.1 Diesel Exhaust Effect

Diesel exhaust did not appear to have any independent effect on the endpoints studied and had only modest augmentation of the significant effects of allergen on adipokine responses measured in the lung. It was hypothesized that diesel exhaust could have an effect based on the allergen synergy literature that exists, and because of diesel exhaust's known effect on pro-inflammatory cytokines (including TNF-α), cytokines that

also influence adipokine concentrations (260,261). There are several reasons why this effect was not observed. One reason could be that the short-term nature of the exposure was not enough to elicit an adipokine response. Animal data has found that $PM_{2.5}$ or other forms of particulate pollution effect on adipokine levels. However, these effects only occur after sustained exposures in rodent models (180,181,191). So, it is possible, that only after long-term diesel exhaust exposure will an effect of diesel exhaust become clear. The alternative of this argument is that diesel is having an impact, but that the 48-hour time point misses the diesel exhaust augmentation occurring in the lung. The final potential explanation for this lack of diesel exhaust effect, relates to the exposure itself. Although the concentration of diesel exhaust exposure (300 $\mu g/m^3$) is comparable to many international controlled human exposure studies utilizing DE, the DE produced at APEL seems less oxidative than the DE used in other models (223), with a reduced capacity to generate reactive oxidative species (262). Therefore, it is possible that the DE exposure used in this study will not have the same augmentation effects noted in other studies.

Although there was no evidence of diesel exhaust augmentation overall, there was evidence of its potential effect in a subset of the sample population. The most interesting diesel exhaust effect was observed after a potential airway responsiveness interaction with exposure was identified ($p = 0.059$). It was not true, as we had originally assumed, that adipokine responses were the same regardless of airway responsiveness. Airway hyperresponsiveness to methacholine or other inhaled stimuli is thought to caused in large part by "fundamentally different inflammatory processes" (263) and therefore, it is conceivable that individuals with hyperresponsiveness would respond differently to allergen and diesel exhaust exposure, and that these differences would be detectable in adipokine responses.

In people with normal airway responsiveness, DEA exposure elevated the adiponectin/leptin ratio (2283 ± 1774) over the levels obtained after DES (880 ± 1034) and FAA exposure (764 ± 672) although these differences are not quite significant, as can be seen in Figure 4.7 the effect size as observed by large differences between the mean values, is noteworthy. This result was completely absent in individuals with airway hyperresponsiveness, whose adiponectin/leptin ratios were similar following all exposures. The increased prevalence of airway methacholine hyperresponsivesness in obese asthmatics, has linked the study of not only asthma and obesity, but also the role of adipokines in this relationship for over a decade (264–266). In mice, leptin infusion increased bronchial hyperresponsiveness in sensitized mice (103), while adiponectin, whose receptors have been identified in airway smooth muscle cells (113,267), acts to prevent bronchial hyperresponsiveness, and airway inflammation in general (178,268). These data are precisely in line with the response observed in this study. In individuals with baseline hyperresponsiveness, no relative increases in adiponectin relative to leptin were observed. This means that these individuals are without the protective effects of adiponectin in the airway and are therefore more prone to experience poor outcomes as

a result of DEA exposure. Conversely, in healthy individuals exhibiting normal airway responses, adiponectin was seen to increase relative to leptin, affording these individuals the anti-inflammatory benefits of adiponectin that acts to counteract the pro-inflammatory effects of leptin (182), thus potentially reducing any negative clinical outcomes following DEA exposure.

It is very interesting that the only endpoint in which airway hyperresponsiveness was seen to alter exposure effect was in this ratio measured in the BAL. Many are interested in the clinical relevance of the adiponectin/leptin ratio to predict various disease outcomes, including insulin resistance (169,269,270), metabolic syndrome (168–172) and even in distantly related inflammatory diseases such as pain severity in osteoarthritis patients (271). It would be worth investigating if adiponectin/leptin ratio measures in the study correlates with clinical symptoms, or lung function decrements experienced by participants following the different exposure conditions. Although no lasting negative outcomes are expected as a result of this study's exposure, this observed difference in ratio response can promote pro-inflammatory and hyperresponsive airway responses, making certain individuals more prone to developing asthma and other inflammatory lung phenomena following repeated, long-term exposure. In the context of this study, the combined exposure of DEA reveals a potentially adaptive adipokine pattern in the BAL, supplementing and building upon previous animal data.

To determine the sample size that would have shown a statistical difference between the BAL ratio following DEA versus FAA exposure, a power calculation was performed. To detect a statistical difference using a two-sided paired t-test, 16 subjects would have been required, an addition of data from three subjects. Therefore, with minimal additional data, a significant effect of co-exposure on the adiponectin/leptin ratio would likely be observed.

5.3.2.2 Sustained Changes in the Lung

This is the first human exposure study to measure adipokine changes in the lung lasting on the order of days. Other studies are relevant in predicting directional changes, however, the time course under study is generally focused on short-term adipokine responses in the serum. The 48-hour time point mirrors data from mice studies, in which sustained adipokine changes in the BAL fluid have been observed (178). It is important to note that adipokine changes lasted at least 48 hours in our study, but may even extend beyond the scope of the observation period.

The measure of these changes in both BAL and BW samples allows for a more complete picture of change. It is intuitive that the trends for adiponectin in the BAL and BW are very similar, as the portions of the lung sampled are physiologically related and connected. The first 40 cc collected from the lung, the BW sample

measures the conducting airways while the BAL is the sample from the following 100 cc measuring the contents of the alveolar spaces and mucosa of the distal conducting airways (272). The larger volume of saline used to collect the BAL samples may be expected to further dilute these samples (273), suggesting that the adipokine concentrations in the distal airways are more concentrated than in the conducting, or mid-order airways. Although not a focus of this study, this may have implications for adipokines relative effect on asthma prevalence or progression, a disease in which responses in the mid-order airways are thought to be critical, verses COPD, in which the distal airway responses are more relevant. The dilution of sample during bronchoscopy collection is a noted limitation of these procedures (273), and it is possible that adipokine concentrations in the lung are, in reality, much higher than the concentrations reported.

5.4 Hypothesis 3: Systemic Adipokine Responses

The majority of studies in the adipokine research field have focused on systemic adipokine responses in various populations, in response to various phenomena. Although these measures are not as relevant to allergen exposure occurring in the lung, it would not be complete to study adipokine responses without also measuring the change in the blood. Additionally, these exists some evidence suggesting that inhaled inflammatory stimuli will alter systemic inflammation as well, and therefore may impact adipokine release. This has been shown in animal models (177,178,181,191,194), but is not always observed in clinical studies (152,204). However, one study found evidence of both systemic and nasal inflammation 24-hours following segmental allergen challenge in the lung (274). It is conceivable therefore, that allergen challenge in the lung could affect serum adipokine levels.

We measured adipokine concentrations in serum samples at baseline, 4, 24 and 48 hours post-DEA and post-FAA exposure to see not only the effect of exposure, but also the time course of adipokine response. It was hypothesized that serum adipokine changes would be seen to change acutely (within 4 hours) and return to baseline within 24 hours. It was hypothesized that leptin would decrease, adiponectin would increase acutely, resistin would increase, and that the adiponectin/leptin ratio would increase. It was hypothesized that diesel exhaust would augment the effect of allergen, causing larger changes in the directions noted above. It was hypothesized, for the same reasons as those noted in the lung, that sex and BMI status would have an effect modification role in these responses. Serum adipokine responses over the 48 hours following FAA or DEA exposure were compared using mixed effects models.

5.4.1 Exposure Effect

In the serum, because the effect of exposure was tested at several time points, the effect of exposure is closely linked to time, therefore the effect of time will also be discussed here. Adipokine concentrations changed

significantly over time, however, the curves for FAA and DEA were almost identical indicating that diesel exhaust had no additional effect. Nevertheless, changes in adipokine concentrations were detected following segmental allergen challenge, and several explanations may be attributed to causing these changes, one of them being allergen exposure itself.

5.4.1.1 Cause of Adipokine Changes Over Time

Leptin was seen to decrease acutely within 4 hours to 9.67 $\pm$ 8.38 ng/mL from 14.5 $\pm$11.6 ng/mL at baseline. This is in line with our hypothesis that was based primarily on the aforementioned study by Sood et al. (204) that observed an immediate decrease in leptin levels following either methacholine or allergen exposure in asthmatics or controls. However, unlike this study and contrary to our hypothesis, leptin levels did not return to baseline within 24 hours or 48 hours (see Figure 4.7). Although it has been demonstrated that serum leptin concentrations may take several weeks to return to baseline during COPD exacerbations (275–277), such responses would not be expected in healthy individuals. One explanation of this result could be because the actual time that serum samples differs between baseline (average 7:31am), 24 hours (average 11:47 am) and 48 hours (average 9:36). Therefore, samples may have been taken during slightly different phases of leptin diurnal variation. See Appendix Table A.2 for this detailed serum collection data. Conversely, resistin levels increased acutely within 4 hours to 12.6 $\pm$ 4.57 ng/mL from baseline levels of 9.95 $\pm$ 2.51 ng/mL, and returns to baseline values at 24 hours as was hypothesized. This hypothesized increase was based only on a study that found elevated plasma resistin levels in asthmatic patients over controls (202), somewhat confirming its role as an pro-inflammatory mediator (278,279). Therefore, in response to a pro-inflamamtory stimulus resistin is expected to increase (146,192,193). This result has even been confirmed in a cohort of healthy individuals exposed to LPS (147), a study population highly relevant to this one. This study also observed a transient increase in resistin from 4 hours, up to a peak concentration at 8 hours, with a return to baseline within 24 hours (147), confirming what was observed in this study.

There are several possible explanations explaining serum adipokine changes, because the study conditions affecting the serum samples are less well controlled than those affecting the lung samples. These include natural diurnal variations, food intake that could affect leptin and resistin levels, or finally the exposure condition as was the goal of the study to determine. Diurnal variations are natural rhythms in adipokine levels that occur on a daily cycle, and cannot be controlled by study design. These diurnal cycles predict that leptin would reach its lowest concentrations between 8:00 am and 5:40 pm (137). Because a decrease is observed in leptin concentrations following exposure at approximately 10:00 am, it is very likely that natural rhythms are driving this change. This was also the conclusion reached by Sood et al after response curves were very similar for relevant and irrelevant allergen exposures, regardless of asthma status (204). Additionally, because

leptin was found to decrease, a directional change that is contrary to data from epidemiological (200,205) and animal studies (103,182,183) that would predict an increase in leptin, it is likely that this change is not occurring because of allergen exposure. Resistin diurnal variations have not been determined in humans, however, mRNA levels measured in mice suggest that resistin levels follow adiponectin rhythms (148). The pattern observed for resistin does not align with observed adiponectin patterns that would predict a peak in late morning followed by a slight decrease in early afternoon (118).

Because this study was designed to investigate other endpoints, fasting status of participants was not rigidly controlled. Subjects were fasted before bronchoscopy procedures, so samples taken at baseline and at 48 hours were under fasting conditions, however samples taken at 4 hours and 24 hours could be affected by eating. It is likely that subjects would have eaten 2-3 hours prior to the blood sample taken at 4 hours. Both leptin and resistin are expected to increase upon feeding (135,149), however the direction of change at 4 hours is opposite indicating that eating is not likely affecting this response change.

Finally, allergen could be driving the observed changes in leptin and resistin. Because allergen is affecting both the exposure curves, and because no comparison to saline exposure alone can be made, it is virtually impossible to confirm that allergen is truly causing this change. For leptin, it is likely diurnal variations and menstrual cycle effects driving the changes observed. For resistin, because the observed transient increase is in line with limited literature, and because the changes observed don't follow the reported diurnal variation, it is somewhat more plausible that these changes are occurring due to allergen exposure. However, further study under a more controlled fasting regime, and with available comparisons to a baseline exposure (FAS) are required to confirm this result.

5.4.1.2 Diesel Exhaust Effect

As in the lung, no additional effect of diesel exhaust was observed. The changes following DEA and FAA exposure showed nearly identical results. Because the time of measurement is much sooner after exposure, missing the potentially acute diesel exhaust augmentation of allergen effect is not a viable explanation in the serum. The remaining reasons stated above about why the hypothesized effect of diesel exhaust was not observed are also relevant in the serum data.

5.4.2 Effect of Demographic Variables

As in the lung, sex, BMI status and airway responsiveness were also evaluated for their overall effect, or for their modification of exposure effect. No effect modification of exposure effect by these variables was observed. As in the lung, sex was significant in predicting leptin responses in the serum, with females having

higher levels than males ($p = 0.008$). Airway responsiveness was significant in predicting serum resistin responses ($p = 0.014$). As was found in the BAL resistin data, those with normal airway responsiveness were predicted to have higher resistin levels than those who are hyperresponsive. It is interesting that this result was replicated in both BAL and serum resistin data. This may provide evidence supporting the alternate direction of resistin association with asthma than was reported by Larochelle et al. (202). It in interesting that serum and lung patterns according to sex and airway hyperresponsiveness, and lack of patterns due to BMI status appear to be consistent.

5.5 Hypothesis 4: Lung and Serum Comparisons

This study provides the beneficial ability to compare adipokine changes in the lung and serum in an effort to understand a more complete picture of adipokine responses. The relationship between systemic and airway inflammation especially in the context of obese individuals remains an important question and a controversial issue in the study of adipokines' role in inflammatory airway pathogenesis and disease (280). Several mechanisms have been hypothesized by which adipokines may have this role, and it is only through studies in which both can be studied by which some insights may be gained. Although changes in airway and serum responses cannot be directly measured in this study, correlations between the two measures after exposure may provide some further clarification.

5.5.1 Correlations of Lung and Adipokine Measures

It was hypothesized that leptin in the serum and in the lung would be correlated 48 hours following FAA exposure. Several studies have found consistent correlation between airway and serum leptin measures in both asthmatics and controls (114), hypothesized to be because leptin can pass freely into the lung, without the need for transportation methods. This is likely due to the fact that leptin does not form multimers and circulates either in a free form, or in a bound form, bound to its soluble receptor (281). A strong, positive, statistically significant correlation was found between serum leptin and BAL leptin measures again confirming what has been found in other studies. No correlations were found between the other measures, again confirming that adiponectin and resistin likely require more sophisticated methods for being transported in the lung, or in the case of adiponectin, is produced in the lung somewhat independently of the serum. It is important to note that the leptin correlation is unadjusted for sex and BMI status. Gender significantly effected both BAL and serum leptin responses, so it is conceivable that this strong correlation may be somewhat diminished by adjustment. An adjusted analysis will be run in the future, correlating the residuals from mixed-effects models that control for all demographic variables. This analysis was not complete in time to be included in this document.

5.5.2 Mechanisms of Adipokine Increase in the Lung

Sustained, and consistent increases in both adiponectin and leptin were seen in the lung as a result of allergen exposure. These results were obtained in spite of unclear adipokine results in the serum. Although perhaps no clear conclusions can be made about how this change occurred, it is necessary to think about mechanisms that may have caused the observed results.

To facilitate this discussion, it is necessary to know if there is any change occurring in serum adiponectin levels. Data from the first triad was analyzed for any exposure or time effects, realizing that only between-subject comparisons can be made at the expense of statistical power. Mixed effects models showed no significant time ($p = 0.096$), or exposure ($p = 0.195$) effects. This data is depicted in appendix Figure B.1. Therefore, no confirmation of change in serum adiponectin concentrations over time as a result of allergen exposure can be obtained. And although serum leptin and resistin were observed to change over time, it is unclear whether this change is due to natural diurnal variation or allergen exposure.

Therefore one hypothesis could be that local production of adipokines may occur in the bronchi or peribronchial fat, for which there is evidence. For adiponectin, that requires specific translocation processes for entry into the lungs, a large increase in serum levels at 24 and 48 hours post exposure would have to have accompanied this change. Therefore, it is believed that the adiponectin increase in the lungs is not merely a reflection of serum adiponectin changes. This was also the conclusion made by Biagioni et al after they found increased adiponectin levels in the sputum without accompanying changes in the serum (152). In contrast, leptin concentrations do change over time in the serum, and remain somewhat depressed below baseline. It is possible that because leptin is suggested to be able to passively diffuse between the serum and the lung (114), that this decrease is a result of leptin movement from the blood into the lungs. It has also been suggested that this diffusion can occur readily during pulmonary inflammation due to increased microvascular permeability during these events (103). Therefore it is possible that leptin can pass easily into the lungs during the initial inflammatory events, but its passage back into the serum may be more difficult once microvascular permeability starts to decrease some time after the initial allergen effect is over. The transport of resistin into the lung is less well characterized, but it is known that resistin forms higher order multimers in circulation, as does adiponectin (144). This structure may impede its movement into the lung. And as serum resistin levels return to baseline at 48 hours, it is unlikely that the elevation of BW resistin is due to translocation from the blood, and it is possible that local production by monocytes/macrophages in the lung caused this increase (144,147,182).

Another possibility is that a feedback mechanism between systemic and pulmonary inflammation is occurring that is not directly mediated by adipokines. Some believe that the initial local allergic reactions taking place in

our case in the lung, cause systemic inflammatory events that can both potentiate the original reaction, and cause distant local reactions (282). This feedback mechanism, an idea also presented in the introduction diagram (100), could be mediated by other systemic cytokines and immune cells , and could be in this study an unmeasured cause of increased adipokine responses in the lung. This suggested late-phase perpetuation of allergic responses also would explain why these adipokines remain elevated even at 48 hours after exposure. It would also be interesting to see if there are other distant manifestations of adipokine changes occurring in the body, for example in the nose, or in the adipose tissue.

5.6 Biological Relevance of the Observed Adipokine Changes

It is important always to be able to distinguish between statistical and biological or clinical significance of results. Many statistically significant results were obtained in this study likely afforded by the powerful mixed effects model that accounted for within-subject variability and the random subject effect. In looking at the effect estimates obtained from the mixed effects analysis, most in the lung are between 2.5 and 6 units of the response variable (in most cases ng/mL). Adipokine concentrations in the lung are significantly lower than in the serum, and therefore these effect estimates indicate a biological significance of the effect of allergen in the lung. Similarly, the effect estimate of airway responsiveness was 4.40 ng/mL, a change likely to be physiologically relevant. This physiologic relevance is illustrated in the Holguin et al. study in which significant differences in BAL leptin levels were found between lean, overweight and obese asthmatics with median (interquartile range) ng/mL values of 0.3 (0.15 – 0.27), 0.7 (0.3 – 1.3) and 2 (0.3 – 5) respectively (114). These differences are within the absolute range of changes we saw due to exposure and airway responsiveness. The effect of sex in predicting overall adiponectin and leptin responses in the lung has very small effect estimates (between 0.2-0.3 units of response variable) indicating a biologically insignificant result. Similarly in the Holguin et al. study, BAL adiponectin differences amongst lean, overweight and obese subjects was not found to be significant, with median (interquartile) ng/mL values of 0.2 (0.2 – 0.3), 0.1 (0.07 – 0.2) and 0.06 (0.03 – 0.2) respectively (114).

Results in the serum had ~1 ng/mL or ~1 pg/mL effect estimate magnitudes. Serum adipokine concentrations were much higher than in the lung, and vary over wider ranges. Therefore, changes of 1 ng/mL are relatively insignificant and unlikely to have any biological relevance. Similarly, airway responsiveness had a predicted effect estimate of 1.3 ng/mL, a change not likely to be physiologically relevant. This conclusion is illustrated in the fact that Biagioni et al. did not find significant change in serum adiponectin concentration after plicatic acid exposure, with an observed increase from 11.02 μg/mL to 11.96 μg/mL (152). Therefore, the differences detected in serum leptin and resistin concentrations are likely not as dramatic or biologically relevant as the changes observed in the lung

Chapter 6: Conclusions

6.1 Place in Adipokine Literature

This study provides adipokine response data from a crossover, controlled human exposure context. To our knowledge this is the first investigation of its kind. It exists in a field in which a paucity of human exposure studies and interventional studies of adipokine responses exist. Adipokine study has been dominated thus far by epidemiological studies investigating associations between adipokine measures and disease outcomes, with a dearth of proper longitudinal studies with the ability to determine causality (187). There have been a few studies to date involving allergen challenge in human populations, and even fewer that use segmental allergen challenge to exposure to lung specifically to allergen. This study is also unique is that it allows for individual differences to be controlled internally because of the crossover study design, and it investigates the potential augmentation of allergen effect by diesel exhaust using a co-exposure model. The study population is also quite unique within the adipokine literature. The study population consists of atopic individuals, allowing for the inflammatory effects of allergen to be observed without the effects of complicating underlying conditions, like obesity, asthma or other metabolic syndrome. And, perhaps more importantly, this study allows for responses among healthy individuals to be observed that can perhaps serve as a benchmark against which future studies can be compared. Finally, measurement of adipokine responses both in the serum and in the lung provides additional insight into the study of adipokine responses that is not often seen among adipokine studies.

6.2 Strengths

In general, human crossover studies have several strengths. These include internal control of individual confounding factors, and the provision of biological plausibility as well as confirmation of a temporal sequence of exposure causing adipokine responses. Although epidemiological studies can identify associations at a population level in large study populations, human controlled studies are necessary to provide mechanistic evidence that can explain such associations. Our study provides a novel method of allergen challenge (bronchoscopy with segmental allergen challenge) that hasn't been used within the human air pollution exposure context before. This allowed for two lung samples comparing exposure + saline and exposure + allergen to be obtained for each bronchoscopy procedure, and allows for insight into the effect of co-exposure on adipokines. Additionally, broad inclusion criteria allowed for the results to be more generalizable to a healthy, atopic population.

6.3 Limitations

As with all human controlled exposure studies, sample size and statistical power is a major limiting factor. This is especially true in this study when effect modification by sex and BMI status further reduced the sample size of the groups being compared. There are several other study design limitations that exist within the serum data that occurred because this study was not designed to study adipokine responses specifically. These include the control of fasting status, menstrual cycle timing, and exact time of blood draws. These inconsistencies prevent solid conclusions regarding the effect of inhaled allergen on systemic adipokine responses.

The two significant occurrences of carryover effect that were observed in the serum adipokine data, and the large number of nondetects in the BW leptin data, limited the comparisons that could be made between the BAL and BW, and between the serum and lung samples. This reduced the confirmation of trends in the data, and somewhat reduces confidence in the results obtained. Additionally, the fact that the BAL leptin data set had 20% of its values under the LOD is a possible limitation. However, because the values under the LOD were consistently obtained following DES or FAS exposure increases certainty in the conclusion that allergen exposure is associated with greater concentrations of leptin. Finally, multiple comparisons is always an important limitation to consider, especially when a large number of statistical comparisons were calculated. Many of these comparisons were not independent, and post-hoc pairwise analyses were limited to statistically significant fixed effects only.

6.4 Suggestions for Future Research

There are several avenues that could be explored to gain further insight into adipokine responses within a healthy study population. First, adipokine responses at the protein level should be explored. Immunohistochemistry has been performed for RELM-beta in the context of a human controlled exposure, however, this group did not look at adiponectin, leptin or resistin responses (222) for which there exist several commercial antibodies, and well-defined staining protocols. Biopsy samples also collected at the 48-hour time point could be stained for these same three adipokines to provide further confirmation of the responses identified by ELISA. Additionally, measurement of adipokine responses in the sputum samples could also confirm the patterns observed in this study. The changes in the different isoforms of adiponectin could also be explored in the BAL and BW. The high molecular weight form may have more biological relevance than the measurement of total adiponectin. This could be easily accomplished by using commercial ELISA kits (ALPCO or R&D Biosystems) that specifically measure certain forms of adiponectin.

To address the effect that these adipokine changes may be having on clinical outcomes, there are other data sources available in this study that could also be used. For example, various cytokine measurements and cell counts have already been collected, and could be correlated with adipokine levels to identify associations between pro- and anti-inflammatory markers. Of particular interest is the adiponectin/leptin data measured in the BAL. To determine possible clinical significance of this data, associations between ratio measures and lung function decrements, dose response slopes or symptom data would be extremely interesting to further define the importance of this potentially adaptive adiponectin increase relative to leptin.

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Appendices

Appendix A Study Procedure Details

A.1 Detailed Participant Data

Individual clinical participant data is shown below in Table A.1.

Table A.1. Individual participant data at baseline.

Subject ID	Age (yr)	Sex	Weight (kg)	Height (cm)	BMI (kg/m^2)	PC_{20} (mg/mL)	Airway Responsiveness (Y/N)	Allergen Exposure
37	22	F	70	175	22.9	>16	N	Birch
52	22	F	65	165	24	3.5	Y	Timothy grass
53	20	F	54.6	158	21.9	13.9	N	House dust mite
58	31	F	70.1	173	23.6	>16	N	House dust mite
70	24	F	65.8	155	27.6	>16	N	House dust mite
81	32	M	67.6	161	26.1	>16	N	Timothy grass
90	34	F	55	157	22.3	0.2	Y	Timothy grass
94	27	M	70.4	178	22.2	>16	N	House dust mite
96	25	F	73.2	173	24.5	>16	N	House dust mite
103	46	M	97.2	155	40.5	0.3	Y	Birch
105	27	M	85	186	24.6	>16	N	Pacific grasses
106	46	F	65	165	23.9	>16	N	House dust mite
107	20	F	50.2	162	19.1	2.1	Y	Pacific grasses
109	31	F	49.6	146	23.4	0.3	Y	Birch
112	28	M	89.5	176	28.9	>16	N	Pacific grasses
114	23	M	83	169	29.1	2.4	Y	Pacific grasses
115	23	F	96	172	32.4	>16	N	House dust mite
116	23	M	74	175	24.2	>16	N	House dust mite

A.2 Time of Blood Collection

Time of individual blood collection for each participant over the two exposure triads is shown below in Table A.2.

Table A.2. Exact time (24-hour time), average over each triad, and overall average time when blood was collected for each participant and time point.

Subject ID	Triad 1				Triad 2			
	Baseline (-4h)	+4h	+24h	+48h	Baseline (-4h)	+4h	+24h	+48h
37	7:55	16:05	11:30	10:13	8:05	15:45	11:30	10:05
52	7:47	16:50	11:11	10:00	7:55	16:15	11:14	11:15
53	7:17	16:38	11:40	9:15	5:11	14:35	9:55	8:35
58	7:40	16:20	12:30	9:40	7:15	15:43	12:05	11:10
70	4:50	14:30	10:05	7:35	5:11	14:20	10:00	8:22
81	8:40	16:30	11:30	10:20	8:28	15:50	11:05	9:55
90	7:34	15:50	12:35	8:18	7:40	15:45	11:43	8:05
94	7:30	16:10	11:12	9:51	7:30	15:48	11:20	9:35
96	7:30	16:12	10:33	9:38	7:41	15:31	11:47	9:50
103	7:55	16:10	12:15	9:40	7:40	15:30	12:20	9:30
105	7:37	15:55	12:17	9:30	7:30	16:00	12:15	9:20
106	7:15	16:00	13:05	9:15	7:55	15:55	13:20	9:07
107	–	–	–	–	–	–	–	–
109	7:40	15:55	12:25	9:55	7:30	15:55	12:15	9:28
112	7:20	16:02	12:00	9:18	8:00	16:00	12:28	9:35
114	8:25	15:55	12:20	9:30	7:40	16:00	12:07	9:50
115	7:33	16:10	12:00	9:32	7:25	16:15	12:10	9:40
116	7:30	16:15	11:20	11:45	7:30	15:55	12:10	9:45
Triad Average	**7:31**	**16:05**	**11:47**	**9:36**	**7:25**	**15:42**	**11:02**	**9:35**
Overall Average	**7:28**	**15:53**	**11:24**	**9:35**	**–**	**–**	**–**	**–**

A.3 Adipokine Protein Stability

To test the stability of leptin, a freeze-thaw experiment was performed. This experiment was performed due to concerns for sample integrity after the -80°C freezer where the samples were stored broke down overnight causing the temperature to rise over a period of several hours. The PBS control sample spiked with 100 pg/mL leptin, and serum, BAL and BW samples were thawed to aliquot and re-frozen prior to the start of the experiment. Samples were left at room temperature for 0, 6, 12 and 24 hours before they were re-froze and later assayed. The percent change in leptin was calculated compared to the value obtained in the 0 hour

sample and are shown below in Figure A.1.

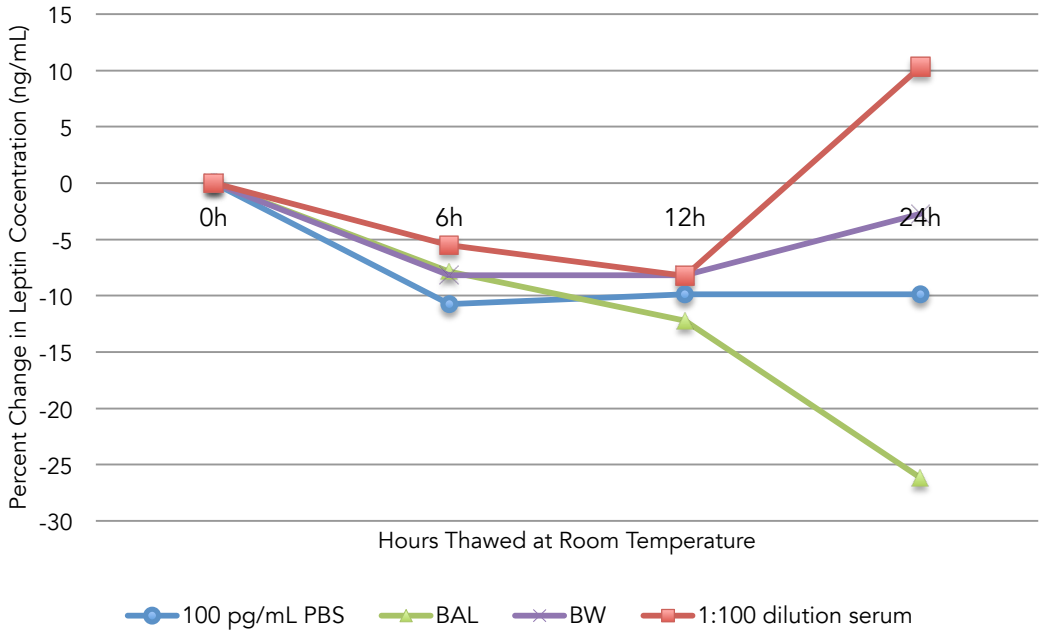


Figure A.1. Percent change in leptin measured in 4 different samples as compared to the 0 hour sample after 6, 12 and 24 hours of thaw at room temperature.

The percent change after 6 and 12 hours were similar and quite consistent for all types of samples. At 6 and 12 hours all samples dropped between 5 and 10%. The data measured at 24 hours are much less consistent. Leptin concentrations in BAL appeared to drop down to 25% of the original concentration. Concentrations in the BW and serum appear to increase after 24 hours. The stability of leptin appears to be fairly robust, and was unlikely to be largely altered when the freezer slower warmed overnight, as the temperatures were unlikely to reach room temperature. Additionally, the decreases in all sample types are fairly consistent so all samples were likely to be equally affected. It is of note that concentrations of 64.1 pg/mL was measured in the 0 hour PBS sample spiked with 100 pg/mL leptin. It is possible that the ELISA method is not measuring the true concentration of leptin, or that the PBS sample was most affected by the initial freeze-thaw causing a loss of protein.

A.4 Sample ELISA Calculation

For serum adiponectin quantified using the R&D Systems Human Total Adiponectin/Acrp30 Quantikine ELISA kit, the following procedures were used to calculate adiponectin concentration:

1. The 540 nm optical density values were subtracted from the 450 nm values.

2. The average of the zero standard (0.0085 ng/mL) was subtracted from every 450 nm value.

3. A standard curve of the averaged log O.D. values against the log adiponectin standard concentrations (log of 0, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250 ng/mL) was plotted. A linear line of fit was plotted through the data, as is shown with its equation and R^2 value in Figure A.2.

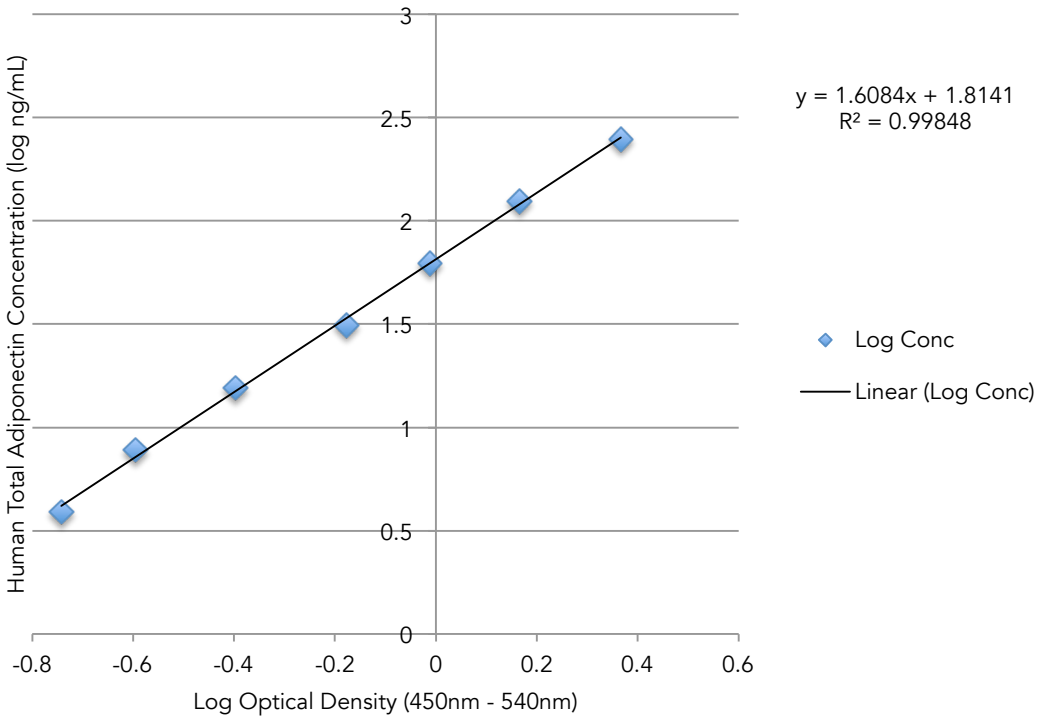


Figure A.2. Serum adiponectin standard curve with a linear line of best fit.

4. The log-450nm values obtained for each sample were plugged into the x values in the equation: y = 1.6084x + 1.8141. The value obtained above x 100 (dilution factor) was applied to each value to obtain the log serum adiponectin concentration (log ng/mL). 10 to the power of this value was used to calculate the final serum adiponectin concentration (ng/mL).

A.5 Distribution of Dependent Variables

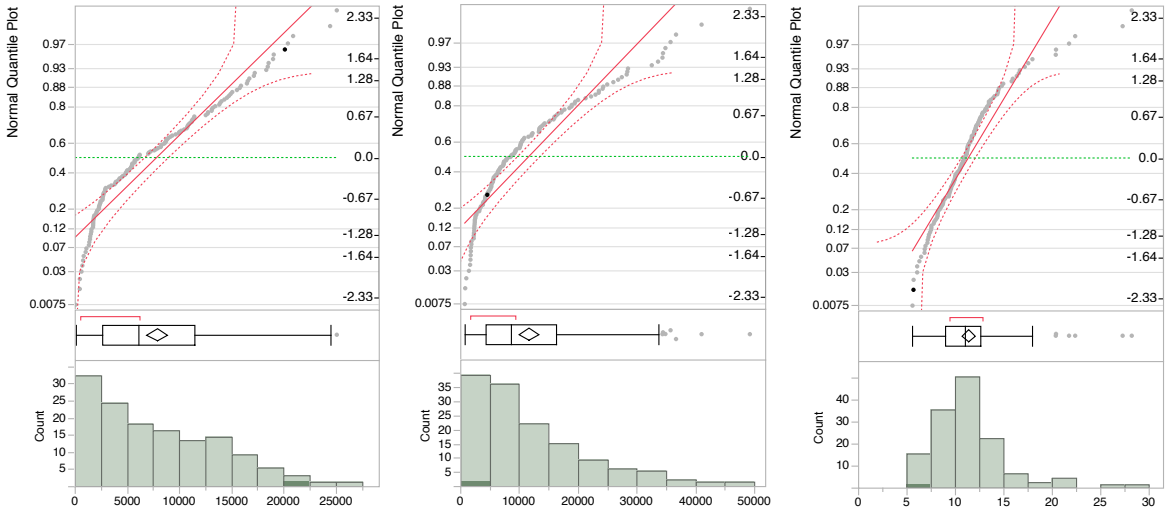
As was discussed in the main text, several considerations went into determining if the nine dependent adipokine variables had lognormal distributions, and if transformation using the natural log (ln) was an appropriate method for approximating normal. These data presented in the appendix support the data in Table 4.3. First, the histograms and normal quantile plot of each raw data set were examined, and are

included in this appendix. Several descriptive statistics were also used to make this decision including: the mean, median and mode, the standard deviation, the geometric mean and the geometric standard deviation. Two statistical tests were also used including: the Kolmogorov-Smirnov test for lognormality (used on un-transformed data) and the Shapiro-Wilk test for normality (used on ln-transformed data). Each piece of data observed will be listed below and numbered from 1-4.

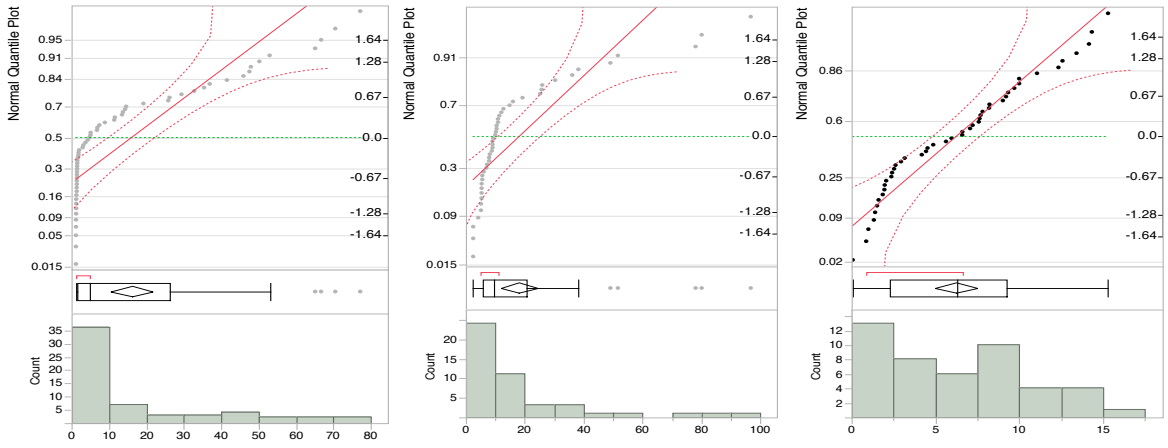
1. Histograms and normal quantile plots of untransformed data:

The histograms and normal quantile plot of each independent variable's raw data are shown below in Figure A.3. These histograms do not include values below the LOD.

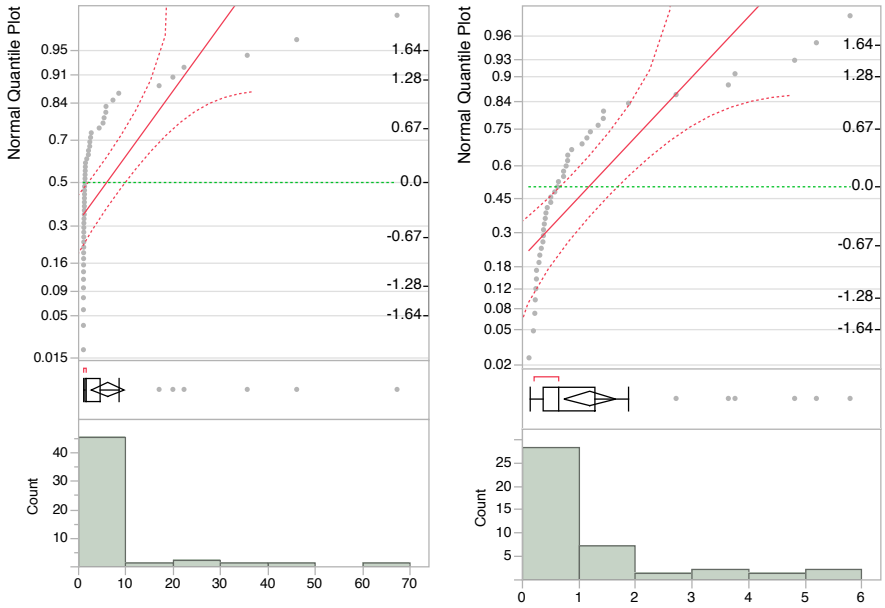
A) Serum (From left to right: Adiponectin, Leptin, Resistin)



B) BAL (From left to right: Adiponectin, Leptin, Resistin)



C) BW (From left to right: Adiponectin, Resistin)



D) Adiponectin/Leptin Ratio (From left to right: BAL and Serum)

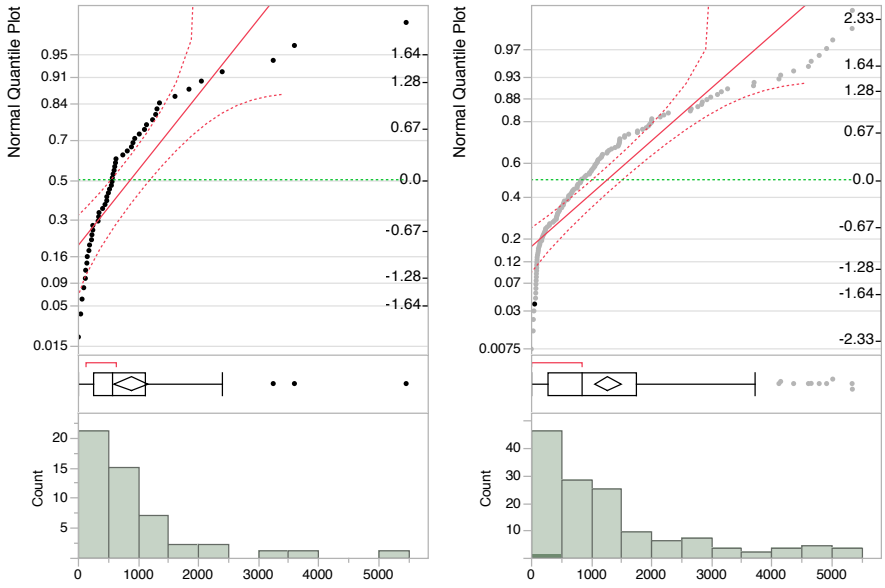


Figure A.3 Histogram and Normal Quantile plot of untransformed data for each adipokine variable.

As can be observed from the histogram and Normal Quantile plot visualization of the data, most of the distributions are highly "skewed right" with the majority of values falling close to zero, and the tail of the distribution (higher values) stretching towards the right side of the graph. As can be seen on the Normal

Quantile plots, most of the data sets vary from the straight line (indicating a normal distribution) with points at the bottom and top of the distribution deviating the most from normal. Serum resistin and BAL resistin are the only data sets whose points fall somewhat on the normal line, while the rest appear to be highly skewed.

2. Mean, median, mode:

In a normal distribution, the mean, median and mode are identical and can all serve as a measure of central tendency. However, in a skewed distribution, this is not true. In a skewed distribution, the mode, median and mean fall from left to right along the curve (mode < median < mean). Because most of the dependent variables didn't have a mode, however, the median and mean were compared. In all data sets, except for BAL resistin, the mean was much greater than the mode. The mean and median were very close in the serum resistin data set (11.4 ng/mL and 11.0 ng/mL). Again this indicates that the majority of data are highly skewed.

3. Geometric standard deviation (s_g):

The geometric standard deviation is perhaps the most widely used parameter to assess the distribution of the data. The natural logarithm was taken of each variable. The log standard deviation was exponentiated to calculate the geometric standard deviation of each variable. For some context, normal distributions generally have $s_g < 1.5$, while data collected in the field of hygiene (worker exposure sampling) have s_g values that range from 1.2 up to 10, but most consistently fall within the range of 1.5 to 4.5 (34). The s_g of the adipokine variables ranged from 1.35 (serum resistin) up to 4.35 (BAL adiponectin). From examination of the s_g only the serum resistin data set appears to be normally distributed, while the rest are likely log-normal.

4. Comparison of geometric mean and median:

In a perfectly lognormal distribution, the geometric mean and the median from the untransformed data will be approximately equal. These two values have similar values in all the adipokine data sets, with the notable except of the BAL resistin and the serum adiponectin/leptin ratio data in which these values are quite different.

5. Statistical tests:

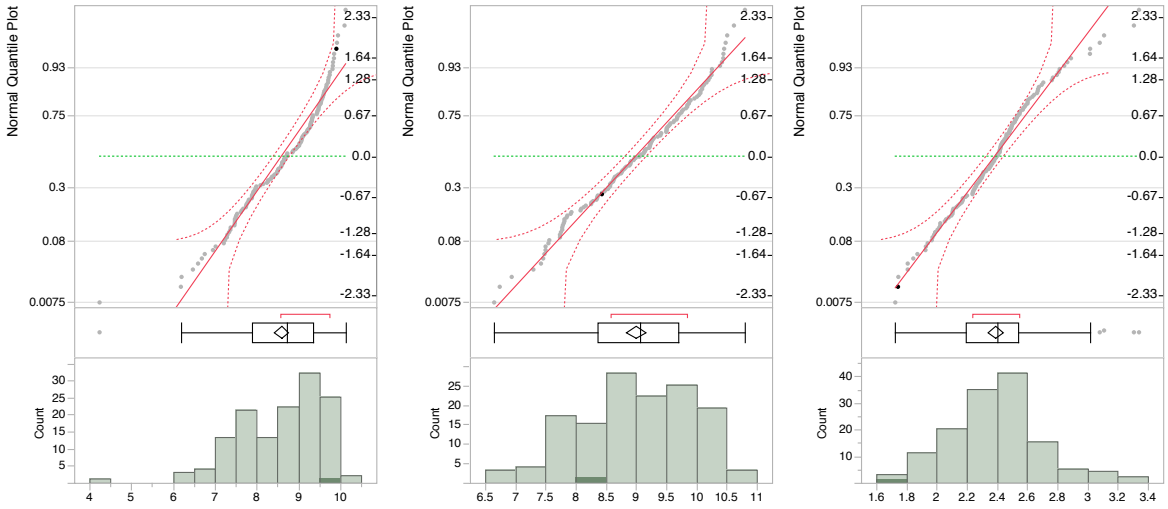
The Kolmogorov-Smirnov test was used to assess if untransformed data had a lognormal distribution, and the Shapiro-Wilk test was used to assess if transformed data approximated a normal distribution. Although not used as an absolute test (because often the distributions failed both tests), these tests were compared and used as further indication of the distribution's character. Some variables were determined to be from a

lognormal distribution (p-values >0.05 that don't reject the null hypothesis), but most had p-values less than 0.05. However, when a Shapiro-Wilk test was used on untransformed data, this hypothesis was more strongly rejected in most cases (lower p-value). Again, most variables failed the Shapiro-Wilk test for normality once they were log-transformed, but most had higher p-values then when the Shapiro-Wilk test was used on untransformed data.

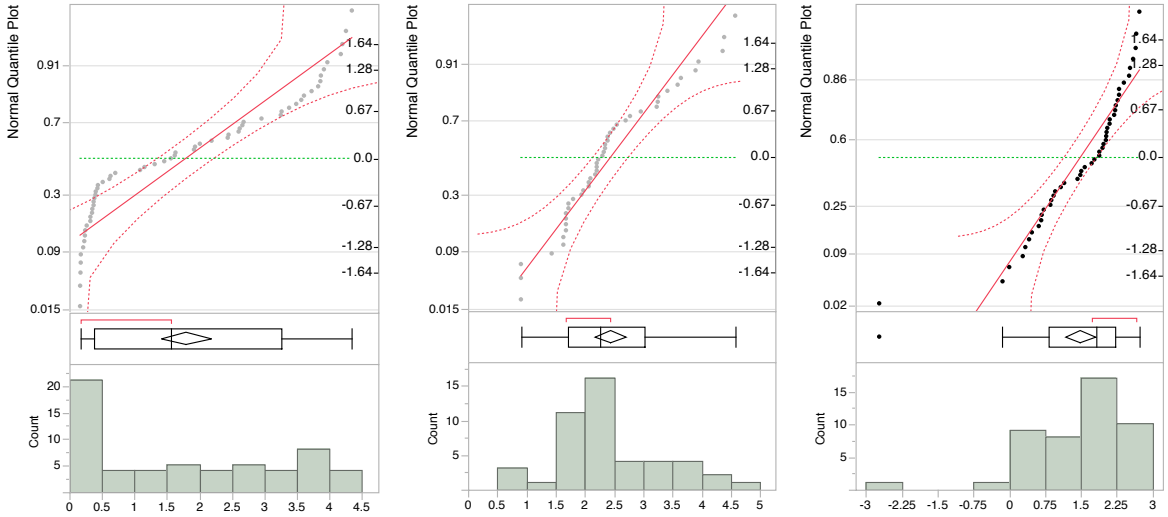
6. Histograms and normal quantile plots of log-transformed data:

Finally, the histograms and the normal quantile plots of the log-transformed data were viewed to see how well the transformed distributions approximated normal. These can be seen below in Figure A.4.

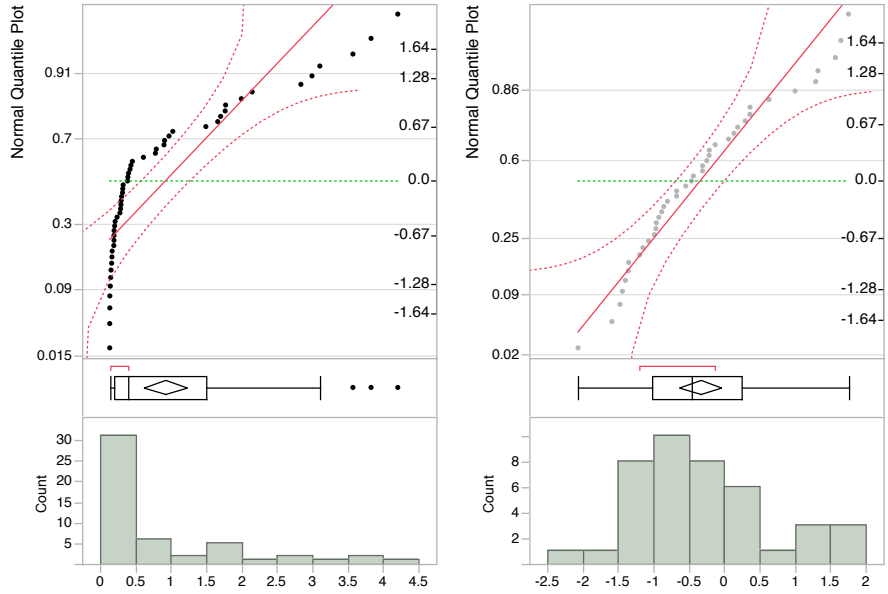
A) Serum (From left to right: Adiponectin, Leptin, Resistin)



B) BAL (From left to right: Adiponectin, Leptin, Resistin)



C) BW (From left to right: Adiponectin, Resistin)



D) Adiponectin/Leptin Ratio (From left to right: BAL and Serum)

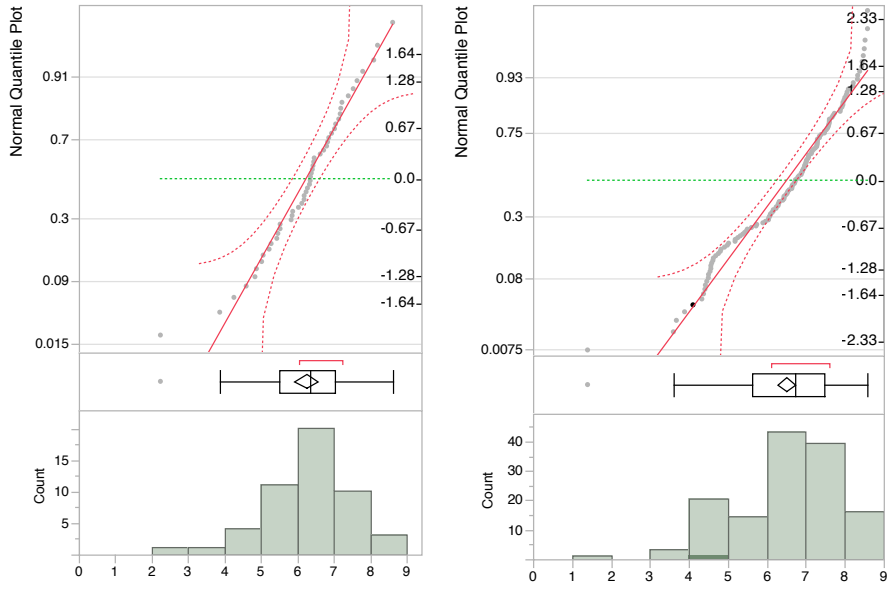


Figure A.4. Histogram and Normal Quantile plot of log-transformed data for each adipokine variable.

Although not perfect, the use of the natural logarithm to transform each variable seems to better approximate normal, especially in the serum data, in the BW resistin data, and in the ratio data sets. However, because BAL and BW adiponectin and BAL leptin data are so highly skewed to begin with, the log-transformed data only improves the approximation of normal marginally. However, with the influence of all the other data previously presented in this appendix, the log-transformation of all variables, except BAL resistin, seems appropriate. The BAL resistin data better approximates normal before it was log-transformed, and therefore the raw data was used in statistical analysis. Serum resistin can be seen to better approximate normal after log-transformation and although the distribution has a s_g of only 1.35, log-transformation was deemed appropriate for statistical analysis.

A.6 LOD Substitution

The LOD for each dependent variable was the sensitivity of the commercial ELISA kit used to quantify each adipokine. Each dependent variable measured in the lung created left censored data, that included small non-zero values reported to be close to, or under, the LOD. The two methods proposed by Hornung and Reed in 1990 were considered for LOD substitution: Method 1 (LOD/2) and Method 2 (LOD/$\sqrt{2}$) (247). The other two methods of dealing with values under the LOD, omitting the values or setting them to zero, were not considered because of the biased value of the mean that they produce (247).

Hornung and Reed conclude that the LOD/2 substitution method should be used when the data is highly skewed, with a s_g larger than 3.0. They also conclude that the LOD/√2 method is better for less skewed data, with s_g between 1.5 and 2.5. These methods best estimate the geometric mean and standard deviation with proportions of nondetects < 30% (247).

According to these recommendations and taking into account the data previously presented about the distribution of each variable, the values under the LOD were substituted. Method 1 (LOD/2) was used for the nondetects in the BAL adiponectin (s_g = 4.35), BW adiponectin (s_g = 2.97) and the BW resistin (s_g = 3.11) data sets. Method 2 (LOD/√2) was used for nondetects in the BAL leptin (s_g = 2.31) and BAL resistin (s_g = 2.75) data sets.

Appendix B Serum Adiponectin Data: First Exposure

This is adiponectin serum data from the first exposure triad. Each subject is therefore only exposed to either FAA or DEA.

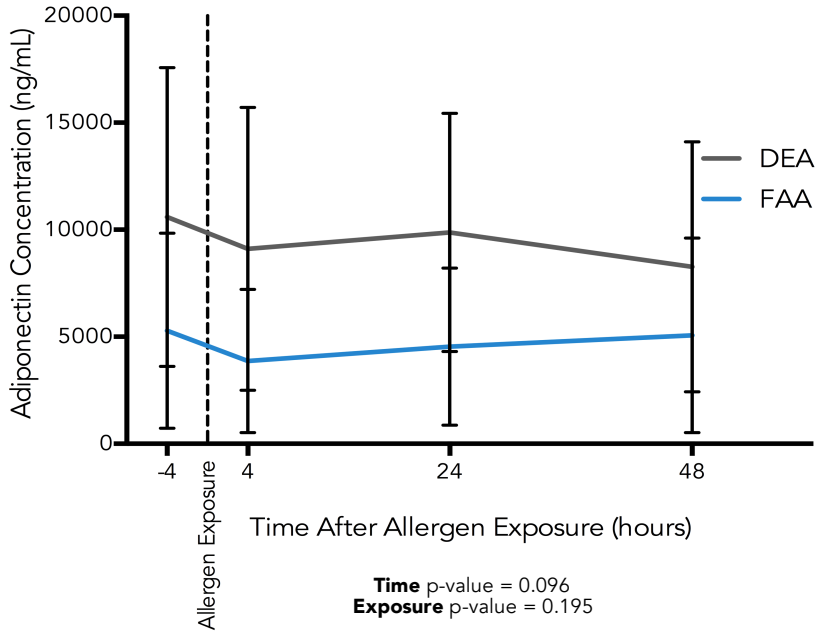


Figure B.1. Serum adiponectin data from the first exposure triad. Each point is the mean value obtained at that timepoint, and the error bars show standard deviation. The dotted vertical line indicates the time of allergen exposure.

Appendix C R Data Analysis Code

C.1 Data Sets Used For Statistical Analysis

> str(lung)

```
'data.frame':       72 obs. of  21 variables:
 $ Subject.ID            : Factor w/ 18 levels "37","52","53",..: 1 1 1 1 2 2 2 2 3 3 ...
 $ Order                 : Factor w/ 2 levels "0","1": 2 2 2 2 2 2 2 2 1 1 ...
 $ Sex                   : Factor w/ 2 levels "0","1": 2 2 2 2 2 2 2 2 2 2 ...
 $ Age                   : int  22 22 22 22 22 22 22 22 20 20 ...
 $ BMI                   : num  22.9 22.9 22.9 22.9 24 24 24 24 21.9 21.9 ...
 $ BMI.median            : Factor w/ 2 levels "above","below": 2 2 2 2 2 2 2 2 2 2 ...
 $ BMI_25                : Factor w/ 2 levels "above","below": 2 2 2 2 2 2 2 2 2 2 ...
 $ Exposure.Condition.Lung: Factor w/ 4 levels "DEA","DES","FAA",..: 1 2 4 3 1 2 4 3 3 4 ...
 $ ADN.BAL               : num  NA NA NA NA 1.42 ...
 $ LnADN.BAL             : num  NA NA NA NA 0.348 ...
 $ LEP.BAL               : num  19.21 10.19 5.11 9.27 6.03 ...
 $ LnLEP.BAL             : num  2.96 2.32 1.63 2.23 1.8 ...
 $ RES.BAL               : num  NA NA NA NA 0.018 ...
 $ LnRES.BAL             : num  NA NA NA NA -4.02 ...
 $ ADN.BW                : num  35.69 4.47 1.38 17.11 NA ...
 $ LnADN.BW              : num  3.575 1.497 0.322 2.839 NA ...
 $ LEP.BW                : logi  NA NA NA NA NA NA ...
 $ RES.BW                : num  13.774 0.258 3.769 4.824 NA ...
 $ LnRES.BW              : num  2.62 -1.35 1.33 1.57 NA ...
 $ ADN.LEP.Ratio         : num  NA NA NA NA 235 ...
 $ Ln.ADN.LEP.Ratio      : num  NA NA NA NA 5.46 ...
```

> str(blood)

```
'data.frame':       136 obs. of  13 variables:
 $ Subject.ID            : Factor w/ 17 levels "37","52","53",..: 1 1 2 2 3 3 4 4 5 5 ...
 $ Sex                   : Factor w/ 2 levels "0","1": 2 2 2 2 2 2 2 2 2 2 ...
 $ BMI.median            : Factor w/ 2 levels "0","1": 1 1 1 1 1 1 1 1 2 2 ...
 $ Exposure.Condition.Blood: Factor w/ 2 levels "DEA","FAA": 1 2 1 2 1 2 1 2 1 2 ...
 $ Timepoint             : Factor w/ 4 levels "0","4","24","48": 1 1 1 1 1 1 1 1 1 1 ...
 $ ADN                   : num  7044 8768 735 1769 1051 ...
 $ LnADN                 : num  8.86 9.08 6.6 7.48 6.96 ...
 $ LEP                   : num  5572 10371 20042 19559 17327 ...
 $ LnLEP                 : num  8.63 9.25 9.91 9.88 9.76 ...
 $ RES                   : num  9.96 11.43 12 12.67 9.59 ...
 $ LnRES                 : num  2.3 2.44 2.49 2.54 2.26 ...
 $ ADN.LEP.Ratio         : num  1264.3 845.5 36.7 90.4 60.6 ...
 $ Ln.Ratio              : num  7.14 6.74 3.6 4.5 4.1 ...
```

> str(blood_B)

```
'data.frame':       17 obs. of  10 variables:
 $ Subject.ID: Factor w/ 17 levels "37","52","53",..: 1 2 3 4 5 6 7 8 9 10 ...
 $ Sex       : Factor w/ 2 levels "Female","Male": 1 1 1 1 1 2 1 2 1 2 ...
 $ BMI.median: Factor w/ 2 levels "above","below": 2 2 2 2 1 1 2 2 1 1 ...
 $ BMI_25    : Factor w/ 2 levels "above","below": 2 2 2 2 1 1 2 2 2 1 ...
 $ ADN_B     : num  7906 1252 1085 15748 9676 ...
```

```
 $ LnADN_B   : num  8.98 7.13 6.99 9.66 9.18 ...
 $ LEP_B     : num  7.97 19.8 15.37 18.81 9.72 ...
 $ LnLEP_B   : num  2.08 2.99 2.73 2.93 2.27 ...
 $ RES_R     : num  10.69 12.33 12.72 9.94 12.62 ...
 $ LnRES_B   : num  2.37 2.51 2.54 2.3 2.54 ...
```

> str(FAS)

```
'data.frame':       18 obs. of  21 variables:
 $ Subject.ID            : int  37 52 53 58 70 81 90 94 96 103 ...
 $ Order                 : int  1 1 0 0 0 0 1 0 0 0 ...
 $ Sex                   : Factor w/ 2 levels "Male","Female": 2 2 2 2 2 1 2 1 2 1 ...
 $ Age                   : int  22 22 20 31 24 32 34 27 25 46 ...
 $ BMI                   : num  22.9 24 21.9 23.6 27.6 26.1 22.3 24.6 24.5 40.5 ...
 $ BMI.median            : Factor w/ 2 levels "above","below": 2 2 2 2 1 1 2 1 1 1 ...
 $ BMI_25                : Factor w/ 2 levels "Overweight","Normal": 2 2 2 2 1 1 2 2 2 1 ...
 $ Exposure.Condition.Lung: Factor w/ 4 levels "DEA","DES","FAA",..: 4 4 4 4 4 4 4 4 4 4 ...
 $ ADN.BAL               : num  NA 13.73 25.84 1.27 4.76 ...
 $ LnADN.BAL             : num  NA 2.62 3.252 0.238 1.561 ...
 $ LEP.BAL               : num  5.11 10.42 15.97 NA 5.34 ...
 $ LnLEP.BAL             : num  1.63 2.34 2.77 NA 1.67 ...
 $ RES.BAL               : num  NA 7.22 12.59 NA 1.96 ...
 $ LnRES.BAL             : num  NA 1.977 2.533 NA 0.671 ...
 $ ADN.BW                : num  1.38 NA NA 1.27 NA ...
 $ LnADN.BW              : num  0.322 NA NA 0.242 NA ...
 $ LEP.BW                : logi  NA NA NA NA NA NA ...
 $ RES.BW                : num  3.769 NA NA 0.426 NA ...
 $ LnRES.BW              : num  1.327 NA NA -0.852 NA ...
 $ ADN.LEP.Ratio         : num  NA 1318 1618 NA 893 ...
 $ Ln.ADN.LEP.Ratio      : num  NA 7.18 7.39 NA 6.79 ...
```

> str(Corr)

```
'data.frame':       18 obs. of  21 variables:
 $ Subject.ID       : int  37 52 53 58 70 81 90 94 96 103 ...
 $ Exposure.Condition: Factor w/ 1 level "FAA": 1 1 1 1 1 1 1 1 1 1 ...
 $ ADN.BAL          : num  NA 36.9 29.4 77.1 19.2 ...
 $ LnADN.BAL        : num  NA 3.61 3.38 4.35 2.96 ...
 $ LEP.BAL          : num  9.27 36.09 25.68 NA 14.82 ...
 $ LnLEP.BAL        : num  2.23 3.59 3.25 NA 2.7 ...
 $ RES.BAL          : num  NA 9.3438 8.2035 NA 0.0655 ...
 $ ADN.BW           : num  17.1 NA NA 67.3 NA ...
 $ LnADN.BW         : num  2.84 NA NA 4.21 NA ...
 $ RES.BW           : num  4.82 NA NA 1.89 NA ...
 $ LnRES.BW         : num  1.574 NA NA 0.635 NA ...
 $ ADN.LEP.Ratio    : num  NA 1023 1144 NA 1296 ...
 $ LnADN.LEP.Ratio  : num  NA 6.93 7.04 NA 7.17 ...
 $ ADN              : num  13237 1833 650 13846 8675 ...
 $ LnADN            : num  9.49 7.51 6.48 9.54 9.07 ...
 $ LEP              : num  7.47 13.46 6.43 7.8 7.39 ...
 $ Ln.LEP           : num  2.01 2.6 1.86 2.05 2 ...
 $ RES              : num  10.9 10.6 11.3 11.1 15 ...
 $ LnRES            : num  2.39 2.36 2.42 2.41 2.71 ...
```

$ ADN.LEP.Ratio.1   : num 1.772 0.136 0.101 1.774 1.173 ...
$ Ln.ADN.LEP.Ratio : num 0.572 -1.993 -2.291 0.573 0.16 ...

C.2 Hypothesis 1: Evaluation of Differences at Baseline

Blood

> t.test(ADN_B~Sex, data = blood_B)

Welch Two Sample t-test

data: ADN_B by Sex
t = -0.3745, df = 9.843, p-value = 0.716
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -8536.502 6084.326
sample estimates:
mean in group Female   mean in group Male
          7789.265   9015.353

> t.test(LnADN_B~Sex, data = blood_B)

Welch Two Sample t-test

data: LnADN_B by Sex
t = -0.359, df = 14.06, p-value = 0.725
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -1.127021 0.803750
sample estimates:
mean in group Female   mean in group Male
          8.636727   8.798363

Lung

> FAS <- subset(data, Exposure.Condition.Lung =="FAS")
> t.test(ADN.BAL~Sex, data = FAS)

Welch Two Sample t-test

data: ADN.BAL by Sex
t = 1.5843, df = 8.335, p-value = 0.1503
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -2.003561 11.000482
sample estimates:
mean in group Female   mean in group Male
          6.088223   1.589763

> t.test(LnADN.BAL~Sex, data = FAS)

Welch Two Sample t-test

data: LnADN.BAL by Sex

t = -1.5828, df = 8.847, p-value = 0.1485
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -2.7727309 0.4934366
sample estimates:
  mean in group Male mean in group Female
       -0.006071391   1.133575747

Figures

For each measured adipokine, in each sample type:

> ADN_BAL_box <- ggplot(data = FAS, aes(x = Sex, y = ADN.BAL, fill = Sex)) + geom_boxplot(outlier.colour = c("grey40") , outlier.size=2.0) + theme_bw(base_family = "Avenir") + scale_fill_manual(values=wes_palette(n=2, name ="Royal1")) + labs(x="Sex", y="Adiponectin Concentration (ng/mL)") + guides(fill=FALSE)

> ADN_BAL_box_BMI <- ggplot(data = FAS, aes(x = BMI_25, y = ADN.BAL, fill = BMI_25)) + geom_boxplot(outlier.colour = c("grey40") , outlier.size=2.0) + theme_bw(base_family = "Avenir") + scale_fill_manual(values=wes_palette(n=2, name ="Rushmore")) + labs(x="BMI status", y="Adiponectin Concentration (ng/mL)") + guides(fill=FALSE)

To print the high resolution figure:

> png(file="BAL_B.png",width=11,height=6, units = "in", res = 400)
> BAL_B <- grid.arrange(ADN_BAL_box, LEP_BAL_box, RES_BAL_box, ADN_BAL_box_BMI, LEP_BAL_box_BMI, RES_BAL_box_BMI, nrow=2)
> dev.off()

C.3 Hypothesis 2: Lung Adipokine Responses

Mixed Effects Model

1. Determine if interaction between Sex, BMI status or Responsiveness with exposure exist.

> ADN <- lmer(LnADN.BAL~Exposure.Condition.Lung*Sex + (1|Subject.ID), data=lung)
> anova(ADN)
Analysis of Variance Table of type III with Satterthwaite
approximation for degrees of freedom
                          Sum Sq Mean Sq NumDF DenDF F.value   Pr(>F)
Exposure.Condition.Lung   44.649 14.8831   3   39 11.9922 **1.052e-05 *****
Sex   4.964 4.9636   1   13 3.9994   **0.06686** .
Exposure.Condition.Lung:Sex 1.147 0.3822   3   39 0.3080   **0.81944**

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> ADN <- lmer(LnADN.BAL~Exposure.Condition.Lung*BMI_25 + (1|Subject.ID),data=lung)
> anova(ADN)
Analysis of Variance Table of type III with Satterthwaite
approximation for degrees of freedom
                          Sum Sq Mean Sq NumDF DenDF F.value   Pr(>F)

Exposure.Condition.Lung 43.574 14.5246 3 39 11.4385 1.61e-05 ***
BMI_25 0.007 0.0075 1 13 0.0059 0.9401
Exposure.Condition.Lung:BMI_25 0.026 0.0088 3 39 0.0070 0.9992

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

There are no interactions with exposure present for Sex or BMI, so no analysis of exposure effects within those categories needs to occur. (can interpret between effects instead of within)

2. Determine the effect of Exposure Condition alone.

> ADN <- lmer(LnADN.BAL~Exposure.Condition.Lung + (1|Subject.ID), data=lung)
> anova(ADN)
Analysis of Variance Table of type III with Satterthwaite
approximation for degrees of freedom
Sum Sq Mean Sq NumDF DenDF F.value Pr(>F)
Exposure.Condition.Lung 45.405 15.135 3 42 12.829 **4.358e-06 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Post hoc Tests

> lsmeans(ADN,pairwise~Exposure.Condition.Lung)
$lsmeans
Exposure.Condition.Lung lsmean SE df lower.CL upper.CL
DEA 2.7233826 0.3494989 40.57 2.01732557 3.429440
DES 1.0364685 0.3494989 40.57 0.33041151 1.742526
FAA 2.4034199 0.3494989 40.57 1.69736287 3.109477
FAS 0.6777169 0.3494989 40.57 -0.02834012 1.383774

Confidence level used: 0.95

$contrasts
contrast estimate SE df t.ratio p.value
DEA - DES 1.6869141 0.3966066 42 4.253 **0.0006**
DEA - FAA 0.3199627 0.3966066 42 0.807 0.8509
DEA - FAS 2.0456657 0.3966066 42 5.158 <.0001
DES - FAA -1.3669514 0.3966066 42 -3.447 0.0069
DES - FAS 0.3587516 0.3966066 42 0.905 0.8025
FAA - FAS 1.7257030 0.3966066 42 4.351 **0.0005**

P value adjustment: tukey method for comparing a family of 4 estimates

3. Determine the effect of Sex and BMI on the Outcome.

> ADN <- lmer(LnADN.BAL~Exposure.Condition.Lung + Sex + BMI_25 + Order + (1|Subject.ID), data=lung)
> summary(ADN)
Linear mixed model fit by REML t-tests use Satterthwaite approximations to degrees of freedom [merModLmerTest]
Formula: LnADN.BAL ~ Exposure.Condition.Lung + Sex + BMI_25 + Order + (1 | Subject.ID)

Data: lung

REML criterion at convergence: 188.5

Scaled residuals:
```
    Min      1Q  Median      3Q     Max
-2.20794 -0.55115  0.08068  0.63993  1.76751
```

Random effects:
```
 Groups     Name        Variance Std.Dev.
 Subject.ID (Intercept) 0.4862   0.6973
 Residual               1.1797   1.0862
Number of obs: 60, groups:  Subject.ID, 15
```

Fixed effects:
```
                              Estimate Std. Error      df t value Pr(>|t|)
(Intercept)                     2.5637     0.5239 17.5000   4.894 0.000126 ***
Exposure.Condition.LungDES     -1.6869     0.3966 42.0000  -4.253 0.000115 ***
Exposure.Condition.LungFAA     -0.3200     0.3966 42.0000  -0.807 0.424355
Exposure.Condition.LungFAS     -2.0457     0.3966 42.0000  -5.158 6.37e-06 ***
Sex1                            1.1671     0.5203 11.0000   2.243 0.046447 *
BMI_25below                    -0.5822     0.5203 11.0000  -1.119 0.286949
Order1                         -0.4098     0.4584 11.0000  -0.894 0.390392
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Correlation of Fixed Effects:
```
            (Intr) E.C.LD E.C.LFAA E.C.LFAS Sex1   BMI_25
Exps.C.LDES -0.379
Exps.C.LFAA -0.379  0.500
Exps.C.LFAS -0.379  0.500  0.500
Sex1        -0.346  0.000  0.000    0.000
BMI_25below -0.346  0.000  0.000    0.000   -0.443
Order1      -0.449  0.000  0.000    0.000    0.034  0.034
```

> anova(ADN)
Analysis of Variance Table of type III with Satterthwaite
approximation for degrees of freedom
```
                          Sum Sq Mean Sq NumDF DenDF F.value    Pr(>F)
Exposure.Condition.Lung 45.405 15.1351     3    42 12.8294 4.358e-06 ***
Sex                      5.936  5.9359     1    11  5.0316   0.04645 *
BMI_25                   1.477  1.4774     1    11  1.2523   0.28695
Order                    0.943  0.9432     1    11  0.7995   0.39039
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Figures

For each adipokine:

> ADN_BAL <- ggplot(data = lung, aes(x = Exposure.Condition.Lung, y = ADN.BAL, fill = Exposure.Condition.Lung, mainTitle = "Female")) + geom_boxplot(outlier.colour = c("grey40") , outlier.size=3.5) + theme_bw(base_family = "Avenir") + scale_fill_manual(values = c("#525252", "#cccccc", "#6baed6", "#bdd7e7")) + labs(x="Exposure Condition", y="BAL Adiponectin (ng/mL)") + guides(fill=FALSE)

For separating by another independent variable:

> ADN_BAL_facet_Sex <- ggplot(data = lung, aes(x = Exposure.Condition.Lung, y = ADN.BAL, fill = Exposure.Condition.Lung, mainTitle = "Female")) + geom_boxplot(outlier.colour = c("grey40") , outlier.size=3.5) + theme_bw(base_family = "Avenir") + scale_fill_manual(values = c("#525252", "#cccccc", "#6baed6", "#bdd7e7")) + labs(x="Exposure Condition", y="BAL Adiponectin (ng/mL)") + guides(fill=FALSE) + facet_wrap(~Sex)

C.4 Hypothesis 3: Systemic Adipokine Responses

Analysis as above, with time as an added fixed effect.

C.5 Hypothesis 4: Lung and Serum Correlations

Pearson Correlation

> Corr <- read.csv("Corr_Data.csv")
> cor.test(Corr$LnADN.BAL, Corr$LnADN, use = "complete.obs", method = "pearson")
Pearson's product-moment correlation

data: Corr$LnADN.BAL and Corr$LnADN
t = 0.3446, df = 12, p-value = 0.7364
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
 -0.4555134 0.5981538
sample estimates:
      cor
0.09899078