EFFECTS OF TRAFFIC-RELATED AIR POLLUTION EXPOSURE ON OLDER ADULTS WITH AND WITHOUT CHRONIC OBSTRUCTIVE PULMONARY DISEASE

by

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

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

June 2021

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Effects of traffic-related air pollution on older adults with and without chronic obstructive pulmonary disease

submitted by Min Hyung Ryu in partial fulfillment of the requirements for

the degree of Doctor of Philosophy

in Experimental Medicine

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Abstract

Our world is populated with numerous urban and occupational hotspots where air pollution levels exceed acceptable levels. Due to our sustained reliance on fossil fuels, many millions of people around the world are breathing in polluted air daily. There is growing evidence that chronic obstructive pulmonary disease (COPD) can be caused and exacerbated by air pollution exposure, but the mechanisms by which this occurs, specifically in COPD, are poorly understood. To fill this knowledge gap, we conducted a controlled human exposure study of older adults to diesel exhaust (DE)—a model for traffic-related air pollution. We recruited thirty research participants (10 ex-smokers with mild-moderate COPD, 20 healthy (9 ex-smokers, 11 never-smokers)) who completed the double-blinded, crossover, controlled human exposure study. Each participant was exposed to filtered air and DE, each for a two-hour period, in a randomly assigned order. Bronchoscopy was performed post-exposure to collect airway samples. Airway lavage samples were probed for changes in cell composition, acute inflammatory proteins, matrix proteinases, and anti-proteases. This investigation revealed that participants with COPD were uniquely susceptible to an inflammatory response not seen in ex-smokers without COPD or never-smoking healthy controls. Next, we evaluated the impact of DE exposure on airway mucosal transcriptome using RNA-seq. We showed that exposure to DE increased expression of antioxidant genes in the airway epithelium *in vivo*, and this expression was modified in the COPD epithelium. Last, we investigated the impact of DE exposure on airway microbiome using 16S rRNA sequencing. Although we did not observe a significant impact on alpha diversity of respiratory microbiome by DE exposure, we discovered that the effect of DE exposure on lung function and cytokines were modified by the respiratory microbiome richness, a simple count of the species of bacteria found in airways. Taken together, my research provides unique and novel insights into how air pollution impacts those with COPD. While epidemiological studies clearly demonstrate a relationship between air pollution and COPD morbidity and mortality, this work adds novel experimental human evidence to support this concept, demonstrating that COPD patients are more susceptible to the harmful effects of air pollution.

Lay Summary

Due to our sustained reliance on fossil fuels, millions of people around the world are constantly breathing in polluted air. It is unclear whether short-term exposure to air pollution, typically found in congested urban areas, elicits a distinct inflammatory response in the airways of chronic obstructive pulmonary disease (COPD) patients. A robust scientific understanding of how traffic-related air pollution affects COPD could improve policies and regulations and advance our understanding of COPD. My thesis work provides direct scientific evidence that people living with COPD, in comparison to healthy individuals, accumulated more inflammatory proteins and proteases in their airways upon exposure to air pollution. The molecular response of airway tissue to air pollution was detailed for a deeper insight. Finally, my work also showed that the microbial community in the airways can modify the impact of air pollution on our lungs.

Preface

The work presented in this thesis was approved by the University of British Columbia (UBC) Clinical Research Ethics Board (H14-00821) and Vancouver Coastal Health Research Institute (V14-00821).

I was the main person responsible for the research participant recruitment and screening. Nafeez Syed, a PhD student with Dr. Jordan Guenette (UBC), shared the responsibility of participant recruitment and screening with me, as a part of his doctoral work. I scheduled and coordinated each participant's research visit. Agnes Yuen (AY), research manager in the Carlsten lab, provided logistical support for the research coordination.

Chapter 1 was written by me and reviewed by my supervisor, Dr. Chris Carlsten (CC), AY, and my PhD supervisory committee members. Shane Murphy (SM), who worked with me on drafting a review paper, helped me with some of the literature searches for this chapter.

Chapter 2 was written by me and reviewed by Kevin Lau (KL), AY, CC and my PhD supervisory committee members. A shortened version of chapter 2 was submitted for peer-reviewed publication at the time of thesis submission and was edited by the manuscript co-authors: Tina Afshar (TA), Hang Li (HL), Denise Jill Wooding (DJW), Juma Orach (JO), Jin Sheng Zhou (JSZ), SM, KL, Carley Schwartz (CS), AY, Christopher F Rider (CFR) and CC. CFR, CC, and I contributed to the conception and design of the work. TA, HL, DJW, JO, JSZ, SM, and I performed data collection and analysis. TA, JO, and I worked on ELISAs and multiplex assays, and verified the underlying data. JO, CS, and I worked on cell counts and verified the underlying data. HL, DJW, JSZ, SM, and I worked on flow cytometry. SM and I verified the underlying flow cytometry data. CS managed the diesel exhaust exposures. DJW, KL, AY, CFR, CC, and I performed sample collections and managed the clinical study. Statistical analysis and interpretation were performed by CC and me. All the figures and tables presented in this chapter were generated by me.

Chapter 3 was written by me and reviewed in detail by Ryan Huff (RH), KL, AY, CC, and my PhD supervisory committee members. RNA-seq analysis plan was conceived and designed by me with help from RH. I extracted RNA from endobronchial brushes. Sample quality check, cDNA synthesis, library preparation, and RNA sequencing were performed by Génome Québec, a centralized RNA sequencing facility. Shijia Li, a master student in the Carlsten lab, performed the initial bioinformatic pipeline work; I supervised Shijia's work and reviewed his codes. I performed differential gene expression analysis using DESeq2 in RStudio and generated all the figures and tables in this chapter.

Chapter 4 was written by me and reviewed by KL, AY, CFR, CC, and my PhD supervisory committee members. My collaborators, Dr. Sara Adar (University of Michigan) and Dr. Janice Leung (UBC), helped design the study and provided a detailed methodology for sample collection and processing. I collected and processed all the microbiome samples with help from CC and staff at the Vancouver General Hospital Bronchoscopy unit. I optimized and performed microbial DNA extraction and quantification. Julia Yang and Dr. Corey Nislow's lab (UBC) performed PCR amplification and 16S rRNA sequencing. Dr. Illiassou Hamidou Soumana and Dr. Fernando Studart (UBC) performed the bioinformatic analysis and generated raw quantitative data for each sample I generated. Spirometry data was collected by AY and me. Electrochemiluminescence multiplex assays were performed by me with assistance from JO and DJW. The statistical analysis was performed by me. Figures and tables in this chapter were generated by me.

Chapter 5 was written by me and reviewed by AY, CC, and my PhD supervisory committee members.

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List of Acronyms and Abbreviations

BW	Bronchial wash
BAL	Bronchoalveolar lavage
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CVD	Cardiovascular disease
DALYs	Disability-adjusted life-years
DE	Diesel exhaust
DEGs	Differentially expressed genes
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental protection agency
FA	Filtered air
FACS	Fluorescence-activated cell sorting
FDR	False discovery rate
FeNO	fractional exhaled nitric oxide
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
GM-CSF	Granulocyte-macrophage colony-stimulating factor
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon gamma
IHD	Ischaemic heart disease
IL	Interleukin
IP-10	IFN-γ-induced protein 10 kDa
LLOD	Lower limit of detection

LME	Linear mixed-effects
MCP	Monocyte chemoattractant protein
MDC	Macrophage-derived chemokine
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NLRP3	NACHT, LRR and PYD domains-containing protein 3
Nrf2	Nuclear factor erythroid 2-related factor 2
PBS	Phosphate buffered saline
PM	Particulate matter
$PM_{2.5}$	Particulate matter of ≤2.5 microns in aerodynamic diameter
PM_4	Particulate matter of ≤4 microns in aerodynamic diameter
PM_{10}	Particulate matter of ≤10 microns in aerodynamic diameter
PMN	Polymorphonuclear
RNA	Ribonucleic acid
SAA	Serum amyloid A
sCD40L	soluble CD40 ligand
TARC	Thymus and activation regulated chemokine
TIMP	Tissue inhibitor of matrix metalloproteinases
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TRAP	Traffic-related air pollution
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
VO_2-max	Maximum O_2 consumption during exercise

Acknowledgements

I consider myself extremely lucky to have been surrounded by many caring individuals who made this long journey enjoyable. I am grateful that I was given this privilege to pursue PhD at the University of British Columbia. I am thankful to have been supported by Vancouver General Hospital and Vancouver Coastal Health Research Institute staff.

Financial support in the form of scholarships from the Canadian Respiratory Research Network, WorkSafe BC, the Natural Sciences and Engineering Research Council of Canada, and the University of British Columbia helped me focus on conducting my research. I am forever grateful for the financial support I received from Canadian taxpayers and donors to pursue my doctoral degree. I owe a great deal to Canada and its citizens.

I am thankful to all the research participants who made my PhD possible. I met so many grandmas and grandpas who cared deeply about the future and next generation. The life stories they shared with me during their participation in my research inspired me.

Next, I want to acknowledge my PhD supervisory committee—Dr. David Granville, Dr. Don Sin, and Dr. Jeremy Hirota. Their encouragement and critique were very vital to the success of my PhD journey. Also, I would like to thank Dr. Chris Rider, Dr. Aruni Jha, and Dr. Andrew Halayko for the mentorship they provided throughout my PhD. Dr. Rider was particularly helpful in conceptualizing, designing, and conducting the study. I am also thankful for my collaborators—Dr. Jordan Guenette, Dr. Janice Leung, Dr. Fernando Studart, Dr. Sara Adar, Julia Yang, and Dr. Corey Nislow.

Friends and colleagues made the journey enjoyable. The Carlsten lab was always full of laughter and exchange of great ideas. Staff and post-docs in the lab supported me greatly in conducting and completing the study. I am incredibly thankful to Agnes Yuen, Kevin Lau, Chris Rider, Andrew Lee, Carley Schwartz, Parteek Johal, Illiassou Hamidou Soumana, Olga Pena, Shuyu Fang, and Darren Sutherland. Agnes and Kevin, in particular, helped run my clinical study and were my manuscript copy editors. The

clinical research could not have been possible without other graduate and professional students' help. I am particularly thankful to Nafeez Syed, Ryan D. Huff, Tina Afshar, Denise J. Wooding, Hang Li, Juma Orach, Shijia (Lenneth) Li, Astrid van Beirs, Shane Murphy, Robbin Klaassen, and Danay Maestre-Batlle for their help with my thesis work. Nafeez, in particular, was most helpful in recruitment and conducting participant screening. Ryan and Tina were my two fellow PhD students in the lab. They put up with all my crazy ideas and got into trouble doing many experiments with me. Many undergraduate students in the lab contributed significantly to my thesis work as well. I am grateful to Ayman Azhar, Bora Jin, Mitch Syberg-Olsen, Evelyn Gunawan, Adrian Karunaratne, Wayne Tse, Jin Sheng Zhou, and Mimi Nguyen for their help with data entry, freezer organization, sample collection, and participant recruitment. Ayman and Bora were the leading force in entering and verifying most, if not all, of my clinical data.

One person who inspired me to care deeply about science, medicine, and research is Dr. Chris Carlsten, my PhD supervisor. He is one of the most passionate human beings I have gotten to know, and I am grateful to have been under his wings during this journey. Chris fostered a deep sense of belonging and togetherness in the lab and pushed me to perform like an Olympic athlete. He kept me focused on putting in consistent efforts and delivering the best action when the peak performance mattered.

I am thankful to my family and friends for their support and encouragement. I am grateful that I got to travel the world and be with many loved ones. I am genuinely thankful to my mom, who has been there for just about everything in life and has given me unconditional love and support in this journey. Life has been full of adventures and good fortune, even in times of global pandemic. For this, I am thankful to God for his love, guidance, and oversight.

Finally, I would like to acknowledge my wife, Heather, and my son, Ian. They were the source of inspiration and energy to finish this work. Heather was the motivation to start this work, and she has supported me throughout my entire PhD and gave me many reasons to smile during ups and downs of my PhD.

Dedication

For my wife, Heather Hyein Kim, who has been there by my side for this whole journey

Chapter 1. Introduction and background

1.1 Study overview

My thesis work is based on "COPD originates in polluted air" study which is the first documented controlled human exposure to traffic-related air pollution (TRAP) that includes chronic obstructive pulmonary disease (COPD) patients and healthy participants and also includes examinations of the lower airways. This study is one of the largest controlled human pollution exposure studies to date to have focused on older adults and includes bronchoscopies—a clinical procedure where a flexible tube is passed down the airway tract, and clinical specimens are collected from the lower airways. Results of this crossover study of short-term exposure to moderate concentrations of diesel exhaust (DE) support the notion that having COPD may confer susceptibility to air pollution-induced negative healthy impacts. While epidemiological studies demonstrate a relationship between TRAP and COPD morbidity and mortality, our work adds mechanistic evidence to support this concept, demonstrating that COPD patients and older adults are more susceptible to airway inflammation following acute exposure to TRAP. Our work provide further insight into what impact COPD has on tissue response to air pollutants by evaluating airway mucosal transcriptomic response to air pollution *in vivo*. This investigation may explain why COPD patients may be more susceptible to airway damage due to air pollution exposures. Finally, my thesis further provides key insights by evaluating the relationship between airway microbiome and air pollution exposure effects.

1.2 Air pollution and mortality

Air pollution kills. The Global Burden of Disease study estimated that in 2015, 4.2 million people worldwide died prematurely from exposure to ambient particulate air pollution.[1] Together with ambient ozone and household air pollution—that caused 0.3 and 2.9 million premature deaths, respectively, in 2015—air pollution was the largest contributor to premature death among all pollution-related mortality risk factors.[2] In comparison, infectious diseases such as HIV/AIDS and malaria were responsible for 1.2

and 0.73 million premature deaths, respectively, and mortalities attributable to HIV/AIDS and malaria are decreasing.[3] Among the top leading non-communicable diseases, cardiovascular diseases and cancers (to each of which air pollution is a major contributor) were responsible for 17.9 and 8.8 million premature deaths in 2015.[3] Tracheal, bronchus, and lung cancer were the leading causes of cancer deaths in the same year; together, they resulted in 1.7 million premature deaths.[3] While substantial progress has been made in reducing premature deaths globally in terms of lowering age-specific mortality,[3] in the absence of drastic global effort to reduce air pollution, premature death due to air pollution is projected to increase to 9 million per year by 2060.[4]

Short-term exposure to outdoor particulate matter ≤2.5 μm in aerodynamic diameter ($PM_{2.5}$) is associated with all-cause mortality; a 10 $\mu g/m^3$ increase in $PM_{2.5}$ was associated with 1.04 [95% confidence interval (CI): 0.52 to 1.56] % increase in the risk of death.[5] In Atkinson and colleagues' meta-analysis of over 110 peer-reviewed time-series studies, associations for respiratory causes of death were larger than those for cardiovascular causes.[5] In a more recent study that utilized mortality data from over 600 cities across the globe, an increase of 10 $\mu g/m^3$ in the 2-day moving average of outdoor $PM_{2.5}$, was associated with increases of 0.68 [0.59 to 0.77] % in daily all-cause mortality, 0.55 [0.45 to 0.66] % in daily cardiovascular mortality, and 0.74 [0.53 to 0.95] % in daily respiratory mortality.[6] Of note, the association between $PM_{2.5}$ level and daily mortality was stronger in less polluted cities with lower annual mean PM concentrations. The concentration-response curves showed a consistent increase in daily mortality with increasing PM concentration, with steeper slopes at lower PM concentrations (Figure 1.1).[6] The shape of the concentration-response curve supports the need for global effort to reduce anthropogenic air pollution—even in less polluted cities.

Short-term exposure to NO_2, another major air pollutant from anthropogenic activities, is also associated with all-cause, cardiovascular, and respiratory mortality. A recent study utilizing data from over 398 cities around the globe, found that, on average, a 10 $\mu g/m^3$ increase in NO_2 concentration was associated with 0.46 [0.36 to 0.57] %, 0.37 [0.22 to 0.51] %, and 0.47 [0.21 to 0.72] % increase in total, cardiovascular,

and respiratory mortality, respectively.[7] Of note, these associations remained after adjusting for PM_{10}, $PM_{2.5}$, ozone, sulfur dioxide, and carbon monoxide. The concentration-response curve showed a linear relationship between NO_2 concentration and daily mortality (Figure 1.2).

In terms of premature death due to air pollution in different age groups, young children (<4 years old) and older adults (>49 years old) are most impacted by air pollution (Figure 1.3).[2] The disease burden of air pollution as measured by disability-adjusted life-years (DALYs)—the sum of the years of life lost due to premature mortality and the years lived with a disability—is also greatest in young children and older adults (Figure 1.4).[2] Notably, the number of premature deaths and DALYs due to air pollution increase with age in adults between 19–65 years old (Figure 1.4),[2] suggesting that total cumulative lifetime exposure to air pollution and other environmental insults, in the context of overall ageing, may increase one's susceptibility to the harmful effects of air pollution. Moreover, chronic diseases that are more prevalent in the older population, such as cardiovascular disease (CVD) and COPD,[8,9] may increase an older adult's susceptibility to the harmful effects of air pollution. Without a doubt, air pollution is a major cause of disease, disability, and premature death,[1] and laws and regulations enacted to adequately protect the general public from the harmful effects of air pollution ought to consider sensitive subpopulations.

1.3 Impact of air pollution exposure on people living with chronic cardiorespiratory conditions

Individuals living with chronic cardiorespiratory conditions, such as CVD, asthma, and COPD, are the potentially susceptible population with increased risk to air pollution toxicity—a wealth of epidemiological data supports this notion.

An increase in $PM_{2.5}$ over days is associated with increased CVD mortality, heart attack, and stroke.[10] Death due to ischaemic heart disease (IHD) had been shown to increase during days with higher air pollution in polluted cities.[11] A large time-series study from Beijing—where mean daily $PM_{2.5}$ concentration can range between 3.9 and 493.9 $\mu g/m^3$—revealed that 10 $\mu g/m^3$ increase in the daily

average concentration of $PM_{2.5}$ was associated with a 0.27 [0.21 to 0.33] % increase in IHD morbidity and a 0.25 [0.10 to. 0.40] % increase in IHD mortality.[12]

A short-term increase in ambient air pollution is associated with an increase in asthma mortality risk.[13] A recent study from China revealed that increase of 47.1 $\mu g/m^3$ (one interquartile range in the study) in $PM_{2.5}$, 26.3 $\mu g/m^3$ in nitrogen dioxide (NO_2), and 52.9 $\mu g/m^3$ ozone (O_3) were associated with increase in asthma mortality, with odds ratio [95% CI] of 1.07 [1.01-1.12], 1.11 [1.01-1.22], and 1.09 [1.01-1.18], respectively.[13] In asthmatics, short-term exposure to air pollution was also associated with asthma-related emergency room visits and hospitalization.[14,15] In one experimental exposure study, exposure of individuals with asthma to high levels of diesel vehicle pollution encountered on London city streets led to a fall in FEV_1 and an increased airway inflammation.[16] My colleagues and I also have shown that exposure to DE in asthmatic individuals resulted in worsening of lung function decline due to allergen exposures and increase in proteins markers and immune cells that are associated with asthma pathophysiology.[17–22]

Concerning COPD, long-term exposure to pollution over the years can lead to an increase in the rate of decline of lung function in non-smoking elderly adults,[23] and those with COPD.[24–26] Long-term exposure to elevated TRAP in Vancouver, BC, Canada—where the level of air pollution has been relatively lower than other industrialized cities worldwide—was associated with an increased risk of COPD hospitalization and mortality.[27] A recent study from Ontario also found that long-term exposure to air pollution ($PM_{2.5}$, NO_2, and O_3) was positively associated with a higher incidence of COPD.[28] In other cities around the world, both long-term and short-term exposure to a higher level of particulate air pollution are associated with increased COPD exacerbation, hospitalization, and mortality—implicating exposure to a high level of air pollution to worsening of COPD morbidity and mortality.[26,29–36]

One recent systematic review of published data quantifying the association between short-term exposure to air pollutants and COPD hospitalization and emergency visits found that a 10 $\mu g/m^3$ increase in daily $PM_{2.5}$ and NO_2 concentration increase the risk of COPD hospitalization and emergency room visits by 2.5

[1.6 to 3.4] % and 4.2 [2.5 to 6.0] %, respectively.[37] A recent study that followed 115 COPD patients for an average continuous period of 128 days carrying a personal air pollution monitor found that increase in gaseous air pollutants (i.e., NO_2, NO, and CO) was associated to an increase in the odds of COPD exacerbation.[35] Few experimental exposure studies have documented the impact of TRAP on individuals living with COPD.[38,39] These studies demonstrated that symptoms related to COPD—such as cough, sputum, shortness of breath, and wheeze—were increased after acute and short exposure to TRAP. However, to the best of my knowledge, no published study to date has evaluated the impact of TRAP on COPD by examining clinical samples from the lower respiratory tract.

1.4 Chronic obstructive pulmonary disease

COPD is a progressive inflammatory disease characterized by airway obstruction that is not fully reversible and is most frequently caused by repeated exposure to noxious particles or gases.[40] In clinics, the standard practice is to use the FEV_1 divided by the forced vital capacity (FVC) below a specific cut-off as a diagnostic criterion of COPD. The Global Initiative for Chronic Obstructive Lung Disease constitutes a case as a post-bronchodilator FEV_1/FVC of <0.70.[40] The American Thoracic Society and European Respiratory Society guidelines recommend the use of the lower limit of normal,[41] whereby the lower fifth percentile of FEV_1/FVC values in a healthy population would be considered to have COPD.

COPD is estimated to affect 174.5 million people globally and result in more than 3.2 million annual deaths.[42] While COPD prevalence varies from country to country, a 12-country study surveying over 100,000 households found that approximately 7-12% of the population have COPD, and prevalence is seen to increase with age and is found at a higher rate in men.[43] In Canada, among those aged 40 years and older, the prevalence of COPD as estimated in the general population using spirometry is 6.4% in never-smokers and 15.3% in ever-smokers.[44] Close to 30% of Canadians diagnosed with COPD have never smoked,[44] and this proportion is consistent with reported proportions of 25-30% seen in Americans, Europeans, and Chinese with COPD.[45–48]

Global Burden of Disease Study estimated that, in 2015, COPD caused 2.6 % (63.9 million) of all global DALYs, representing a significant burden to individuals and society.[42] When considering risk factors for these DALYs in COPD, tobacco smoking and ambient particulate matter were the main risk factors, with age-standardized DALY rates of approximately 400 and 250 DALY rate per hundred thousand people, respectively.[42] Household air pollution, occupational particulates, ozone, and second-hand smoke were the other risk factors identified by the 2015 Global Burden of Disease Study to contribute to DALYs in COPD.[42] Together, these six top risk factors explained 73.3% of DALYs due to COPD.

1.5 Economic burden of COPD

COPD causes a tremendous economic burden to individuals and health care systems. In the US, the total national medical costs attributable to COPD and its sequelae were estimated to be $36 billion USD in 2010,[49] and this cost was estimated to have grown to $49 billion in 2020, according to the US Centers for Disease Control and Prevention.[50] In the US healthcare system, the biggest driver of COPD's healthcare cost is hospitalization.[51] Notably, the cost of severe exacerbation-related hospitalization ranged from $7,000 to $39,200 USD per visit.[52] Similarly, in other nations, hospitalization due to COPD exacerbations and its comorbidities is the key driver of healthcare cost.[53–56] Thus, decreasing the hospitalization of COPD patients by reducing the modifiable risk factors, such as cigarette smoking (including second-hand smoking) and air pollution exposure, may significantly decrease COPD's economic burden.

In British Columbia, the estimated excess cost of COPD was $667 million CAD per year, or $5,196 CAD per person-year between 2001 and 2010.[53] During this period, inpatient, outpatient, medication, and community care costs were responsible for 57%, 16%, 22%, and 5% of the total excess costs in British Columbia, respectively (Figure 1.5).[57] In other words, most of the excess cost of COPD in British Columbia resulted from providing direct care to COPD patients. Therefore, reducing the modifiable risk factors, such as exposure to particulate matter associated with COPD hospitalization, would have a direct impact on lowering overall COPD cost in British Columbia.[26] Interestingly, 26% ($1,343 CAD per person-year) of the excess cost in COPD patients was attributed to the direct care of COPD, and 51%

($2,673 CAD per person-year) was attributable to care of its comorbidities (i.e., cardiovascular diseases), suggesting that the prevention and appropriate management of comorbidities in COPD patients may also reduce the overall economic burden of COPD.[53] Indeed, short-term air pollution exposure has been associated with cardiovascular disease events,[10,58] and long-term exposure to air pollution increases cardiovascular mortality among adults with COPD.[59] Therefore, reducing exposure to ambient air pollution in COPD may also reduce significant excess cost by reducing cardiovascular diseases in those with COPD.[59]

1.6 A case for the need to decrease COPD exacerbations

In addition to the economic impact of acute exacerbations of COPD, several clinical factors call for efforts to reduce exacerbations in COPD. First and foremost, the reason for the need to decrease COPD exacerbation is that moderate and severe acute exacerbations increase morbidity and mortality in COPD patients.[9,60] This is because exacerbations contribute to an increase in the risk of subsequent exacerbations (Figure 1.6). A study from Quebec—examining over 73,000 COPD patients who had at least one severe exacerbation of COPD between 1990–2005—found that the risk of subsequent severe exacerbation was increased three-fold after the second severe exacerbation and 24-fold after the 10th exacerbation, relative to the first.[61]

Secondly, the reason for the need to decrease COPD exacerbation is that exacerbation may have an impact on lung function decline in COPD patients although there is still controversy on whether or not exacerbations cause a rapid lung function decline. A recent retrospective analysis compared the annual decrease in FEV_1 and FVC values in COPD patients.[62] Patients who did not have an acute exacerbation during the first half of the period, demonstrated a post-bronchodilator decline rate of 39.1 mL/year in FEV_1 and 34.7 mL/year in FVC. After these patients suffered a moderate to severe acute exacerbation, they were re-evaluated in the second half of the study and were found to have a post-bronchodilator decline in FEV_1 and FVC of 76.5 and 106.5mL/year, respectively. These data highlight the importance of

the effort to decrease COPD exacerbations. One way to do so would be to reduce COPD patients' exposure to a high level of air pollution.

1.7 Air pollution may contribute to COPD exacerbation and worsening by increasing airway inflammation

Morbidity and mortality arising from air pollution in urban area are attributable to air pollutants originating primarily from motorized traffic, such as $PM_{2.5}$, NO_2, and O_3.[63,64] To date, beyond epidemiological findings, *in vitro* cell culture models and *in vivo* animal toxicology studies have demonstrated that exposure to PM can dose-dependently increase cellular inflammatory markers. For instance, Leclercq and colleagues showed that PM_4 could induce human bronchial epithelial cells—differentiated *in vitro* using air-liquid interface culturing technique to mimic human bronchial epithelium *in vivo*—to produce granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-α, interleukin (IL)-1β, IL-6, and IL-8.[65] In their study, Leclercq and colleagues also demonstrated that cytokine production and release increase with repeated exposure to PM_4, and the cells from COPD donors produce more GM-CSF, TNF-α, TGF- α, IL-1β, and IL-8 than cells from healthy donors.[65]

Exposure of human airway macrophages to PM *ex vivo* showed that PM altered macrophage phagocytic activity, increased their oxidant production, and induced releases of pro-inflammatory mediators such as TNF-α and IL-1β that are important in mediating the local and systemic inflammatory response.[66] In mice, exposure to low dose PM_{10} at 5 μg/day for three weeks was shown to increase macrophages and lymphocyte infiltration into the airways, accompanied by increases in NACHT, LRR and PYD domains-containing protein 3 (NLRP3) and IL-1β in bronchoalveolar lavage.[67] In rabbits, exposure to PM led to an increase in cytokine productions by airway macrophages, which accelerated the transit of neutrophils and monocytes in bone marrow,[68] demonstrating that exposure to PM has systemic inflammatory effects.

In healthy humans, short-term exposure to DE, a TRAP exposure model, had been shown to increase plasma concentration of TNF-α and IL-6 at 24 hours after 1 hour DE exposure (300 μg/m3 $PM_{2.5}$).[69] Another study found that healthy volunteers exposed to DE (300 $\mu g/m^3$ $PM_{2.5}$) for 3 hours showed increased blood monocyte and leukocyte counts and a trend towards an increase in serum IL-6 with no change to IL-8, CRP, and fibrinogen concentration in blood.[70] In our lab, exposure of healthy young participants to DE (300 $\mu g/m^3$ $PM_{2.5}$) had no impact on serum IL-6 and TNF-α.[71] However, a recent study from China found that a higher level of daily $PM_{2.5}$ concentration was associated with an increase in blood inflammation markers (i.e., IL-1β, C-reactive protein (CRP), macrophage inflammatory protein-1α and -β, soluble receptor for advanced glycation end products, and insulin-like growth factor-binding proteins-1 and -3).[72] Xu and colleagues also found that increased $PM_{2.5}$ concentration was associated with increases in circulating MMPs (matrix metalloproteinases; MMP-1,-2,-3,-7,-8, and -9) and decreases in TIMPs (tissue inhibitors of MMPs; TIMP-1 and -2).[72] It is also important to note that exposure to a high level of $PM_{2.5}$ concentration was associated with increases in markers of thrombogenicity (i.e., soluble CD40 ligand (sCD40L), soluble P-selectin, and fibrinogen/fibrin degradation products),[72] supporting the epidemiological findings that short-term exposure to air pollution is linked with higher cardiovascular events and mortality.

In older adults with chronic respiratory diseases (i.e., asthma, COPD, or Asthma-COPD overlap syndrome), exposure to a higher level of $PM_{2.5}$—measured by wearable personal air pollution monitor and fixed-site monitoring station—was associated with an increase in fractional exhaled nitric oxide (FeNO), suggesting that PM exposure may increase airway inflammation in those with chronic respiratory diseases.[73] In COPD patients, acute exposures to $PM_{2.5}$, NO_2, SO_2, and CO were associated with reduced levels of serum Th2 cytokines (i.e., eotaxin, IL-4 and IL-13) and increased serum Th1 and Th17 cytokines (i.e., IL-2, IL-12, IL-17A, interferon γ (IFNγ), monocyte chemoattractant protein (MCP)-1 and sCD40L),[74] suggesting that short-term air pollution may be contributing to systemic inflammation often observed in COPD patients. Indeed, our research group showed increased activation of neutrophils in

airways and blood of ex-smokers following DE exposure.[75] DE exposure was also associated with an increase in neutrophil extracellular traps—web-like structures released from neutrophils that are thought to be increased in COPD airways and contribute to COPD pathogenesis.[75] However, to the best of my knowledge, there have not been any published controlled exposure studies demonstrating that airway inflammation can be worsened by exposure to TRAP or DE in COPD. Therefore, there is a critical knowledge gap in understanding how air pollution exposures lead to increased COPD exacerbations and worsening.

1.8 Airway mucosal transcriptome in the context of air pollution exposures

The first line of defence of the respiratory tract to inhaled pollutants and other atmospheric contaminants is the respiratory mucosa. The respiratory mucosa lines the internal surface of the respiratory tract and consists of a high percentage of epithelial cells (~90%) and immune and stromal cells.[76,77] With recent advances in bulk and single-cell RNA profiling using next-generation sequencing, investigators around the globe have begun to characterize the respiratory mucosa in health and disease in much greater detail.[76] For example, single-cell RNA sequencing (scRNA-seq) has led to a recent discovery of pulmonary ionocyte—a novel, disease-associated, rare epithelial cell type expressing a high level of *CFTR* genes.[77,78] With bulk RNA-seq analysis, COPD researchers have identified networks of genes associated with progression of COPD, unveiling that the loss of epithelial coherence and decline of the regenerative repair process may be the driving forces for COPD progression.[79]

RNA-seq technology allows us to investigate the tissue transcriptome—all the messenger RNA (mRNA) expressed in a population of cells. With the cost of RNA-seq considerably decreased to ~$300 per sample, many labs can now use RNA-seq to probe and understand the transcriptional response of cells and tissue in health and disease.

In the context of air pollution studies, several human molecular epidemiological studies and *in vitro* epithelial cell exposure studies have been conducted using RNA-seq to date. For instance, O'Beirne and colleagues had shown that an increase in thirty-day ambient $PM_{2.5}$ levels was associated with significant

dysregulation of the small airway epithelial transcriptome in smokers.[80] Exposure of immortalized human bronchial epithelial cells (16HBE) to $PM_{2.5}$ collected from Beijing, China had detailed the transcriptomic response of epithelial cells to urban $PM_{2.5}$.[81] The Beijing study revealed that a large number of genes—involved in cellular response to xenobiotic stimuli, metabolic response, and inflammatory and immune response pathways—were activated following exposure to $PM_{2.5}$.[81] *In vitro* exposure of primary nasal airway epithelial cells from healthy donors to $PM_{2.5}$ extract showed that PM exposure led to differential expression of 424 genes, including activation of aryl hydrocarbon receptor signalling, mucus secretory expression program, and an IL-1 inflammatory program.[82] Similarly, another i*n vitro* epithelial cell exposure study revealed that $PM_{2.5}$ from biomass combustion resulted in differential expression of genes functionally involved in cytokine and immune responses, angiogenesis, inflammation, and detoxification.[83]

Although epidemiological and *in vitro* studies have detailed mucosal transcriptomic response to air pollution, to the best of my knowledge, no published research to date has examined the mucosal transcriptomic response to air pollution in human airways *in vivo*. Moreover, there is a critical knowledge gap in understanding whether the mucosal transcriptomic response of COPD airways to air pollution is different than that of healthy airways.

1.9 Airway microbiome in the context of COPD

The human microbiome is the genetic material of all the microbes that live on and inside the human body. By studying the diversity and functionality of the human microbiome, scientists are working to gain insight into how human-microbial interactions shape health and disease. In recent years, the mechanisms through which the respiratory microbiota—the community of microbes present in our airways—exerts functional effects on the pathogenesis, exacerbation, and comorbidities of COPD have become better understood.[84] Studies have documented differences in the composition of respiratory microbiota in individuals living with chronic respiratory diseases (e.g., COPD, asthma, and cystic fibrosis) in comparison to healthy individuals.[84–86] It is now known that COPD exacerbation events are associated

with decreased microbial alpha diversity and an increased proportion of *Proteobacteria* phylum in sputum samples.[87] Use of oral corticosteroids, a treatment for COPD exacerbations, can alter the lung microbiome and is associated with increased airway bacterial richness (i.e., the number of different bacterial species) and diversity (i.e., total abundances of each species and their relative abundance distributions) in sputum of COPD patients.[84,87]

Currently, investigators have begun examining the relationship between the microbial community in the lower respiratory tract and clinical features of COPD by examining the lung microbiome sampled by bronchoscopies.[88–90] Of note, Opron and colleagues showed that BAL bacterial composition was associated with several clinical features—including bronchodilator responsiveness, peak expiratory flow rate, and symptom scores—in mild COPD patients.[88] However, the respiratory microbiome's role in the human response to inhaled air pollutants remains largely unknown.[91] As we continue to explore how microbial community of distal airways shape our immune responses to external stimuli in the lungs, and ultimately have an influence on COPD exacerbations and pathogenesis, a careful examination of host-microbe-environment interactions will be important.

1.10 Controlled human exposure studies involving COPD participants

I searched PubMed for articles published between database inception (1984) and February 25, 2020. I searched with key terms "controlled exposure," "exposure chamber," "diesel exhaust," and "concentrated ambient particles." Initial searches were narrowed using filters for "clinical trials," "clinical study," "randomized controlled trials," and "human studies." The majority of controlled human exposure studies of air pollution to date included young and healthy participants. Studies demonstrated that acute air pollution exposure led to acute lung function decline and increased blood biomarkers of inflammation and vascular dysfunction. Several large controlled human exposure studies (with a similar sample size as this study) investigated the effect of air pollution exposure on asthmatics—another susceptible population—and demonstrated that air pollution exposures exacerbate allergen-induced airway inflammation and acute lung function decline. In my literature search, there were only four published controlled human exposure

studies that included individuals with COPD, and those studies examined acute air pollution exposure effect on lung function, blood biomarkers (related to systemic inflammation), cardiovascular endpoints (e.g. hypercoagulability), and upper airway samples (induced sputum).[39,75,92,93] Currently, there is a wealth of observational data supporting the assertion that COPD and its associated symptoms can be both caused and accelerated by air pollution exposure and that short-term air pollution exposure leads to exacerbations that often require extra medication or hospitalization.[29–31] However, there is only limited evidence from controlled human exposure studies to buttress the information from epidemiologic and toxicologic studies that can facilitate an integrated evaluation of the evidence to support establishing or revising emission guidelines. Regulatory agencies such as the US Environmental Protection Agency have relied on controlled human exposure studies to inform policy decisions and establish or revise standards to protect public health and improve air quality.[94] From the regulators' perspective, the role of controlled human exposure studies is to produce transient and reversible biomarker or physiological responses that inform about biological mechanisms of pollution effect but do not cause clinical effects.[94] To this end, there have been no direct observations of the impact of TRAP exposure on those with COPD or those at risk for developing COPD in a controlled human exposure experiment; such direct evidence is critical to deepening support for the association between TRAP and COPD.

1.11 Thesis hypothesis and objectives

The overarching objective of my thesis is to detail and document the impact of DE exposure on the respiratory system of older adults with and without COPD. The overarching hypothesis of my thesis is that *having COPD confers susceptibility to air pollution-induced negative health impacts*.

Specifically, Chapter 2 will evaluate the impact of DE exposure on airway and systemic inflammatory markers. I will test the hypothesis that:

1. *DE exposure increases inflammatory proteins and cells in the airways and in circulation, and this increase is greater in those with COPD.*

Chapter 3 will evaluate the airway mucosal transcriptomic response to DE exposure. I will test the hypotheses that:

2. *DE exposure leads to upregulation of genes involved in the antioxidant response.*
3. *DE exposure leads to upregulation of mucin genes (MUC5AC, MUC5B, MUC1, and MUC16).*
4. *COPD epithelium will mount different transcriptional responses to DE exposure compared to healthy epithelium.*

Chapter 4 will explore the relationship between airway microbiome and air pollution exposure effects. I will test two hypotheses:

5. *Acute exposure to DE alters the alpha diversity of the respiratory microbiome.*
6. *The effect of DE exposure on lung function and cytokines is modified by the respiratory microbiome richness.*

1.12 Figures

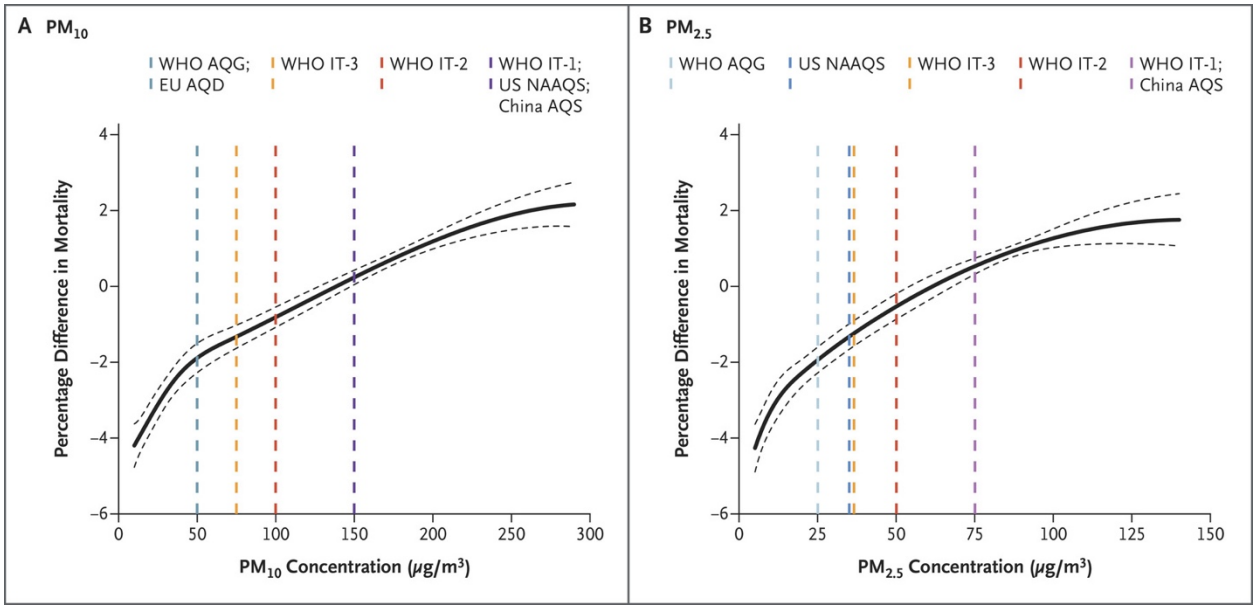

Figure 1.1. Concentration-response curves for the associations of 2-day moving average PM_{10} and $PM_{2.5}$ with daily all-cause mortality. The y axis represents the percentage difference from the pooled mean effect (as derived from the entire range of PM concentrations at each location) on mortality. Zero on the y axis represents the pooled mean effect, and the portion of the curve below zero denotes a smaller estimate than the mean effect. The dashed lines represent the air-quality guidelines or standards for 24-hour average concentrations of PM_{10} or $PM_{2.5}$ according to the World Health Organization Air Quality Guidelines (WHO AQG), WHO Interim Target 1 (IT-1), WHO Interim Target 2 (IT-2), WHO Interim Target 3 (IT-3), European Union Air Quality Directive (EU AQD), U.S. National Ambient Air Quality Standard (NAAQS), and China Air Quality Standard (AQS). Reproduced with permission from Liu *et al* NEJM 2019,[6] Copyright Massachusetts Medical Society.

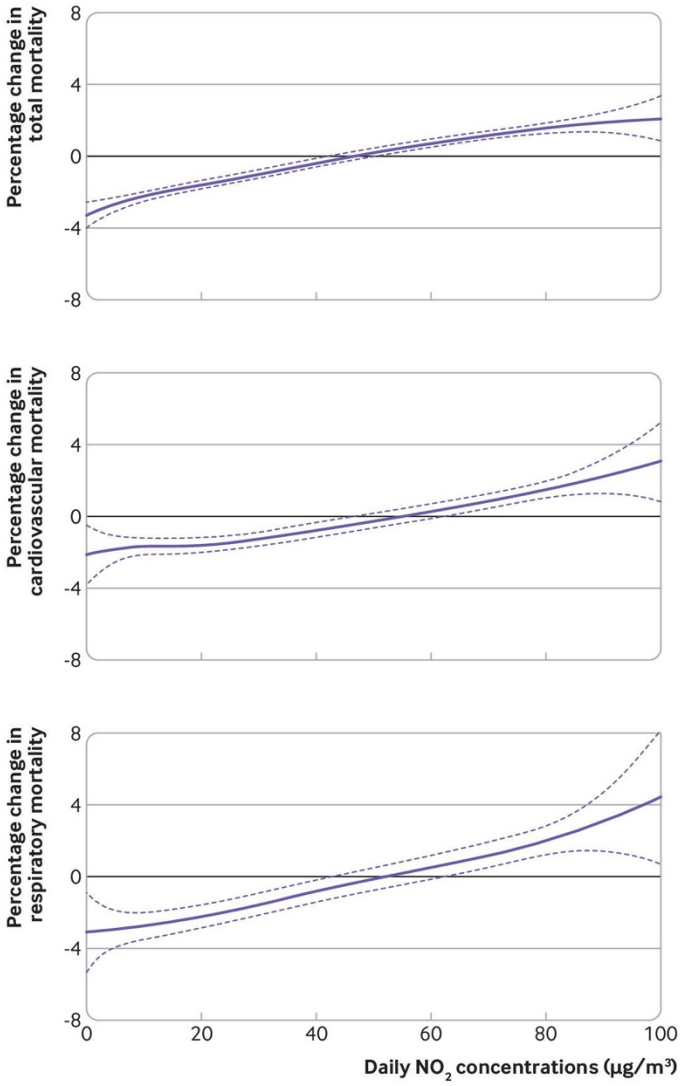

Figure 1.2. Concentration-response curve between nitrogen dioxide (NO_2) concentrations (lag 1) and total, cardiovascular, and respiratory mortality. The vertical scale can be interpreted as the relative change of the mean effect of NO_2 on mortality; the fraction of the curve below zero denotes a smaller estimate than the mean effect. Reprint from Meng *et al* BMJ 2021 with permission.[7]

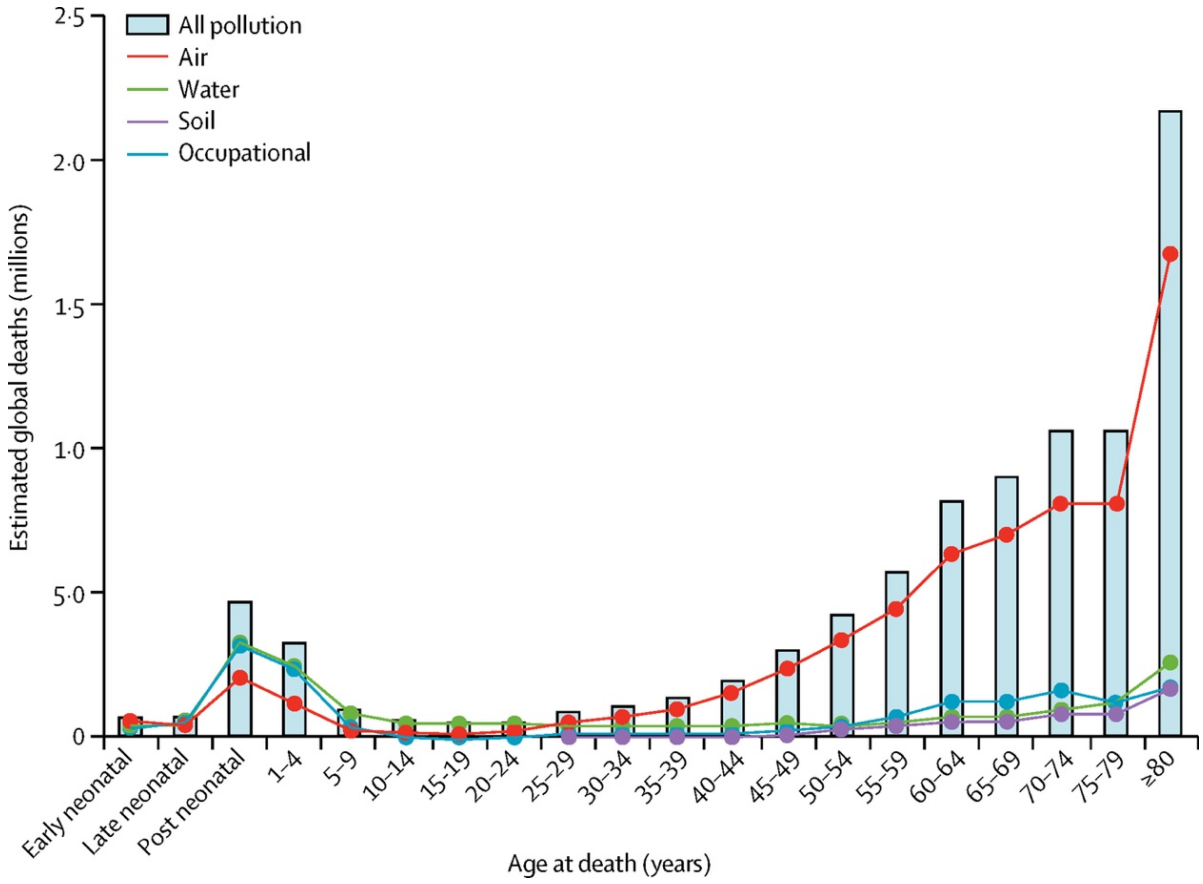

Figure 1.3. Estimated global deaths by pollution risk factor and age at death. Reprint from Landrigan *et al* 2018 with permission.[2]

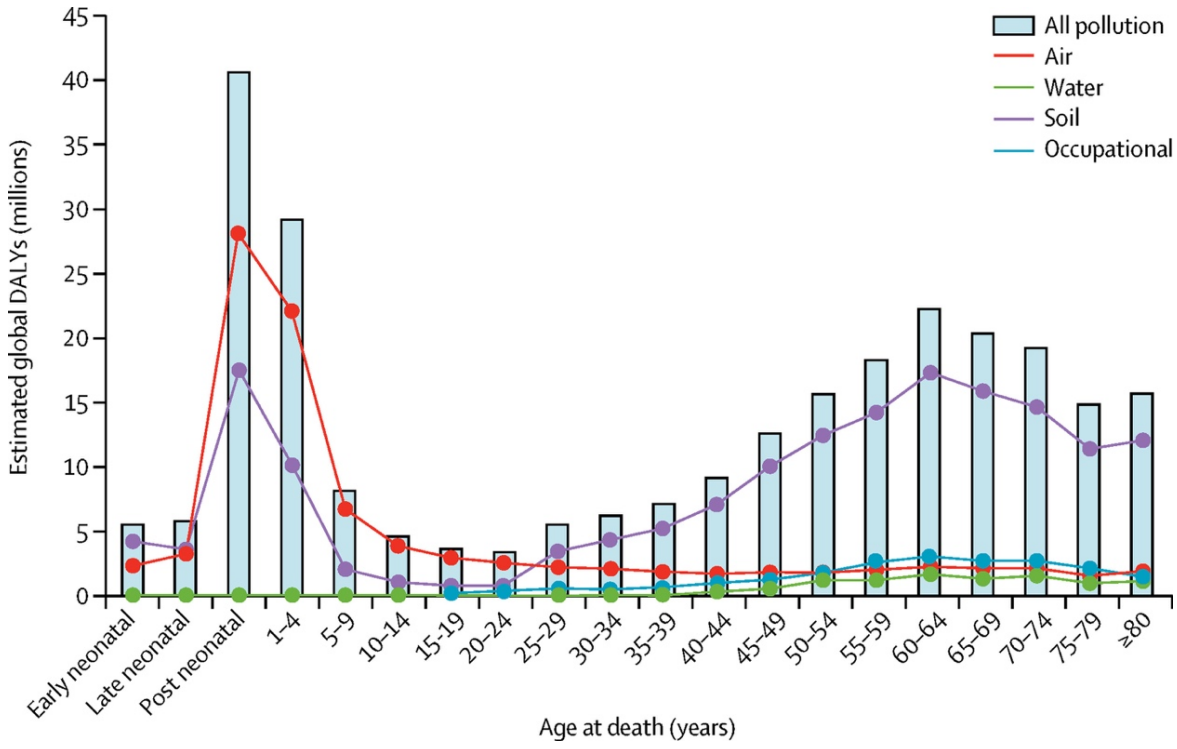

Figure 1.4. Estimated global disability-adjusted life-years by pollution risk factor and age at death. Reprint from Landrigan *et al* 2018 with permission.[2]

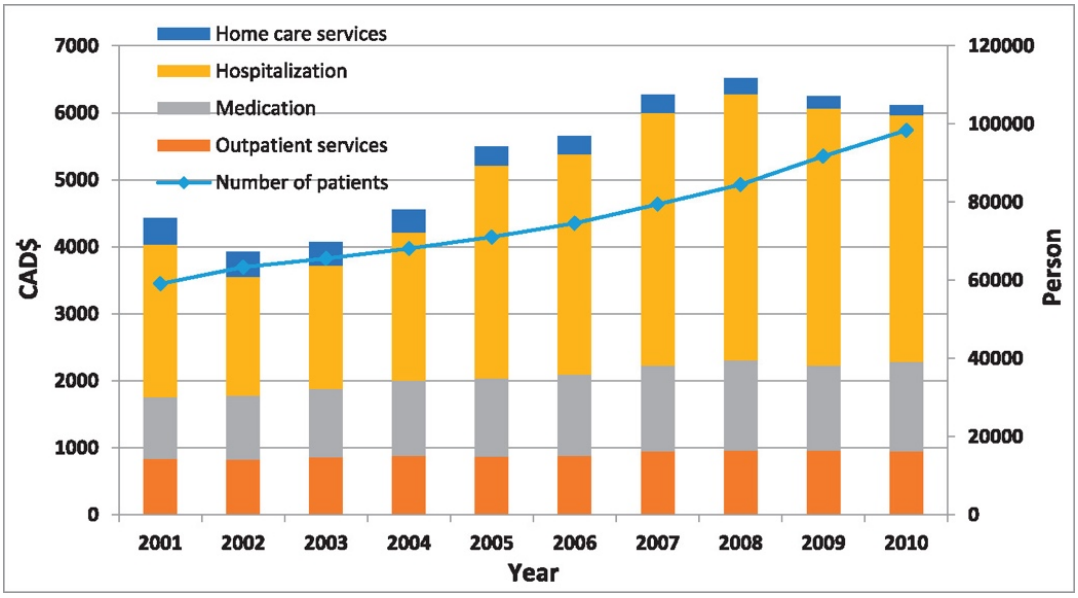

Figure 1.5. Number of patients with, and annual excess costs (total and by component) of, COPD in British Columbia, Canada from 2001 to 2010. Reprint from Khakban *et al* 2015 with permission.[57]

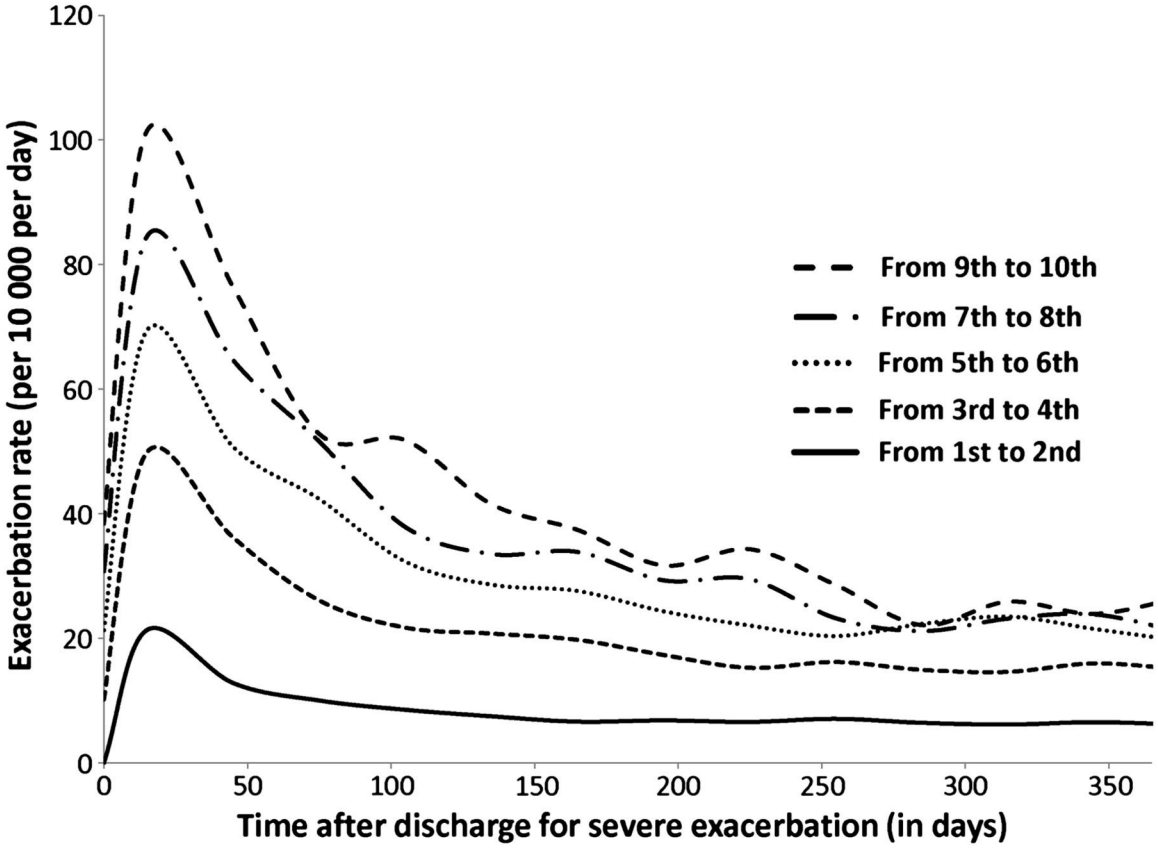

Figure 1.6. Hazard function of a subsequent hospitalized COPD exacerbation in the 1-year period after discharge from the previous one. Reprint from Suissa *et al* with permission under the terms of the Creative Commons Attribution Non-commercial License.[61]

Chapter 2. COPD patients are more susceptible to the effects of traffic-related air pollution than are healthy individual

2.1 Introduction

Chronic obstructive pulmonary disease (COPD), a disease characterized by chronic lung inflammation with persistent airway obstruction,[95] is estimated to affect 174.5 million people and result in more than 3.2 million annual deaths globally.[42] Growing evidence supports the assertion that COPD and associated symptoms can be both caused and exacerbated by air pollution exposure. Indeed, short-term exposure to increased ambient air pollution is associated with increased COPD exacerbations that often require extra medication or hospitalization.[29–31] A randomized study demonstrated that COPD patients experience greater respiratory symptoms—including cough, sputum induction, shortness of breath, and wheezing—after walking down a polluted city street for 2 hours, compared with walking through a cleaner city park.[38]

Despite growing evidence linking increased COPD exacerbation frequency and associated symptoms to acute air pollution exposures, the mechanisms through which air pollution exerts its harmful effects in COPD are incompletely understood. To date, it is unclear whether or not short-term exposure to traffic-related air pollution (TRAP), as is typically found in congested urban areas, elicits a distinct inflammatory response in the lower respiratory tract of COPD patients; no study has directly sampled bronchoalveolar lavage in those with COPD in this context nor whether such responses are different from those of healthy individuals. To fill this knowledge gap, we conducted a controlled human exposure study that compared groups believed to have higher susceptibility to air pollution (COPD patients and ex-smokers with significant tobacco smoking history (>10 pack-years)), to healthy individuals. To the best of my knowledge, this is the first documented study of controlled human exposure to air pollution that includes COPD patients, healthy participants, and examination of the lower airways.

To provide insight into the differential susceptibility between these groups and buttress the notion that the cellular response to TRAP exposure contributes to phenotypic variation in this context, I tested the hypothesis that *DE exposure increases inflammatory proteins and cells in the airways and in circulation, and this increase would be greater in those with COPD.* Results of this crossover study of short-term exposure to moderate concentrations of DE suggest that having COPD confers susceptibility to negative healthy impacts.

2.2 Methods

2.2.1 Participant recruitment and selection

This controlled exposure study took place between January 2015 and May 2019 (ClinicalTrial.gov ID: NCT02236039). This study was approved by the University of British Columbia Clinical Research Ethics Board (H14-00821) and Vancouver Coastal Health Research Institute (V14-00821). All participants provided written informed consent prior to enrollment. Research participants (aged 40–80) were recruited via local online classified advertisements, flyers, clinical referrals, and newspaper advertisements. Figure 2.1 summarizes the recruitment process and numbers.

We recruited three groups of participants: never-smokers without COPD, ex-smokers (minimum 10 pack-years of smoking history) without COPD, and ex-smokers with mild or moderate COPD ($FEV_1/FVC < 0.7$ and FEV_1% predicted ≥ 50). Prior to study enrolment, all participants self-reported ≥6 months of smoking cessation, which continued throughout the study. Serum cotinine tests were used to exclude active and passive tobacco smoking.

An initial telephone screening identified potentially eligible participants. During the telephone screening, detailed information about the study was provided and the potential participants were given a chance to clarify any concerns and ask questions. During the phone interview, information about tobacco smoking history, smoking status, medications, and co-morbidities were collected using a pre-set study

questionnaire approved by the ethics board. Telephone screening information was reviewed by the study physician, and eligible participants were invited for an in-person screening.

At the in-person screening visit (Visit 1), participants provided written, informed consent, and completed medical history, recent physical activity, physical activity readiness, and common cold questionnaires, as well as a COPD Assessment Test.

Participants had baseline spirometry and impulse oscillometry assessed, and an electrocardiogram was obtained to rule out any cardiac arrhythmias. In addition, an exercise test was performed to determine VO_2-max, which was used to set exercise intensity during exposures.

Exclusion criteria were as follows: pregnant and/or breastfeeding, allergy to anesthetics and other study medications, participation in another clinical study with a medication intervention arm, unstable COPD symptoms, a body mass index <19.5 or > 34.9 kg/m^2, current smoker or quit smoking <6 months prior to study screening, or a smoking history of 0-10 pack-years (in the ex-smoker group), diagnosed with asthma, regular and frequent use of inhaled corticosteroids, bronchodilators, antihistamines, non-steroidal anti-inflammatories, anticoagulants or decongestants, or significant comorbidities considered by the principal investigator to increase risk.

2.2.2 Medications and dietary supplements

Participants were also asked to withhold: 1) short-acting β_2-agonists (SABAs) for 4 hours prior to testing; 2) anti-cholinergic for 24 hours prior to testing; 3) long-acting β_2-agonists (LABAs) for 12 hours prior to testing if taken in a separate inhaler (e.g. not combined with an inhaled corticosteroid inhaler); 4) LABAs and ICS for 24 hours prior to testing, if these two medication groups were combined in one inhaler; 5) inhaled corticosteroids (if taken alone as a mono-therapy, or if taken with either a LABA or SABA, but in two separate inhalers) for 24 hours prior; and 6) any other lung-active medications at the discretion of the study physician.

Participants reported any medication use (prescribed or over-the-counter) throughout the study, at each visit. Participants were advised to stop taking dietary supplements during the study and were asked to continue all of their regular prescribed medications (other than lung active medications) as directed by their primary physician, and to report any changes in usage.

2.2.3 Participant smoking status

All participants self-reported ≥6 months of smoking cessation prior to study enrolment, which was maintained throughout the study. Serum cotinine levels were measured at each exposure visit to verify abstinence from first-hand smoking and remained <10 ng/mL for all but two participants (Cotinine ELISA Kit; OriGene Technologies). The two participants who exceeded our cotinine threshold were on nicotine replacement therapy (nicotine gum) or reported exposure to second-hand smoke and displayed blood cotinine levels below 21 ng/mL.

2.2.4 Human exposure

This randomized, double-blinded, crossover, controlled exposure study took place at the Air Pollution Exposure Laboratory in Vancouver, Canada, using the exposure facility and protocols described previously.[22,75,96] Each participant was exposed for 2 hours on separate occasions to DE (at a nominal concentration of 300 $\mu g/m^3$ of particulate matter of ≤2.5 microns in aerodynamic diameter [$PM_{2.5}$]) and filtered air (FA) (Figure 2.2). Exposure order (i.e., FA first vs. DE first) was computer-randomized, and there was a minimum 4-week washout between exposures. Participants abstained from short- and long-acting bronchodilator and inhaled corticosteroid use for 48 hours, and caffeine for 12 hours prior to exposures. Visits were rescheduled if participants exhibited signs of upper respiratory tract infection (i.e., Common Cold Questionnaire score greater than 3).[97]

To generate diesel exhaust, a constant load of 2.5 kW was applied to a 6.0 kW Coliseum GY6000 generator with a 406 cc Yanmar L 100 EE 4-stroke diesel engine (the U.S Environment Protection Agency Tier 3-compliant).[96] Diesel exhaust was diluted using the dilution system previously described in

detail.[96] Concentrations of $PM_{2.5}$ (mass concentration), CO, NO, NO_2, NO_x, CO_2, total volatile organic compounds, and particle size distribution were monitored in real-time (data recorded every 2 seconds) throughout each exposure. The exposure booth (dimensions 4' (width) x 6' (depth) x 7' (height)) was maintained in negative pressure during the exposure, and exhaust air flow was controlled in real-time by the staff engineer to maintain the level of $PM_{2.5}$ nominally at 300 $\mu g/m^3$. For the filtered air (FA; control) condition, the diesel generator was in operation with the same load conditions as the DE exposure, with the exhaust diverted from the dilution and exposure system. Instead, during the FA condition, incoming air to the exposure booth was twice filtered (with high-efficiency particulate air filters) room air.

Each participant was exposed for 2 hours on separate occasions to DE and FA. The order of exposure was computer randomized and there was a minimum 4-week washout period between exposures. During the exposure, participants completed two 15-minute cycles on a stationary bike inside the booth (30% VO_2-max load). The 15-minute cycling period occurred 20 minutes into every hour of the exposure. Blood pressure and blood oxygen saturation were measured every 20 minutes. Spirometry was monitored every 60 minutes.

2.2.5 Blood sample collection and cell count analysis

Whole blood was collected in 27 out of 30 participants (3 participants had difficulties with venipuncture), in EDTA tubes at baseline and 24 hours post-exposure and analyzed at the Vancouver General Hospital pathology lab for a complete blood count. Cell differentials of the number of neutrophils, eosinophils, macrophages, lymphocytes, and basophils per litre of blood were reported.

2.2.6 Bronchoscopy sample collection and processing

Research bronchoscopies were performed 24 hours after each exposure, in 27 out of 30 participants, to collect bronchial wash (BW) and bronchoalveolar lavage (BAL). 9 were healthy never smokers, 8 were ex-smokers without COPD, and 10 were ex-smokers with COPD. Immediately prior to each bronchoscopy, participants were given nebulized salbutamol as part of the routine research bronchoscopy

procedure. To collect BW and BAL, the bronchoscope was wedged at a 4-5 generation bronchus on either the right medial lobe or the left lingula. For BW, 20 mL of saline (sterile 0.9% NaCl) was instilled and suctioned twice. For BAL, 50 mL of saline was instilled and suctioned twice. BW and BAL were transferred back to the research laboratory on ice and immediately processed. BW and BAL were put through a mesh filter (40 μm) and centrifuged for 15 minutes at 475 relative centrifugal force (rcf) at 4ºC. Supernatants were separated and aliquoted for storage at -80ºC. BW and BAL cells were counted on a hemocytometer to determine total cell number, and cytospins were prepared and stained for differential cell counts.

2.2.7 Flow cytometry for T cells

Cells were processed immediately after collection. 200,000 BAL cells were resuspended in 50 μL of phosphate buffered saline (PBS) and used for BAL cell staining. 50 μL of whole blood from the EDTA tube was used for blood staining. Antibodies were purchased from BD. 28.5 μL of antibody cocktail (antibody concentrations summarized in Table 2.1 was added to 50 μL of the resuspended BAL and EDTA blood samples. Cells were incubated with antibodies for 30 minutes at room temperature in the dark. The BAL cells were then washed with PBS once and resuspended in 250 μL of PBS. For blood, 1 mL of BD FACS lysing solution (1x) was added to the blood sample and incubated at room temperature for 15 minutes. The blood sample was then centrifuged at 320 rcf for 5 minutes and resuspended in 250 μL of PBS. 60,000 events were collected on a BD FACS Canto II. Gates were set using fluorescence minus one controls. Flow analysis was performed using FCS Express 6 Flow Research Edition (De Novo Software). T cells were gated (i.e., singlets > CD45+ > CD3+ and CD4+/CD8+ gates).

2.2.8 Total and differential cell counts for BW and BAL

BAL and BW cells were resuspended in PBS and counted on a hemocytometer to estimate total cell number. Cytospins were then prepared for differential cell counts. Cytospins were stained with the HARLECO Hemacolor stain set (EMD Millipore). Two observers counted at least 500 cells for every

sample. The slides were counted for macrophages, neutrophils, ciliated bronchial epithelial cells, eosinophils, basophils, and lymphocytes. Differential cell counts are expressed as number of specific leukocytes per total leukocytes. For epithelial cells, the ratio between epithelial and total cells was used for statistical analysis.

2.2.9 Enzyme-linked immunosorbent assays

Matrix metalloproteinase (MMP)-12 and neutrophil elastase were assayed with Human MMP-12 ELISA Kit (ab213811; Abcam) and Human PMN Elastase ELISA Kit (ab119553; Abcam), respectively, following the manufacturer's protocol. Samples were assayed without dilution and incubated overnight at 4ºC. Samples were assayed in duplicates, and the mean of the duplicate values was used in statistical analysis.

2.2.10 Electrochemiluminescence assays

A commercially available multiplex kit (V-Plex Vascular Injury Panel 2 Human Kit, Meso Scale Diagnostics) was used to assay serum amyloid A (SAA), C-reactive protein (CRP), vascular cell adhesion protein 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1).

This assay was done according to the manufacturer's protocol with minor modifications. BW and BAL samples stored at -80ºC were thawed on ice and centrifuged at 2,000 rcf for 3 minutes prior to being diluted 5-fold with Diluent 101 (MSD). Samples (in singlicate) were incubated with primary antibodies overnight on a plate shaker (300 rpm) at 4ºC. The following day, samples were washed 3x with PBS containing 0.05% Tween 20. 25 µL of the detection antibody solution was then applied to each well. Samples were incubated at room temperature with shaking for 1 hour. Plates were then washed 3x with the wash buffer. 150 µL of Read Buffer T (MSD) was added to each well. The plates were read immediately after adding the read buffer, on Meso® Quick Plex SQ 120, Model 1300 (MSD). The lower limit of detection (LLOD) was set at the signal intensity that was 2.5 standard deviations above the background noise in the blank.

Single-plex kits (R-PLEX (MSD)) were used for the following analytes: MMP-1, -3, -7, -9 and -10, alpha-1 antitrypsin and tissue inhibitor of metalloproteinases (TIMP)-1. All assays were performed according to the manufacturer's protocol with some modifications to the diluents and dilution factors (summarized in Table 2.2). Samples (in duplicate) were incubated with the primary antibodies overnight on a plate shaker (300 rpm) at 4ºC. The following day, samples were washed 3x with PBS containing 0.05% Tween 20. 50 µL of the detection antibody solution was applied to each well. Samples were incubated at room temperature with shaking for 1 hour. The plates were then washed 3x with the wash buffer. 150 µL of MSD GOLD Read Buffer (MSD) was added to each well. The plates were read immediately after adding the read buffer on the MSD plate reader. The lower limit of detection (LLOD) was set at the signal intensity that was 2.5 standard deviations above the background noise in the blank.

2.2.11 Statistical analyses

Effects of DE exposures on the study endpoints were assessed using linear mixed-effects (LME) models (nlme package, v.3.1-148) in R, version 4.0.3 (The R Foundation) implemented in R Studio, (v.1.3.1093, RStudio). No adjustments for multiplicity were made in these analyses. When necessary, to normalize the data distribution, data were $\log_{10}$-transformed. Any effect estimates and associated confidence intervals based on $\log_{10}$-transformed data are presented as back-log transformed values.

In our primary LME model, exposure (FA and DE) and participant age were fixed effects, and participant ID was set as a random effect. To evaluate whether COPD, age, or group (divided into healthy, ex-smokers without COPD, and ex-smokers with COPD) modified the effect of DE on the outcomes, three models each with interaction terms (exposure-by-COPD, exposure-by-age or exposure-by-group) as fixed effects were run. Order effects (i.e., DE first vs. FA first) were assessed by using exposure order as a fixed effect in a separate model. P-values ≤ 0.05 were considered statistically significant and are reported for primary outcomes (as defined a priori in our clinicaltrials.gov registration).

2.3 Results

2.3.1 Study population

Figure 2.1 describes how the study participants were recruited and how many participants underwent and completed exposures and research bronchoscopies. Characteristics of the 30 study participants who underwent exposure study are presented in Table 2.3.

2.3.2 Exposure characteristics

Exposure data are presented as mean±SD. $PM_{2.5}$ mass concentration for FA and DE exposure were 4.5±4.2 and 282.2±44.4 $\mu g/m^3$, respectively. Nitrogen oxide (NO) for FA and DE exposure were 0.18±0.15 and 3.0±1.9 ppm, respectively. Nitrogen dioxide (NO_2) concentration for FA and DE were 10.4±17.4 and 92.3±89.4 ppb, respectively. NO_X concentration for FA and DE were 0.19±0.16 and 3.1±1.9 ppm, respectively. CO concentration for FA and DE were 1.7±0.8 and 12.1±3.0 ppm, respectively. Particle number concentration ($\#/cm^3$) for FA and DE were 18360±24167 and 242496±75096. The geometric mean particle size for DE exposure was 96.0±16.6 nm.

2.3.3 Effect of DE exposure on airway inflammatory protein markers

24 hours post-exposure, DE increased CRP (effect estimate (fold increase) [95% CI]: 4.1 [1.1 to 14.9], p=0.04), SAA (4.5 [1.2 to 16.7], p=0.03), and VCAM-1 (3.9 [1.2 to 13.0], p=0.03), but not ICAM-1, in BW collected from those with COPD (N=10) (Figure 2.4). DE also significantly increased SAA (1.7 [1.2 to 2.3], p=0.006) and VCAM-1 (1.6 [1.0 to 2.3], p=0.05), but not CRP and ICAM-1 in BAL in the COPD group. DE exposure had no detectable effect on CRP, SAA, VCAM-1 or ICAM-1 in BW or BAL from those without COPD (N=16).

There was a significant exposure-by-COPD interaction, such that those with COPD experienced a greater DE-attributable increase in CRP in BW (p=0.006), SAA in BW (p=0.002) and BAL (p=0.005), and also of VCAM-1 in BW (p=0.004) and BAL (p=0.02), compared to those without COPD. There was a

significant exposure-by-age interaction such that age augmented the DE-attributable increase in CRP ($p<0.001$), SAA ($p<0.001$), ICAM-1 ($p=0.009$) and VCAM-1 ($p<0.001$) in BW. Confining this interaction analysis to the group with COPD alone, there was not a significant exposure-by-age interaction in any of these 4 endpoints, suggesting that age did not drive the DE-attributable changes in those with COPD. Notably, there was no significant exposure-by-age interaction for any of the four proteins measured in BAL.

2.3.4 Effect of DE exposure on protease and antiprotease levels in airways

In BAL, DE exposure increased MMP-7, MMP-10 and alpha-1 antitrypsin in the COPD group (Table 2.4), while there was no detectable effect in those without COPD. Indeed, there was significant exposure-by-COPD interaction such that those with COPD experienced a greater DE-attributable increase of MMP-7 ($p=0.04$), MMP-10 ($p=0.02$), and alpha-1 antitrypsin ($p=0.005$), in BAL, and of MMP-9 ($p=0.05$), TIMP-1 ($p=0.04$), and alpha-1 antitrypsin ($p=0.05$) in BW, compared to those without COPD.

There was significant exposure-by-age interaction for MMP-7 ($p<0.001$), MMP-9 ($p=0.001$), and MMP-10 ($p=0.001$), TIMP-1 ($p<0.001$), and alpha-1 antitrypsin ($p<0.001$) in BW and for MMP-10 ($p=0.02$) in BAL. Confining this interaction analysis to the group with COPD alone, there was not a significant exposure-by-age interaction in any of these 7 endpoints. MMP-1 (LLOD = 1.9 pg/mL) and MMP-3 (LLOD = 5.5 pg/mL) were not detectable, in all BW and BAL samples.

2.3.5 Effect of DE exposure on the airway cellularity

Exposure had no significant impact on the total volume of BW and BAL collected, the total number of cells per mL of BW and BAL, percent live cells in BW and BAL, or the differential cell counts (whether as an absolute number of a given cell type per mL or percentage of that cell type amongst total cells) in BAL. As a large proportion (18 out of 26 participants) of BW samples had red blood cells and epithelial cells together representing >30% of the total, we considered BW unreliable for differential cell counts. There were no significant interaction effects of COPD or age on total or differential airway cell counts.

2.3.6 Effect of DE exposure on circulating leukocytes

Complete blood cell counts at baseline and 24 hours after each exposure were analyzed as delta values (24-hour cell count – baseline cell count). Table 2.5 summarizes the DE effect on blood cell counts; there was a significant increase in total lymphocytes (0.14 cells x 10^9/L) attributed to DE, irrespective of COPD status. There was no effect of exposure on the ratio of circulating helper T cells (% $CD4^+CD8^-$ cells / $CD3^+$ cells) and cytotoxic T cells (% $CD8^+CD4^-$ / $CD3^+$) to $CD3^+$ T cells (N=27, 1 participant with missing data for these endpoints). However, there was a significant exposure-by-COPD interaction such that those with COPD had a greater DE-attributable increase in the proportion of helper T cells (% $CD4^+CD8^-$ cells / CD3+ cells) in blood, compared to those without COPD (p=0.05). Neither the ratio between helper and cytotoxic T cells, nor the proportion of B cells ($CD3^-CD19^+$) in $CD45^+$ cells, was affected by exposure. There were no significant interaction effects of COPD or age on circulating lymphocytes.

No significant order effect was found for any of the outcomes.

2.4 Discussion

Short-term exposure to ambient fine PM is associated with increased COPD hospitalizations and mortality.[29–31] Fine PM exposure can lead to acute COPD exacerbations by increasing inflammation, oxidative stress, and immune dysfunction.[34,36] Our data suggest that exposure to short-term moderate concentrations of traffic-related air pollution may be more harmful to individuals with COPD compared to those without COPD, given the differential response in relevant markers as detailed in our results.

Specifically, CRP, SAA, and VCAM-1 were all seen to increase in COPD airways following DE exposure. CRP and SAA are markers of acute inflammation and, as soluble pattern recognition molecules, they can play a critical role in maintaining homeostasis at the airway mucosal surface.[98] VCAM-1 mediates neutrophil infiltration into the airways following PM exposure and has been shown to mediate PM-induced inflammatory lung injury in mice.[99] In the context of COPD exacerbation, SAA can promote

neutrophilic inflammation and blunt resolution of inflammation by opposing the actions of endogenous pro-resolving mediator, lipoxin A_4.[100] Increased baseline serum CRP level has been associated with increased all-cause mortality and accelerated decline in FEV_1.[101] CRP and SAA are biomarkers of COPD exacerbation and have been reported to be produced during acute COPD exacerbations after the release of proinflammatory mediators, such as IL-6, IL-1β, and TNF-α.[100,102,103] We measured IL-6, IL-1β, and TNF-α, in both BW and BAL, and did not detect a significant effect of exposure to DE (data not shown), likely due to the fact that these acute-phase proinflammatory mediators typically return to baseline in BAL in less than 24 hours following an environmental insult in humans.[104]

Nevertheless, we demonstrate that in addition to the inflammatory protein markers, DE exposure can increase MMPs and antiproteases in the airways of those with COPD relative to those without airway disease. Protease and antiprotease imbalance are thought to play a major role in COPD pathogenesis.[95,105] Although the exact role of each MMP in COPD pathology is not completely understood, my data demonstrates that DE can directly elicit an increase in airway MMPs. The sharp contrast between those with and without COPD in terms of exposure-induced airway increase in MMPs suggests one mechanism by which COPD patients may be more susceptible to the effects of air pollution.

Interestingly, there was no effect modification in ex-smokers without COPD, implying that the elevated risk attributable to acute pollution exposure was reserved to those with manifest disease. The presence of airflow obstruction leads to a greater particle deposition rate, such that those with COPD may have experienced greater fine particulate deposition relative to others at the same ambient pollution concentration,[106] effectively leading to increased overall exposure dose and resulting inflammatory effects.[36] It is also possible, as we previously demonstrated, that immune cells such as peripheral neutrophils in those with COPD may be more susceptible to activation by air pollution.[75] Likewise, other immune and structural cells of COPD airways might have been primed, through other pathological changes associated with COPD, to respond to PM exposure more prominently. Alternatively, it is possible that the cells in COPD airways may have impaired endogenous antioxidant and inflammation-resolving

capacity. Future studies to explore this alternative explanation, by looking into the epigenome and transcriptome of cells collected after DE exposures, in those with and without COPD, are encouraged.

To the best of my knowledge, this is the largest controlled human air pollution exposure study to focus on older adults and perform bronchoscopies. Nonetheless, a limitation of the study is that the COPD group was older on average than the groups without COPD, reflecting our difficulty in recruiting younger individuals with COPD who were not active tobacco users (excluded as this would potentially overpower DE exposure effects). Our data suggests that age alone confers some susceptibility independent of COPD status, another important finding that was not an initial focus of our study. Finally, this study was limited in that we measured the total abundance of proteases and antiproteases, as opposed to measuring proteolytic activity in the bronchial samples due to practical and logistical challenges associated with conducting time-sensitive assays. Given our observation of the impact DE exposure had on proteases and antiproteases, future studies should focus on measuring these endpoints in fresh samples and investigate the pathological significance of increased proteases and antiproteases upon exposure to air pollution in COPD.

As highlighted by the Lancet Commission on Pollution and Health report, research exploring emerging causal links between pollution, disease and subclinical impairment is key to combatting the global burden of pollution-related disease.[2] In the global fight against pollution, human exposure studies are critical in providing key mechanistic insights to support legislation for public health protection, and to shed light on future prevention and therapeutic approaches to protect those who are more susceptible to the harmful effects of air pollution. While epidemiological studies clearly demonstrate a relationship between TRAP and COPD morbidity and mortality, our study adds mechanistic evidence to support this concept, demonstrating that COPD patients and older adults are more susceptible to airway inflammation following acute exposure.

2.5 Tables

Table 2.1. Summary of antibodies used for flow cytometry analysis of T cells in bronchoalveolar lavage and blood

Fluorophores	Antigen (isotype)	Dilution factor (stock volume: final volume)	Supplier (Catalogue #)
FITC	CD4 (lgG1k)	1:9.5	BD (555346)
PE	CD19 (lgG1k)	1:1.9	BD (555413)
PerCP-cy5.5	CD3 (lgG1k)	1:14.25	BD (560835)
PE-Cy7	CD69 (lgG1k)	1:14.25	BD (557745)
APC-Cy7	CD45 (lgG1k)	1:11.4	BD (557833)
V450	CD8 (lgG1k)	1:7.125	BD (560347)

28.5 µL of antibody cocktail was incubated with cells (resuspended in 50 µL of PBS) for 30 minutes in the dark.

Table 2.2. Summary of the diluents and dilution factors for MSD assays

Protein target	Catalogue Number	Antibody Diluent	Sample Diluent	Sample Dilution Factor
MMP-1	F210J-3	3	7	< LLOD
MMP-3	F21ZF-3	3	7	< LLOD
MMP-7	F210K-3	12	12	1X
MMP-9	F215Q-3	3	2	1X
MMP-10	F217K-3	3	2	1X
Alpha-1 antitrypsin	F210R-3	12	12	500X
TIMP-1	F21YO-3	37	101	1X
CRP, SAA, ICAM-1, VCAM-1	K15198D	101	101	5X

Abbreviations: MMP = matrix metalloproteinase, LLOD = lower limit of detection, TIMP = tissue inhibitor of metalloproteinases, CRP = c-reactive protein, SAA = serum amyloid A, VCAM-1 = vascular cell adhesion protein 1, ICAM-1 = intercellular adhesion molecule 1.

Table 2.3. Study participant demographics

	Healthy (no COPD)	Ex-smokers (no COPD)	COPD
Participant number N (female:male)	11 (5:6)	9 (2:7)	10 (4:6)
Age Years, median (range)	55 (42-72)	56 (40-73)	67.5 (65-80)
Smoking history Pack-years, median (range)	0	30 (10.5-92.5)	15.8 (3-67.5)
Year since quit smoking Years, median (range)	N/A	16 (0.83-42)	21.5 (2-41)
FEV_1/FVC (%) post-bronchodilator; mean (range)	78.9 (74-86)	78.1 (70-86)	58 (44-69)
FEV_1 (% predicted) post-bronchodilator; mean (range)	118 (92-162)	108 (93-120)	91 (66-116)

Abbreviations: N = number of participants, FEV_1 = forced expiratory volume in 1 second; FVC= forced vital capacity; COPD = chronic obstructive pulmonary disease.

Table 2.4. Effect of DE exposure on proteases and antiproteases in the airways

		No COPD (N=16)		COPD (N=10)	
Protein	Sample	DE effect [95% CI]	P Value	DE effect [95% CI]	P Value
MMP-7	BW	0.6 [0.2 to 1.4]	0.18	4.2 [0.6 to 30.0]	0.13#
	BAL	1.1 [0.6 to 2.0]	0.79	3.1 [1.2 to 8.2]	**0.03**#
MMP-9	BW	0.5 [0.2 to 1.4]	0.18	4.5 [0.3 to 63.3]	0.22#
	BAL	0.9 [0.6 to 1.3]	0.42	1.4 [0.5 to 3.7]	0.50
MMP-10	BW	0.6 [0.2 to 1.6]	0.28	4.2 [0.5 to 38.1]	0.17#
	BAL	0.8 [0.5 to 1.2]	0.23	2.6 [1.4 to 4.9]	**0.008**#
MMP-12	BW	0.7 [0.2 to 1.9]	0.42	1.4 [0.4 to 5.4]	0.98
	BAL	1.2 [0.7 to 2.1]	0.41	0.9 [0.3 to 2.6]	0.82
Neutrophil elastase	BW	0.9 [0.3 to 3.2]	0.86	0.7 [0.1 to 4.7]	0.71
	BAL	1.0 [0.7 to 1.4]	0.83	1.1 [0.6 to 2.1]	0.73
TIMP-1	BW	0.5 [0.2 to 1.5]	0.19	4.1 [0.5 to 35.5]	0.17#
	BAL	0.9 [0.7 to 1.3]	0.64	1.6 [0.7 to 3.6]	0.22
Alpha-1 antitrypsin	BW	0.4 [0.1 to 1.6]	0.19	4.1 [0.5 to 36.4]	0.17#
	BAL	0.7 [0.5 to 1.1]	0.08	2.0 [1.0 to 3.9]	**0.05**#

Abbreviations: CI = confidence interval; COPD = chronic obstructive pulmonary disease; MMP = matrix metalloproteinase; BW = bronchial wash; BAL = bronchoalveolar lavage; TIMP-1 = tissue inhibitor of metalloproteinase-1.

Effect estimates and associated confidence intervals are presented as back-log transformed values. Therefore, a given effect estimate represents the ratio of fold changes induced by DE exposure relative to the control (FA). Significant ($p \leq 0.05$) effect of exposure indicated by **bold**. # indicates significant exposure-by-COPD interaction.

Table 2.5. Effect of diesel exhaust exposure on blood cell counts

	DE effect [95% CI]	P Value
WBC	+0.28 [-0.14 to 0.69]	0.20
Neutrophils	+0.14 [-0.23 to 0.51]	0.45
Lymphocytes	+0.14 [0.05 to 0.23]	**0.005**
Monocytes	+0.14 [-0.09 to 0.38]	0.24
Eosinophils	+0.02 [-0.02 to 0.05]	0.35
Basophils	+0.01 [0.00 to 0.03]	0.16

Definition of abbreviations: CI = confidence interval; WBC = white blood cells. Cell count values are expressed as unit change relative to filtered air (cells x 10^9/L). Significant ($p \leq 0.05$) effect of exposure indicated by **bold**.

2.6 Figures

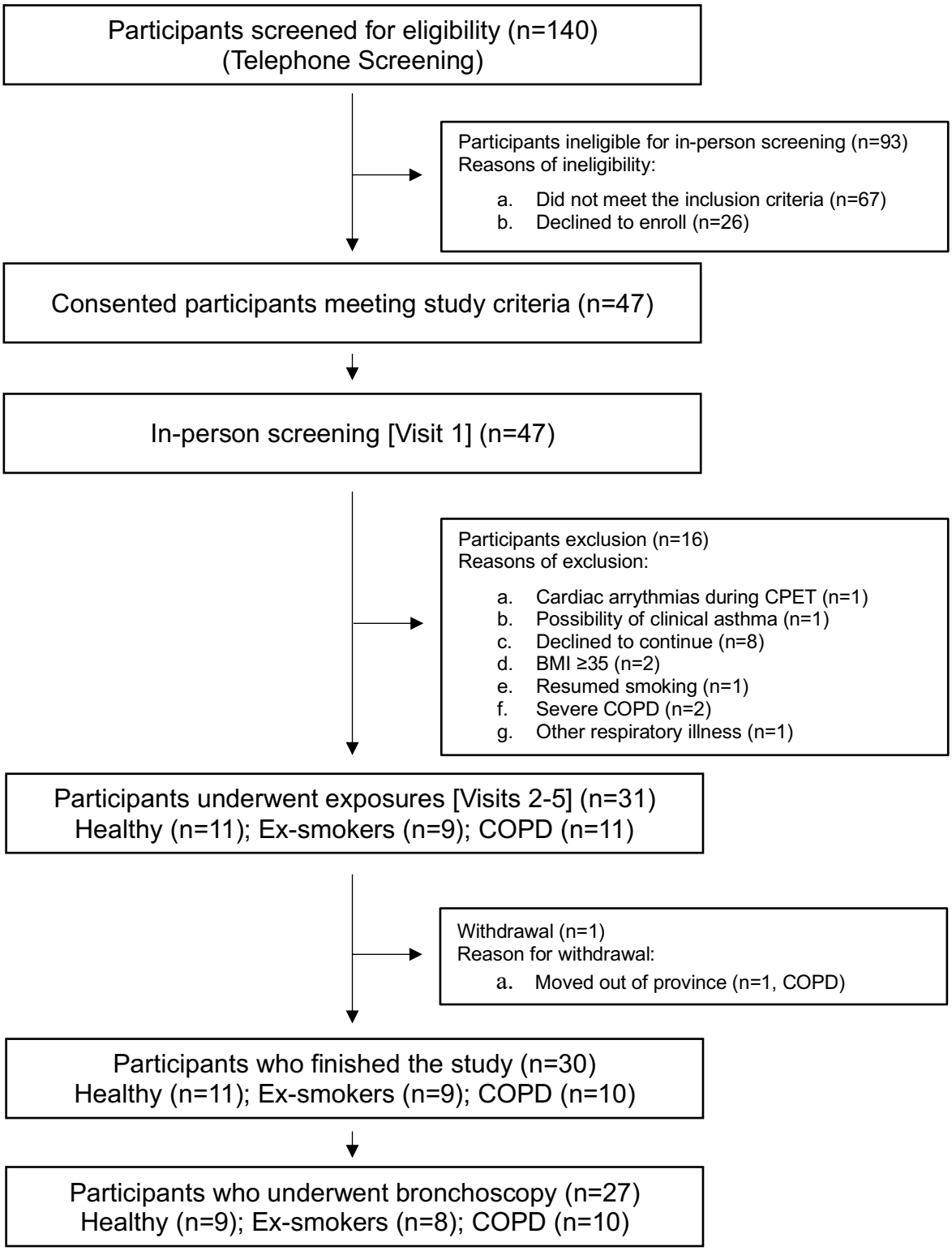

Figure 2.1. Participant recruitment flow chart.

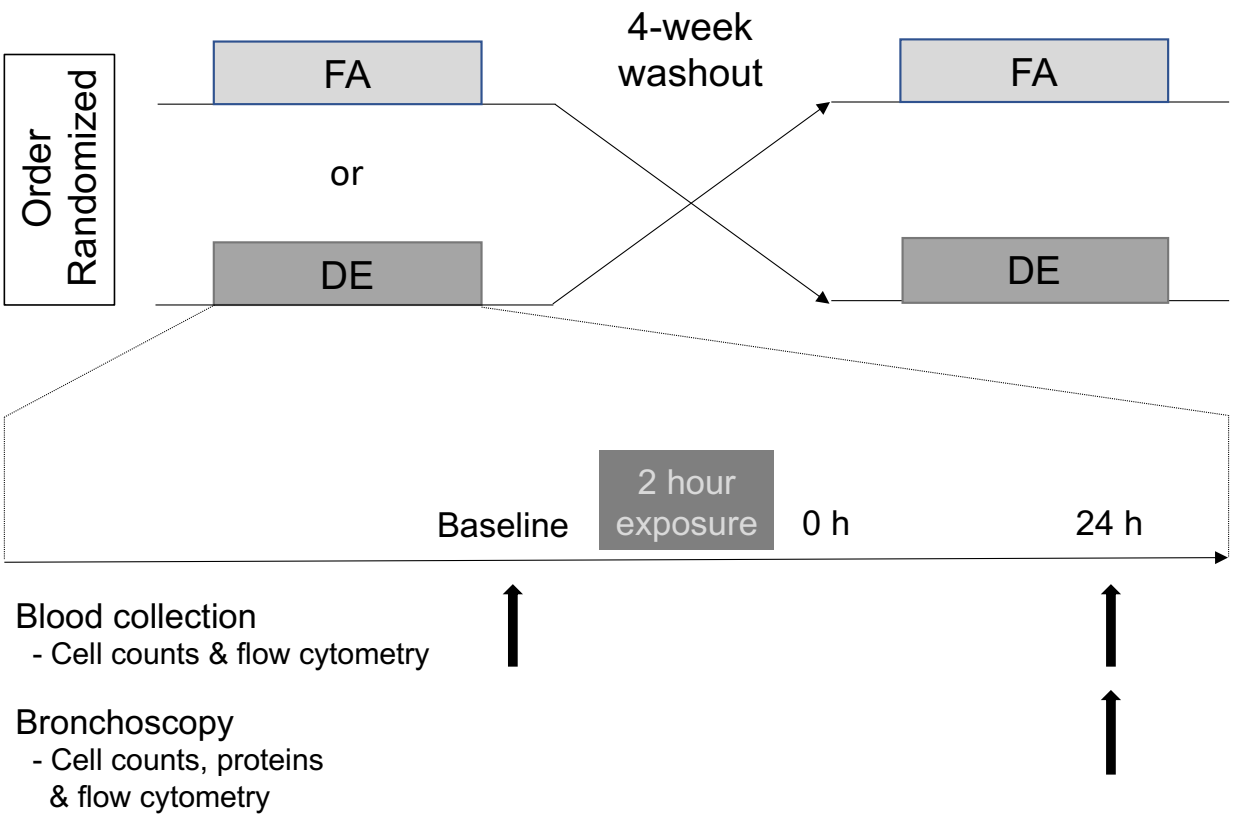

Figure 2.2. Study design and sampling time. We performed a randomized, double-blinded, controlled human exposure crossover study to diesel exhaust (DE) and filtered air (FA). DE was diluted to a nominal $PM_{2.5}$ mass concentration of 300 $\mu g/m^3$. 30 research participants (11 healthy never-smokers, 9 ex-smokers (>10 pack years), and 10 ex-smokers with chronic obstructive pulmonary disease) were exposed for 2 hours on separate occasions to DE and FA, in random order, separated by a ≥4-week washout. Blood was collected before and 24 hours after each exposure just prior to a bronchoscopy.

Chapter 3. Transcriptomic response of COPD epithelium to traffic-related air pollution is different from that of healthy airway epithelium

3.1 Introduction

Chronic obstructive pulmonary disease (COPD) is a complex and heterogeneous disease. COPD is characterized by a limitation in airflow that is not fully reversible, abnormal inflammatory responses in the small airways and alveoli, and immune-mediated destruction of alveolar walls. These three features manifest as emphysema and chronic bronchitis in COPD patients.[107] Acute exacerbation of COPD, which increases symptoms and is associated with increased mortality, can be instigated due to a range of triggers, with cigarette smoke and air pollution exposure being two of the main culprits.[34,108,109]

Short-term exposure to ambient fine particulate matter (PM) is associated with increased COPD hospitalization and mortality.[29–31] Particles with an aerodynamic diameter of 0.1 μm or less can travel deep into the lungs and cause inflammation.[110] The presence of airflow obstruction leads to a greater particle deposition rate, such that those with COPD may experience greater particulate deposition relative to healthy individuals at the same ambient pollution concentration,[106] effectively leading to increased overall exposure dose.[36] In COPD, the respiratory tract's inflammatory response to toxic inhalants appear to have been modified by chronic bronchitis and emphysema, with amplified and prolonged inflammation being observed in COPD patients exposed to chronic irritants.[40,111] However, how diseased tissue induce increased susceptibility to inflammatory triggers and their subsequent harmful effects requires further investigation. In this regard, molecular profiling of the COPD airways after an acute exposure to air pollutants would increase our understanding of the complex gene-environment interactions that may contribute to COPD pathogenesis and progression.

To better understand COPD phenotypes and pathogenesis, large-scale population studies (e.g., COPDGene, ECLIPSE, GenKOLS, NETT/NAS, and UK-BiLevel) utilized genome and transcriptome profiling and analysis tools to identify common molecular mechanisms.[112–118] Early RNA-seq profiling

identified gene expression and pathway signatures associated with COPD and smoking in these studies, further supporting the link between cigarette smoking and COPD pathogenesis.[113,114] In general, genes whose differential expression in airway epithelial cells is associated with tobacco smoking and COPD code for proteins involved in inflammation and oxidative stress responses.[119] Thus, it is critical to detail whether genes involved in inflammation and oxidative stress responses, are differentially expressed following DE exposure in COPD epithelium *in vivo,* and then compare the response between healthy and COPD.

In addition to understanding inflammation and oxidative stress responses, mucin production and its differential gene expression would also be revealing of epithelial function in response to air pollution. Airway mucin concentration is a marker of chronic bronchitis in COPD, and mucin accumulation is believed to induce sputum production, increase airway inflammation, cause infections, and create airflow obstruction.[120,121] Upregulation of *MUC5AC* and *MUC5B* in response to PM has been demonstrated *in vitro*.[122] However, it is not clear whether short-term exposure to traffic-related air pollution (TRAP) can cause upregulation of mucin genes *in vivo*.

To the best of my knowledge, no studies to date have assessed the direct impact of TRAP on the transcriptome profile of COPD epithelium *in vivo.* Moreover, it is unclear whether short-term exposure to TRAP, as is typically found in congested urban areas, elicits a distinct transcriptional epithelial response in the lower respiratory tract of COPD patients. In order to provide insight into the differential susceptibility between healthy never smokers, ex-smokers, and those with mild-moderate COPD, we evaluated the impact of DE exposure on the airway epithelial transcriptome *in vivo*. I will test the hypotheses that:

1. *DE exposure leads to upregulation of genes involved in the antioxidant response.*
2. *DE exposure leads to upregulation of mucin genes (MUC5AC, MUC5B, MUC1, and MUC16).*
3. *COPD epithelium mounts different transcriptional responses to DE exposure compared to healthy epithelium.*

3.2 Methods

This study was approved by the University of British Columbia clinical research ethics board (H14-00821) and Vancouver Coastal Health Research Institute (V14-00821). All participants provided written informed consent before inclusion. This study was registered at ClinicalTrial.gov with identification number NCT02236039. The outcomes reported here were secondary endpoints.

3.2.1 Participant selection

Three groups of participants aged 40 - 80 were recruited: 1) healthy never smokers without COPD (NS group), 2) ex-smokers with >10 pack-year tobacco smoking history and without COPD (ES group), and 3) ex-smokers with mild to moderate COPD (FEV_1/FVC <0.7 and FEV_1% pred ≥50%) (COPD group). Exclusion criteria were as follows: pregnant or breastfeeding, allergy to anesthetics and other study medications, participation in another clinical study with a medication intervention arm, unstable COPD symptoms, a body mass index <19.5 or >34.9 kg/m^2, current smoker or quit smoking <6 months prior to study screening, a smoking history of 0-10 pack-years (in the ES group), and/or diagnosed with asthma. Additional exclusion criteria include regular and frequent use of inhaled corticosteroids, bronchodilators, antihistamines, non-steroidal anti-inflammatories, anticoagulants or decongestants, and/or significant comorbidities considered by the principal investigator to increase risk.

All participants self-reported ≥6 months of smoking cessation prior to study enrolment, which continued throughout the study. Serum cotinine test was used to exclude active and passive tobacco smoking. COPD participants abstained from short- and long-acting bronchodilators and inhaled corticosteroid use for 48 hours prior to visits. Visits were rescheduled if participants exhibited signs of upper respiratory tract infection (Common Cold Questionnaire score greater than 3).[97] Figure 2.1 describes recruitment process and how many participants completed exposures and research bronchoscopies. Only participants who underwent bronchoscopy and had RNA from bronchial brushing samples pass quality control (n=25) were

included for RNA-seq analysis in this chapter. One research participant from the main study opted out of endobronchial sampling and was excluded from RNA-seq analysis.

3.2.2 Human exposure

Controlled human exposure took place at the Air Pollution Exposure Laboratory in Vancouver, Canada, using the facility and protocols described previously.[20,75,96] Each participant was exposed for 2 hours on separate occasions to DE (at a nominal concentration of 300 μg/m^3 of particulate matter ≤2.5 μm in aerodynamic diameter [$PM_{2.5}$]) and filtered air (FA). Figure 3.1 shows study design and laboratory and bioinformatics workflows. DE was generated using a Yanmar L 100 EE4-stroke diesel engine with a constant load of 2.5 kW applied throughout the exposure. The exposure booth was maintained in negative pressure during the exposure, and exhaust airflow was controlled in real-time by the staff engineer to maintain $PM_{2.5}$ nominally at 300 μg/m^3. For FA condition, the diesel generator was in operation with the same load conditions as the DE exposure, with the exhaust diverted from the dilution and exposure system. Instead, during the FA condition, incoming air to the exposure booth was twice filtered (with high-efficiency particulate air filters) room air. Each participant was randomized to the order of exposure (FA first or DE first) at the time of enrollment. The exposure condition was only known to the exposure facility engineer. There was a minimum 4-week washout period between exposures.

3.2.3 Sample collection and RNA extraction

Flexible bronchoscopy was performed 24 hours after each exposure to collect endobronchial brushing samples. Each endobronchial brushing sample was immediately placed in 1 mL of DNA/RNA Shield (Zymo Research) and stored at -80 °C. RNA was extracted using the Zymo Quick-DNA/RNA Miniprep Plus Kit (Zymo Research) following the manufacturer's protocol after the conclusion of the study. Briefly, frozen samples were brought to room temperature and vortexed for 30 seconds. Thawed DNA/RNA Shield solution was transferred to a 15 mL conical tube and replaced with 1 mL of DNA/RNA lysis buffer. The endobronchial brush was vortexed with the lysis buffer, and the solution was

carefully pipetted and combined with DNA/RNA shield in the 15 mL conical tube. The manufacturer's purification protocol was subsequently followed to isolate DNA and RNA. Isolated RNA was eluted in 50 µL DNAse/RNase-Free Water. RNA was initially quantified using NanoDrop (Thermo Fisher Scientific) and stored at -80 °C.

Subsequent procedures, sample quality check using Bioanalyzer (Agilent), cDNA synthesis, library preparation and RNA sequencing (NovaSeq 6000, Illumina), were performed by Génome Québec, a centralized sequencing facility, according to their standard operation procedures. mRNA enrichment was performed using the NEBNext Poly(A) Magnetic Isolation Module (New England BioLabs). cDNA synthesis was achieved with the NEBNext RNA First Strand Synthesis and NEBNext Ultra Directional RNA Second Strand Synthesis Modules (New England BioLabs). The remaining steps of library preparation were done using and the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs). Adapters and PCR primers were purchased from New England BioLabs. Libraries were quantified using the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems). The average size fragment was determined using a LabChip GXII (PerkinElmer) instrument. RNA samples with a RIN value greater than 6.5 were sequenced. RNA samples were sequenced using NovaSeq 6000 (Illumina); 80 libraries were multiplexed per lane on NovaSeq S4 PE 100bp chip to achieve ~30 M reads/sample.

3.2.4 RNA-Seq bioinformatics analysis and statistical comparison

Raw sequencing files (FASTQ files) were quality checked with FastQC (v.0.11.8). Salmon (v.1.4.0, https://combine-lab.github.io/salmon/) based on Ensembl-version 98 reference transcriptome was used to generate counts files. Differential gene expression analysis based on the negative binomial distribution was performed in RStudio (v.1.3.1093, RStudio), using DESeq2 (v.1.30.0, Bioconductor.org) package.[123] R (v.4.0.3, The R Foundation) was used with RStudio. Z-scores, from the DESeq2 outputs, were adjusted for false discovery rate using fdrtool (v.1.2.16) with a cut-off value set at 0.75, and the adjusted p-values

were calculated using the Benjamini & Hochberg method in stats package with a false discovery rate (FDR) of 10% (v.4.0.3).[124] Adjusted p-value less than 0.1 was considered significant.

3.3 Results

Figure 2.1 describes how the study participants were recruited and how many participants underwent and completed exposures and research bronchoscopies. Characteristics of the 25 study participants who were included in the analysis of this chapter are summarized in Table 3.1. These were participants who underwent bronchoscopies and collected samples passed quality control.

3.3.1 DE exposure elicited changes in gene expression of airway epithelium *in vivo*

I first sought to identify gene expression changes in response to DE exposure by comparing DE against FA exposure in each of the three participant groups: NS, ES, and COPD. These comparisons revealed 35, 131, and 10 differentially expressed genes (DEGs) in response to DE exposure in NS, ES, and COPD epithelium, respectively (Figure 3.2). DEGs in each group are listed in Table 3.2, 3.3, and 3.3. Of note, none of the 10 DE exposure-induced DEGs in the COPD group were altered in the ES and NS groups. In addition, 9 out of 10 DEGs in the COPD epithelium were down-regulated. Three common genes that significantly changed in both NS and ES epithelium were *GPX2, PSMC1P1,* and *TRIM16L* (Figure 3.2D). Table 3.4 lists DE exposure-induced DEGs in all the participants without COPD (ES and NS group combined).

3.3.2 DE exposure increased genes in the Nrf2 pathway in ex-smokers but not in COPD

To identify significant pathways activated by the DE exposure, I performed functional enrichment analysis (https://biit.cs.ut.ee/gprofiler/gost) using DEGs in each group. The functional enrichment analysis mapped DEGs in the ES group (listed in Table 3.3) to known functional information in four pathways (WikiPathways (WP) database): the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway (WP2884, adjusted p-value 0.001), pentose phosphate metabolism (WP134, adjusted p-value 0.002), nuclear receptors meta-pathway (WP2882, adjusted p-value 0.01), and glutathione metabolism (WP100,

adjusted p-value 0.04). DE exposure-induced DEGs in COPD and NS did not map to any significant enriched pathways. Functional enrichment analysis was performed on DE exposure-induced DEGs in non-COPD participants, listed in Table 3.4; Figure 3.3 summarizes the result of functional enrichment analysis for non-COPD epithelium.

Genes included in the curated Nrf2 pathway are available online in the WikiPathways (https://www.wikipathways.org/instance/WP2884_r106658). Notably, DE exposure induced significant increases in the following Nrf2 pathway related genes: *GSTP1*, *PRDX1*, *NQO1*, *G6PD*, *GPX2*, *ME1*, *ADH7*, *ALDH3A1*, and *GCLC* (Figure 3.4). None of these genes were significantly changed following DE exposure in COPD epithelium.

3.3.3 DE exposure did not significantly change the expression of mucin genes in the airway epithelium

Based on the normalized count of transcripts, *MUC5AC*, *MUC5B*, *MUC1*, and *MUC16* were the most expressed mucin genes in the airway epithelium. However, there were no significant changes observed in any one of the four mucin genes in the airway epithelium 24 hours after DE exposure (Figure 3.5).

3.3.4 DE exposure elicited a divergent transcriptional response by COPD airway epithelium compared to the non-COPD epithelium

As seen in Figure 3.2D, there was no overlap of DEGs between COPD and the other control groups. To test the hypothesis that COPD status modified DE effects on the transcriptome of bronchial cells, I performed exposure-by-COPD status interaction analysis using DESeq2. In this interaction analysis, I identified that COPD status modified DE effects on 156 genes. Of these genes, 17 genes had low or no expression (< 20 normalized count/sample) in COPD or non-COPD participants. Of the remaining 139 genes modified by COPD status (Figure 3.6), *PSMB3* was the only gene significantly increased by DE exposure in COPD after false discovery correction.

3.4 Discussion

Although cigarette smoking is a significant risk factor for COPD, only a subset of smokers develops COPD. Likewise, not everyone that is chronically exposed to TRAP develops COPD. Lifetime total exposure to toxic inhalants (e.g., cigarette smoke and air pollutants), genetic factors, and other comorbidities determine whether someone develops COPD.[40] I hypothesize that variation in the mucosal immune response to environmental insults modulate the extent of damage to lung tissue caused by environmental exposures. Thus, probing tissue responses to toxic inhalants in those at risk of COPD (ex-smokers) or those who have mild COPD is crucial to identifying susceptible individuals at the early stages of COPD development and understanding COPD pathophysiology.

Consequently, we evaluated the change in epithelial transcriptome upon exposure to DE *in vivo* in older adults with and without COPD. We then compared the transcriptomic responses of COPD airways to that of healthy airways. In participants without COPD (NS and ES groups combined), our data confirmed the upregulation of antioxidant genes involved in the Nrf2 pathway following DE exposure (Figure 3.3 & 3.4). This transcriptional response was attenuated in participants with COPD, in part because some antioxidant genes were upregulated at baseline in some COPD patients.

In vitro, epithelial cells from COPD donors have less antioxidant capacity than epithelial cells from healthy donors, as measured by *in vitro* total antioxidant response to free radical reactions and glutathione disulfide/reduced glutathione ratio.[65,125] As a result, repeated exposures to particulate matter elicits a greater increase in oxidative damage markers in COPD cells than in healthy cells.[65] Large cohort transcriptome studies had shown that *GPX2*, a gene in the Nrf2 pathway, is upregulated in smokers with COPD compared to smokers without COPD, and other Nrf2 pathway genes—*G6PD*, *GPX2*, and *NQO1*—are elevated in active smokers in comparison to nonsmokers.[119] Methylation of DNA CpG islands are broadly increased in small airway epithelia of COPD patients.[126,127] The genes targeted by differential methylation in COPD airways downregulate Nrf2 expression, resulting in the loss of phase II metabolizing enzymes that provide antioxidant protection.[127] Moreover, downregulation of histone

deacetylase 2 by tobacco smoke impairs Nrf2 activation in the lung by decreasing the half-life of Nrf2.[128] Taken together, evidence to date suggests that COPD epithelium may have alterations in the genetic and epigenetic regulation of antioxidant genes leading to suppressed antioxidant responses. My study also indicates that COPD epithelium may have less antioxidant capacity and supports the notion that having mild-moderate COPD may confer increased risk to oxidative stress damage in the airways from subsequent exposure to air pollutants. In chapter 2, I showed an increase in C-reactive proteins, serum amyloid A, and matrix metalloproteinase-7 and -10 following DE exposure in the COPD group only. Combined with transcriptome data, we can speculate that aberrant inflammatory response to DE in COPD may in part be due to the compromised antioxidant response.

I did not see any significant changes in *MUC5AC*, *MUC5B*, *MUC1*, and *MUC16* expression levels in response to DE exposure (Figure 3.5). *In vitro*, exposure to diesel exhaust particles increases *MUC5AC*, *MUC5B* and *MUC4* mRNA expression.[122,129] Our research group have shown in a controlled human exposure study—albeit in asthmatic individuals—that DE exposure increased mucin protein levels in the bronchoalveolar lavage.[19] It is possible that any increase in mRNA peaked at an earlier timepoint. Alternatively, DE exposure may have only impacted the protein level or had no impact even on protein levels. In human airways, mucin mRNA transcript and protein levels vary significantly between individuals. Our analysis revealed that there was one order of magnitude variability in mucin transcript level between individuals. Similarly, a larger COPD cohort showed that total mucin protein concentration in COPD airways could differ in one to two orders of magnitude.[120] Therefore, measuring other characteristics of mucus may be more informative. Future studies can directly measure solid concentration[130] and mechanical properties (stiffness, spinnability, and viscosity) of mucus.[131] To allow for these measurements, unfiltered bronchoalveolar lavage should be collected; we used mesh-filtered in processing BAL in this study to prioritize other endpoints.

Of the 11 genes whose expression was altered by exposure to DE in COPD epithelium, *PSMB3* may be of interest to follow up in future studies. *PSMB3* codes for proteasome subunit beta type-3. The ubiquitin-

proteasome system is the main protein waste disposal and recycling system of the cells but cigarette smoke inhibits this system in airway epithelial cells, partially through increased intracellular oxidative stress.[132,133] In mice, cigarette smoke exposure and impaired proteasomal function enhances apoptotic cell death in the alveolar walls, suggesting that a decrease in proteasomal function may accelerate cigarette smoke-induced pulmonary emphysema.[134] Future studies can probe ubiquitin-proteasome dysfunction in the context of air pollution exposure and whether that may contribute to COPD pathophysiology.

Another DE-induced gene in COPD to be follow up on is *STK36*. *STK36* codes for serine/threonine protein kinase 36, and when mutated, result in primary ciliary dyskinesia.[135] Defective mucociliary clearance seen in primary ciliary dyskinesia results in recurrent infection and inflammation of the upper and lower airways.[135] Indeed, repeated exposure to cigarette smoke impairs respiratory epithelial ciliogenesis.[136] Future studies should test whether *STK36* can be downregulated in COPD epithelial cells by exposure to diesel exhaust particles *in vitro* and test whether the downregulation results in changes in cilia function.

One of the limitations of the study was that we used bulk RNA-seq analysis. Thus, we were not able to capture and explore cellular heterogeneity of the human airway epithelium in COPD compared to healthy. We cannot rule out that the attenuated transcriptome response of COPD epithelium to DE was in part due to differences in cell population distributions. It is well documented that the proportion of club cells—which are specialized epithelial cells that secrete anti-inflammatory proteins and are involved in antioxidant response—is decreased in the COPD eptihelium.[137] Goblet and basal cell hyperplasia resulting in thickening of the epithelial cell layers is also seen in COPD airways.[138] Therefore, it is possible that attenuated transcriptomic responses to DE in COPD may be the result of remodeled airways. Future studies utilizing single-cell RNA-seq analysis would provide much deeper insight into how remodelled airway in COPD may have increased susceptibility to TRAP.

In summary, we demonstrated that exposure to TRAP, typical of congested urban areas, increases expression of antioxidant genes in the airway epithelium *in vivo*, and this expression was modified in the

COPD epithelium. Our future work should aim to integrate the transcriptome data with the DNA methylome data. By interrogating epigenetic perturbation due to DE exposure, we may gain deeper insight into the molecular mechanism contributing to COPD's susceptibility to harmful effects of TRAP exposure. Moreover, future work should continue to assess the role of gene-environment interaction in detailing COPD development and progression in the context of TRAP exposures.

3.5 Tables

Table 3.1. Participant demographics for RNA-seq analysis

	Never-smokers (no COPD)	Ex-smokers (no COPD)	Ex-smokers (with COPD)
Participant number N (female:male)	9 (3:6)	7 (0:7)	9 (3:6)
Age Year, median (range)	55 (47 - 72)	61 (45-73)	67 (65-80)
Smoking history Pack-year, median (range)	NA	47 (10.5-92.5)	19.5 (3-67.5)
Year since quit smoking Year, median (range)	NA	16 (0.83-42)	16 (2-41)
FEV_1/FVC Post-bronchodilator, mean (range)	77.9 (74-80)	76.9 (70-86)	59.1 (44-69)
FEV_1 (% predicted) Post-bronchodilator, mean (range)	122 (101-162)	107 (93-120)	91 (66-116)

Definitions: COPD = chronic obstructive pulmonary disease, N = number of participants, pack-year = number of packs of cigarettes (20 cigarettes/pack) smoked per day multiplied by the number of years the person has smoked, FEV_1 = forced expiratory volume in 1 second, FVC = forced vital capacity.

Table 3.2. Significant gene expression changes after diesel exhaust exposure in NS group's airway epithelium

Gene ID	Gene Symbol	Base Mean	Log_2 Fold Change	p_{adj}
ENSG00000084207	GSTP1	42148	0.27	0.05
ENSG00000130707	ASS1	3793	0.27	0.09
ENSG00000101310	SEC23B	3322	0.18	0.08
ENSG00000051620	HEBP2	2747	0.17	0.03
ENSG00000167085	PHB	2520	0.24	0.09
ENSG00000160932	LY6E	2162	0.50	0.05
ENSG00000228327	AL669831.1	1873	-0.30	0.05
ENSG00000151632	AKR1C2	1835	0.50	0.05
ENSG00000167553	TUBA1C	1739	0.38	0.05
ENSG00000275993	SIK1B	1467	0.58	<0.10
ENSG00000112699	GMDS	1049	0.28	0.08
ENSG00000167656	LY6D	808	0.76	<0.10
ENSG00000171931	FBXW10	799	-0.25	0.08
ENSG00000176153	GPX2	590	0.50	<0.10
ENSG00000204304	PBX2	535	-0.49	0.03
ENSG00000108448	TRIM16L	533	0.35	0.06
ENSG00000205420	KRT6A	467	1.45	0.02
ENSG00000171401	KRT13	328	1.61	<0.001
ENSG00000137440	FGFBP1	324	0.64	0.05
ENSG00000241506	PSMC1P1	274	-0.34	0.05
ENSG00000223839	FAM95B1	267	-0.80	0.06
ENSG00000276935	MBOAT7	248	-1.06	0.03
ENSG00000112473	SLC39A7	209	-11.15	0.06
ENSG00000185304	RGPD2	201	-1.31	0.06
ENSG00000188933	USP32P1	176	-0.92	0.04
ENSG00000226936	B3GALT4	155	-13.38	0.01
ENSG00000276429	PLCH2	145	-13.98	<0.01
ENSG00000154277	UCHL1	126	0.88	0.04
ENSG00000137313	TRIM26	116	-12.15	0.01
ENSG00000278016	TIGD5	110	11.45	0.05
ENSG00000244682	FCGR2C	53	-0.81	0.05
ENSG00000188282	RUFY4	39	-0.90	0.08
ENSG00000169469	SPRR1B	32	2.17	0.04
ENSG00000244398	AC116533.1	29	-10.92	0.08
ENSG00000206290	COLL11A2	7	4.27	0.05

Differential expression analysis based on the negative binomial distribution was performed using DESeq2 package.[123] Adjusted p-values, p_{adj}, were calculated using the Benjamini-Hochberg method implemented in DESeq2 and fdrtool packages in RStudio.

Table 3.3. Significant gene expression changes after diesel exhaust exposure in ES group's airway epithelium

	Gene ID	Gene Symbol	Base Mean	Log_2 Fold Change	p_{adj}
1	ENSG00000117450	PRDX1	19678	0.29	<0.001
2	ENSG00000108602	ALDH3A1	16887	0.86	0.001
3	ENSG00000181019	NQO1	11617	0.38	<0.001
4	ENSG00000090382	LYZ	9491	-0.29	<0.001
5	ENSG00000197446	CYP2F1	9379	-0.22	<0.001
6	ENSG00000102804	TSC22D1	7341	-0.20	0.003
7	ENSG00000001084	GCLC	6444	0.33	0.003
8	ENSG00000196344	ADH7	5987	0.53	<0.001
9	ENSG00000177156	TALDO1	5404	0.27	<0.001
10	ENSG00000163931	TKT	4022	0.31	0.002
11	ENSG00000168710	AHCYL1	3572	0.14	0.04
12	ENSG00000167757	KLK11	3521	-0.28	0.04
13	ENSG00000127954	STEAP4	3244	-0.42	0.02
14	ENSG00000080493	SLC4A4	2205	-0.31	0.003
15	ENSG00000113657	DPYSL3	2091	0.39	0.04
16	ENSG00000184916	JAG2	2015	-0.26	0.05
17	ENSG00000166483	WEE1	1961	-0.23	0.06
18	ENSG00000111912	NCOA7	1912	-0.41	0.09
19	ENSG00000162772	ATF3	1858	-0.54	0.06
20	ENSG00000185950	IRS2	1775	-0.50	0.01
21	ENSG00000196502	SULT1A1	1612	0.23	0.008
22	ENSG00000162402	USP24	1523	-0.28	0.001
23	ENSG00000122420	PTGFR	1448	-0.42	0.001
24	ENSG00000129292	PHF20L1	1399	0.18	0.02
25	ENSG00000160211	G6PD	1263	0.18	0.05
26	ENSG00000166828	SCNN1G	1248	-0.44	<0.001
27	ENSG00000115594	IL1R1	1164	-0.28	0.002
28	ENSG00000176153	GPX2	1110	0.83	<0.001
29	ENSG00000149311	ATM	1098	-0.19	0.06
30	ENSG00000087842	PIR	1098	0.38	<0.001
31	ENSG00000115657	ABCB6	1095	0.42	0.002
32	ENSG00000081320	STK17B	1045	-0.27	0.07
33	ENSG00000101448	EPPIN	956	-0.24	0.08
34	ENSG00000167183	PRR15L	825	0.32	0.002
35	ENSG00000162591	MEGF6	786	-0.43	0.003
36	ENSG00000285437	POLR2J3	763	0.24	0.03
37	ENSG00000197838	CYP2A13	741	-0.46	0.005
38	ENSG00000144476	ACKR3	728	-0.32	0.02
39	ENSG00000148468	FAM171A1	710	0.29	0.09
40	ENSG00000143702	CEP170	674	0.30	0.004
41	ENSG00000157693	TMEM268	642	-0.29	0.05
42	ENSG00000180758	GPR157	630	0.38	0.003
43	ENSG00000275246	TLCD2	591	0.58	0.01
44	ENSG00000108448	TRIM16L	558	0.37	<0.001

	Gene ID	Gene Symbol	Base Mean	Log_2 Fold Change	p_{adj}
45	ENSG00000112303	VNN2	546	-0.32	0.04
46	ENSG00000170485	NPAS2	542	-0.45	0.04
47	ENSG00000143493	INTS7	506	-0.34	0.003
48	ENSG00000065833	ME1	482	0.51	<0.001
49	ENSG00000211592	IGKC	462	-1.68	0.03
50	ENSG00000198074	AKR1B10	454	0.62	0.02
51	ENSG00000168743	NPNT	447	-0.35	0.05
52	ENSG00000204574	ABCF1	438	-0.55	0.06
53	ENSG00000142347	MYO1F	437	-0.41	0.005
54	ENSG00000125375	DMAC2L	432	0.22	0.09
55	ENSG00000282633	IGHA1	430	-1.78	0.01
56	ENSG00000127946	HIP1	428	0.32	0.04
57	ENSG00000049769	PPP1R3F	414	-0.33	0.05
58	ENSG00000178385	PLEKHM3	399	-0.31	0.006
59	ENSG00000150756	ATPSCKMT	392	0.33	0.05
60	ENSG00000113924	HGD	341	0.52	0.09
61	ENSG00000188175	HEPACAM2	340	-0.44	0.05
62	ENSG00000277633	IGHG1	327	-2.22	0.04
63	ENSG00000100234	TIMP3	317	-0.57	0.001
64	ENSG00000274442	PRPF8	291	-11.67	<0.001
65	ENSG00000241506	PSMC1P1	231	0.57	0.01
66	ENSG00000110799	VWF	217	0.65	0.01
67	ENSG00000234798	DXO	215	-0.79	0.05
68	ENSG00000053524	MCF2L2	205	0.38	0.006
69	ENSG00000282184	IGHG3	180	-2.61	0.004
70	ENSG00000236177	HLA-DPA1	170	0.72	0.002
71	ENSG00000164692	COL1A2	166	-1.05	0.02
72	ENSG00000206279	DAXX	158	-1.17	0.05
73	ENSG00000011465	DCN	150	-1.16	0.02
74	ENSG00000142173	COL6A2	142	-1.58	0.0006
75	ENSG00000178226	PRSS36	139	0.80	0.004
76	ENSG00000124224	PPP4R1L	137	0.50	0.09
77	ENSG00000177519	RPRM	130	-0.65	0.04
78	ENSG00000166183	ASPG	121	0.56	0.06
79	ENSG00000204681	GABBR1	115	-0.77	0.007
80	ENSG00000284320	SHMT1	113	13.26	<0.001
81	ENSG00000174059	CD34	111	-0.75	0.01
82	ENSG00000108342	CSF3	100	-0.86	0.05
83	ENSG00000163520	FBLN2	94	-0.64	0.04
84	ENSG00000250264	AL669918.1	90	-1.11	0.09
85	ENSG00000197140	ADAM32	84	-1.07	0.06
86	ENSG00000224180	CCHCR1	78	-1.22	0.05
87	ENSG00000166069	TMCO5A	78	0.63	0.01
88	ENSG00000270136	MICOS10-NBL1	71	0.71	0.04
89	ENSG00000177370	TIMM22	70	-10.89	0.001
90	ENSG00000018236	CNTN1	68	0.74	0.08
91	ENSG00000166482	MFAP4	67	-1.38	0.01

	Gene ID	Gene Symbol	Base Mean	Log_2 Fold Change	p_{adj}
92	ENSG00000211679	IGLC3	65	-2.31	0.001
93	ENSG00000168542	COL3A1	65	-1.12	<0.1
94	ENSG00000173535	TNFRSF10C	55	-0.84	0.05
95	ENSG00000179388	EGR3	55	-1.46	0.01
96	ENSG00000211675	IGLC1	53	-1.36	0.06
97	ENSG00000184156	KCNQ3	50	-0.93	0.02
98	ENSG00000274404	GOLGA6L22	48	1.65	0.05
99	ENSG00000203618	GP1BB	45	-10.10	0.003
100	ENSG00000103426	CORO7-PAM16	45	-1.16	<0.001
101	ENSG00000226560	C2	42	3.78	0.003
102	ENSG00000167210	LOXHD1	40	0.98	0.07
103	ENSG00000182492	BGN	38	-2.05	0.02
104	ENSG00000107317	PTGDS	37	-2.52	0.004
105	ENSG00000113361	CDH6	36	-1.07	0.06
106	ENSG00000239951	IGKV3-20	30	-1.95	0.04
107	ENSG00000173868	PHOSPHO1	29	-1.53	0.01
108	ENSG00000248329	APELA	28	-1.47	0.005
109	ENSG00000274746	ZNF100	27	-10.50	0.002
110	ENSG00000168079	SCARA5	27	-2.53	0.001
111	ENSG00000070729	CNGB1	26	0.92	0.05
112	ENSG00000174348	PODN	25	-2.49	0.001
113	ENSG00000143196	DPT	25	-2.52	0.003
114	ENSG00000278149	RDH13	24	11.37	<0.001
115	ENSG00000075218	GTSE1	24	1.03	0.09
116	ENSG00000281962	IGHV3-23	24	-2.22	0.05
117	ENSG00000172673	THEMIS	22	-2.22	0.04
118	ENSG00000149451	ADAM33	22	-2.23	0.003
119	ENSG00000251546	IGKV1D-39	21	-2.13	0.03
120	ENSG00000276537	TTYH1	20	1.76	0.003
121	ENSG00000275122	THEMIS	20	3.68	0.002
122	ENSG00000121068	TBX2	18	-2.86	<0.001
123	ENSG00000206297	TAP1	15	-3.57	0.001
124	ENSG00000084110	HAL	14	-1.53	0.08
125	ENSG00000089250	NOS1	13	1.31	0.03
126	ENSG00000277897	GSTT2	13	3.04	0.09
127	ENSG00000169894	MUC3A	12	-1.69	0.03
128	ENSG00000228321	TNF	10	-2.26	0.03
129	ENSG00000285708	AC097634.4	9	-3.48	0.08
130	ENSG00000285585	AC069444.2	8	-2.75	0.01
131	ENSG00000244116	IGKV2-28	7	-3.65	0.08

ES group included participants who had tobacco smoking history of >10 pack-year and had FEV_1/FVC >0.7. Differential expression analysis based on the negative binomial distribution was performed using DESeq2 package.[123] Adjusted p values, p_{adj}, were calculated using the Benjamini-Hochberg method implemented in DESeq2 and fdrtool packages in RStudio.

Table 3.4. Significant gene expression changes after diesel exhaust exposure in COPD group's airway epithelium

Gene ID	Gene Symbol	Base Mean	Log_2 Fold Change	p_{adj}
ENSG00000008710	PKD1	4340	-0.18	0.04
ENSG00000165923	AGBL2	3900	-0.26	0.04
ENSG00000158163	DZIP1L	2790	-0.27	0.09
ENSG00000163482	STK36	2483	-0.38	0.006
ENSG00000184949	FAM227A	1870	-0.38	0.02
ENSG00000184465	WDR27	1375	-0.34	0.09
ENSG00000115687	PASK	1364	-0.26	0.09
ENSG00000197283	SYNGAP1	1356	-0.50	0.04
ENSG00000235715	PSMB8	810	-1.99	0.06
ENSG00000277791	PSMB3	220	1.84	0.04

Chronic obstructive pulmonary disease (COPD) group included participants with COPD as defined by having $FEV_1/FVC < 0.7$. Differential expression analysis based on the negative bionmial distribution was performed using DESeq2 package.[123] Adjusted p values, p_{adj}, were calculated using Benjamini-Hochberg method implemented in DESeq2 and fdrtool packages in RStudio.

Table 3.5. Significant gene expression changes after diesel exhaust exposure in non-COPD group's airway epithelium

Gene ID	Gene Symbol	Base Mean	Log_2 Fold Change	p_{adj}
ENSG00000084207	GSTP1	40527	0.19	0.02
ENSG00000117450	PRDX1	19453	0.23	0.003
ENSG00000181019	NQO1	10121	0.35	<0.001
ENSG00000177156	TALDO1	5354	0.26	0.003
ENSG00000163931	TKT	4083	0.30	0.008
ENSG00000151632	AKR1C2	2126	0.39	0.01
ENSG00000196139	AKR1C3	1734	0.21	0.06
ENSG00000160211	G6PD	1272	0.24	0.02
ENSG00000115657	ABCB6	1102	0.33	<0.1
ENSG00000087842	PIR	942	0.37	<0.001
ENSG00000176153	GPX2	823	0.64	<0.001
ENSG00000204304	PBX2	719	-0.31	0.07
ENSG00000108448	TRIM16L	544	0.36	<0.001
ENSG00000171401	KRT13	480	1.55	0.03
ENSG00000065833	ME1	427	0.49	0.007
ENSG00000114648	KLHL18	402	0.23	0.08
ENSG00000198074	AKR1B10	284	0.62	0.03
ENSG00000276429	PLCH2	208	-12.78	0.01
ENSG00000282184	IGHG3	158	-1.97	0.07
ENSG00000137313	TRIM26	155	-10.53	<0.001
ENSG00000284320	SHMT1	152	11.84	0.02
ENSG00000172551	MUCL1	137	0.53	0.08
ENSG00000142173	COL6A2	121	-1.16	0.08
ENSG00000278016	TIGD5	120	8.01	0.008
ENSG00000204681	GABBR1	116	-1.01	0.07
ENSG00000166183	ASPG	112	0.70	0.07
ENSG00000226936	B3GALT4	98	-13.23	0.008
ENSG00000166069	TMCO5A	74	0.47	0.06
ENSG00000275399	NLRP2	47	12.34	0.02
ENSG00000237056	ZBTB22	40	11.80	0.04
ENSG00000169469	SPRR1B	39	1.66	0.02
ENSG00000182492	BGN	38	-1.65	0.07
ENSG00000225190	PLEKHM1	33	12.26	0.02
ENSG00000181135	ZNF707	32	-11.76	0.04
ENSG00000274746	ZNF100	23	-11.42	0.06
ENSG00000174348	PODN	23	-1.73	0.08
ENSG00000121068	TBX2	17	-2.23	0.07
ENSG00000244398	AC116533.1	17	-12.05	0.03
ENSG00000248405	PRR5-ARHGAP8	16	1.19	0.04
ENSG00000149451	ADAM33	16	-1.80	0.08
ENSG00000228321	TNF	12	-2.42	0.02

Non-COPD group included both healthy never-smokers and ex-smokers. Differential expression analysis based on the negative binomial distribution was performed using DESeq2 package.[123] Adjusted p-values, p_{adj}, were calculated using the Benjamini-Hochberg method implemented in DESeq2 and fdrtool packages in RStudio.

3.6 Figures

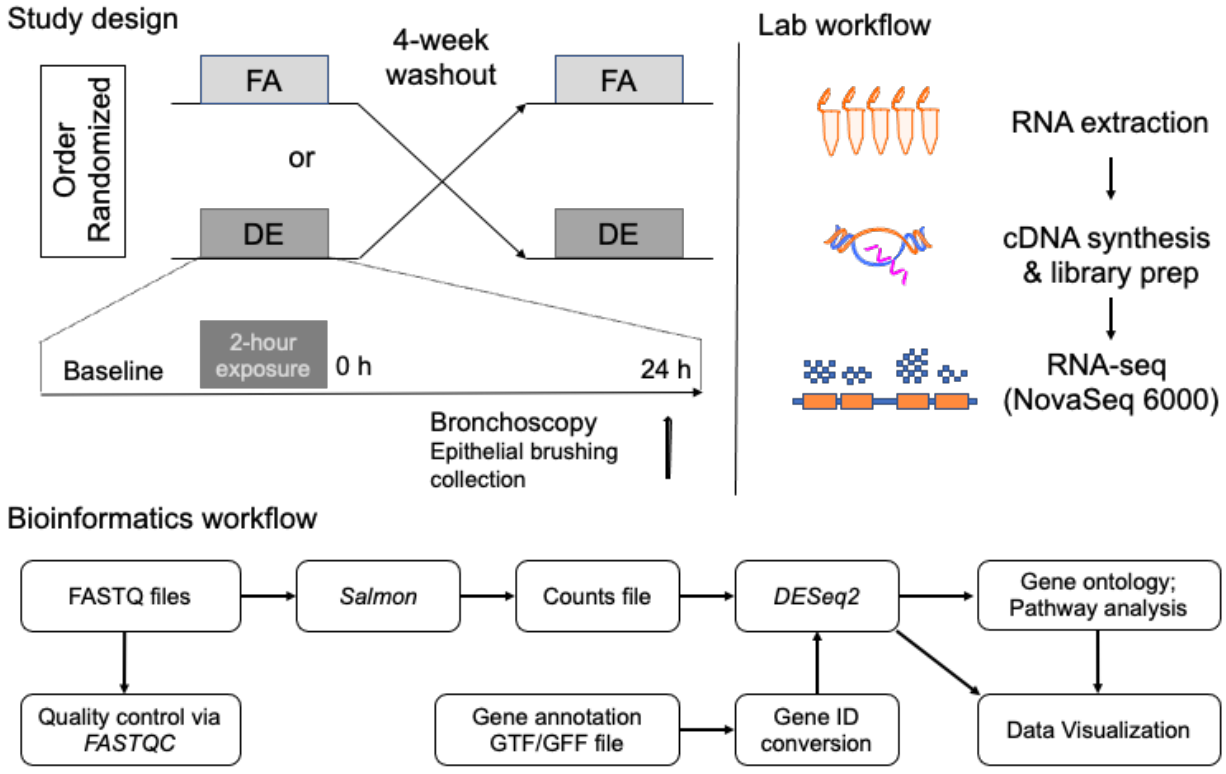

Figure 3.1. Study design, lab workflow, and bioinformatics workflow. Schematic overview of the study. 25 research participants underwent bronchoscopy following exposure to diesel exhaust (DE, at a nominal concentration of 300 µg/m^3 of particulate matter ≤2.5 µm in aerodynamic diameter [$PM_{2.5}$]) and filtered air (FA) for 2 hours on two separate occasions separated by a 4-week washout. Bronchoscopy was performed 24 hours after each exposure and stored at -80 °C. RNA was extracted from the endobronchial brushes and RNA sequencing (NovaSeq 6000) was performed after enriching the samples for poly-A-tail, cDNA synthesis and library prep. mRNA count files were generated using package *Salmon* (v.1.4.0) and differential expression analysis was performed in in RStudio, using package, DESeq2.

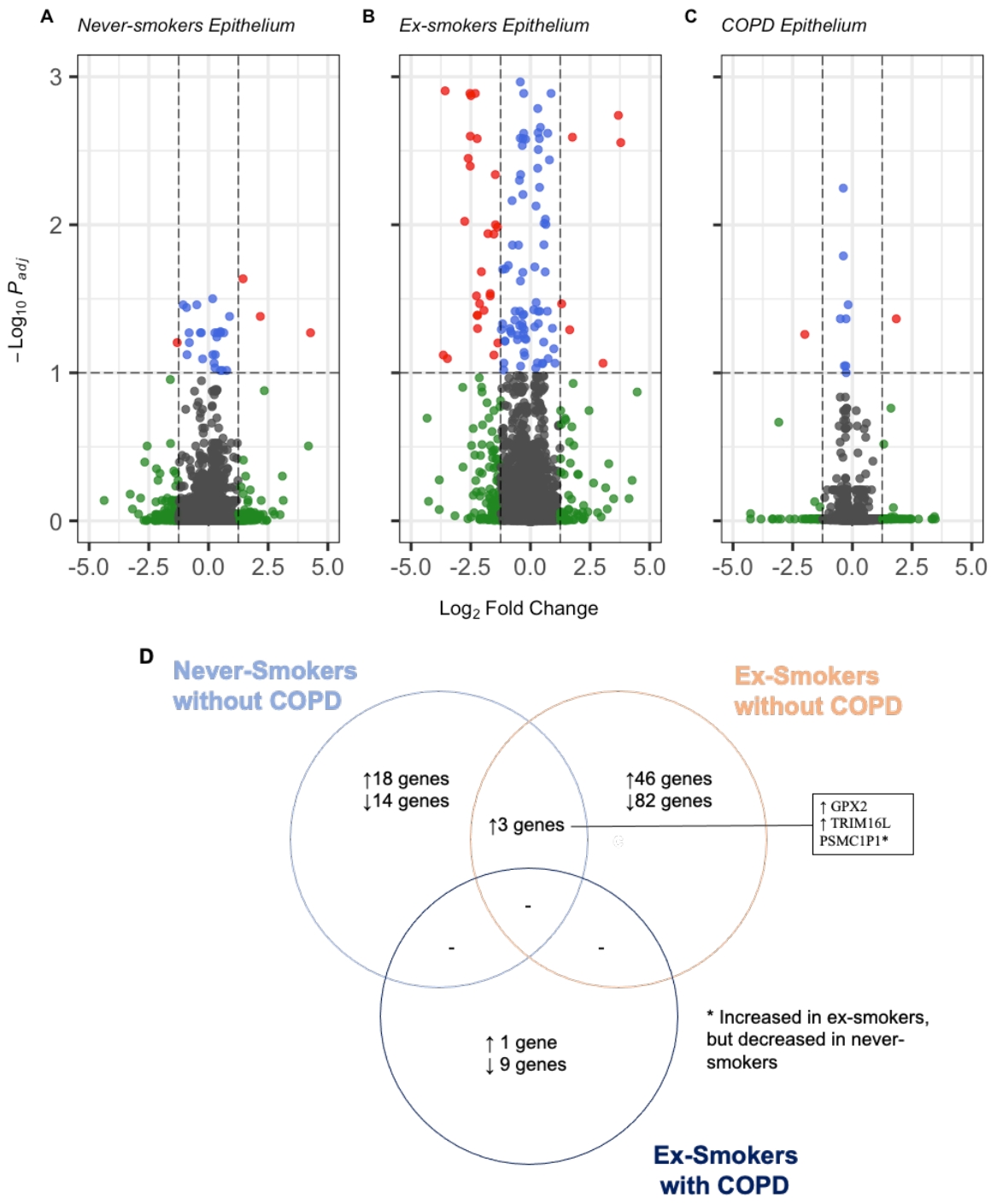

Figure 3.2. Effect of diesel exhaust exposure on airway epithelium transcriptome. Panel [A, B, C] are volcano plots showing differentially expressed genes (DEGs) in the airway epithelium comparing diesel exhaust (DE) to filtered air exposures. In blue and red are genes significantly changed by DE exposure: in blue are genes with p_{adj} <0.1 & $\log_2$ fold change <1.25, and in red are genes with P_{adj} <0.1 & $\log_2$ fold change <0.25. DEGs were calculated using DESeq2 in RStudio. Volcano plots were generated using the EnhancedVolcano (v.1.8.0) package in RStudio. Panel [D] is a Venn diagram showing the direction of change for DEGs in each study group and the overlapping DEGs between the never-smokers and ex-smoker groups.

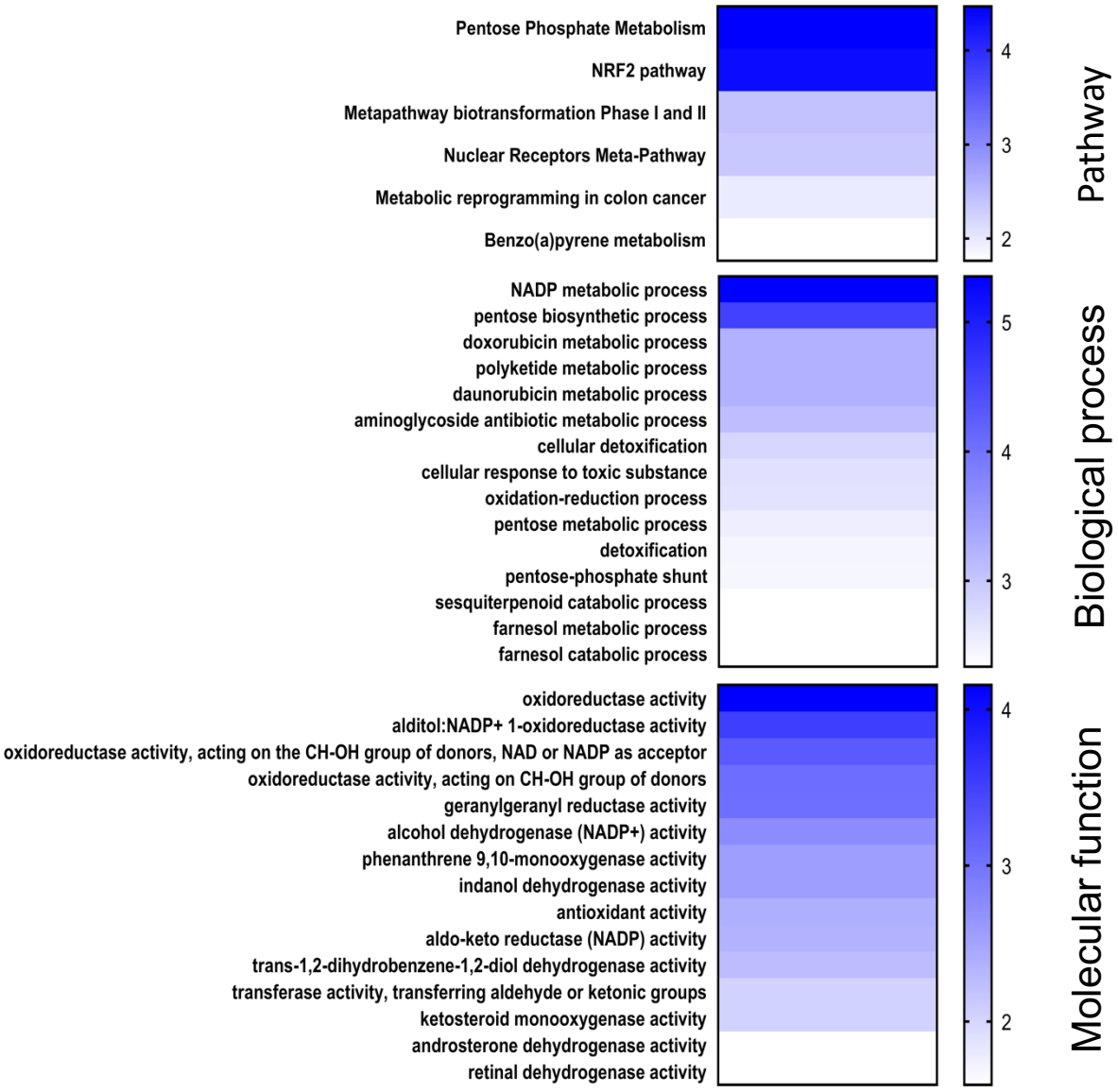

Figure 3.3. Functional enrichment analysis of differentially expressed genes in the epithelium of participants without COPD. Functional enrichment analysis was performed on differentially expressed genes in response to diesel exhaust exposure in participants without chronic obstructive pulmonary disease (COPD). An internet-browser-based interface was used for the analysis (https://biit.cs.ut.ee/gprofiler/gost) and graphical representations were produced in GraphPad Prism 9. WikiPathway (https://www.wikipathways.org) and the Gene Ontology (http://geneontology.org) biological process and molecular function databases were queried. Gradient of blue increases with -Log_{10} of adjusted p-values.

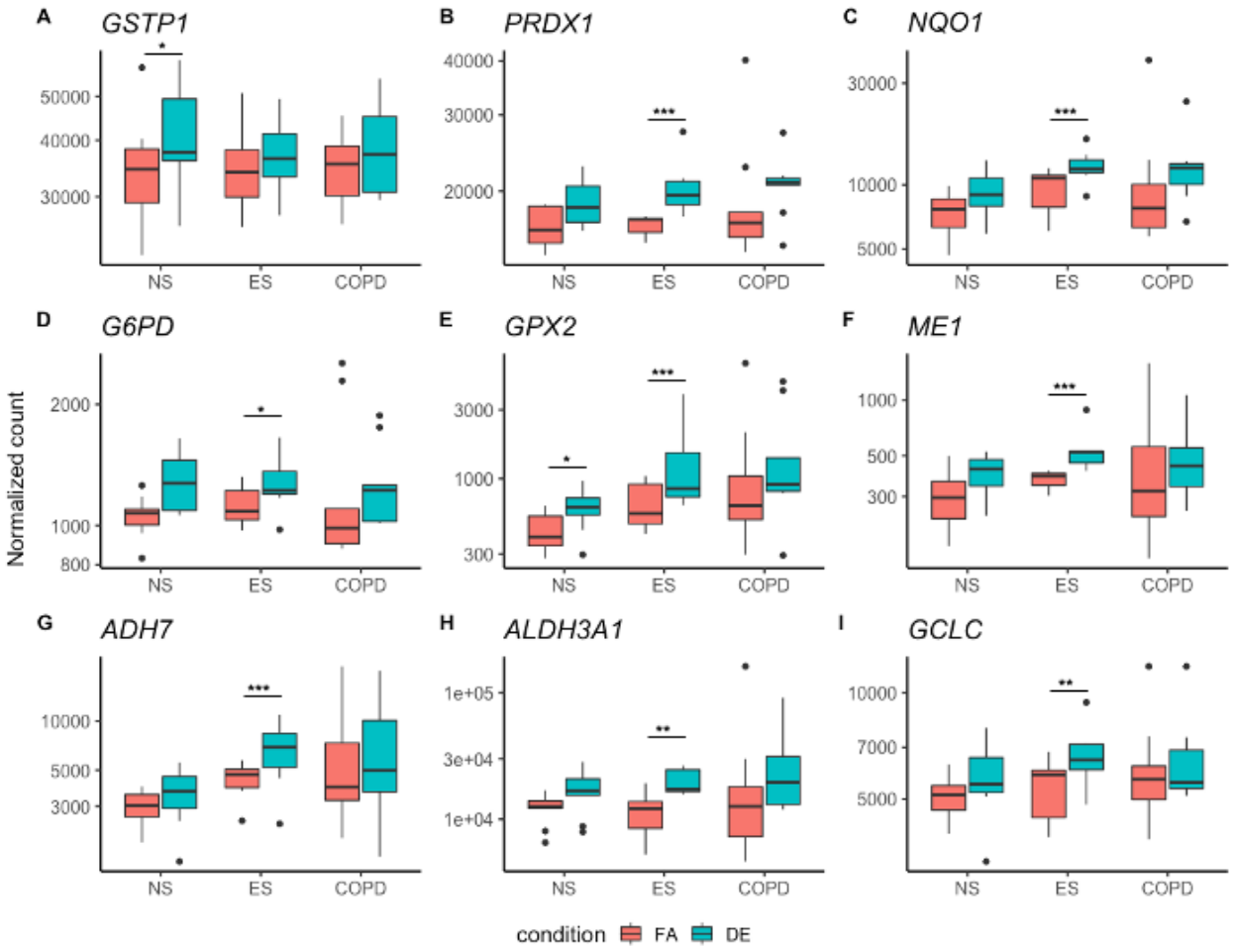

Figure 3.4. Genes in the Nrf2 pathway differentially expressed by diesel exhaust exposure. Three groups of participants: 1) healthy never-smokers without chronic obstructive pulmonary disease (COPD) (NS) group, 2) ex-smokers with >10 pack-year tobacco smoking history and without COPD (ES) group, and 3) mild to moderate COPD patients (FEV_1/FVC <0.7 and FEV_1% pred ≥50%) that are ex-smokers (COPD). Each participant was exposed to filtered air (FA) and diesel exhaust (DE) in this controlled human exposure study. *p<0.1, **p<0.01, *** p<0.001. Plots were generated using DESeq2 in RStudio. Boxplots show median, two hinges for 25th and 75th percentile, and two whiskers. The upper whisker extends from the hinge to the largest value no further than 1.5 x IQR from the hinge (where IQR is the inter-quartile range, or distance between the first and third quartiles). The lower whisker extends from the hinge to the smallest value no lower than 1.5 x IQR of the hinge. Data beyond the end of the whiskers are classified as outliers and are plotted individually.

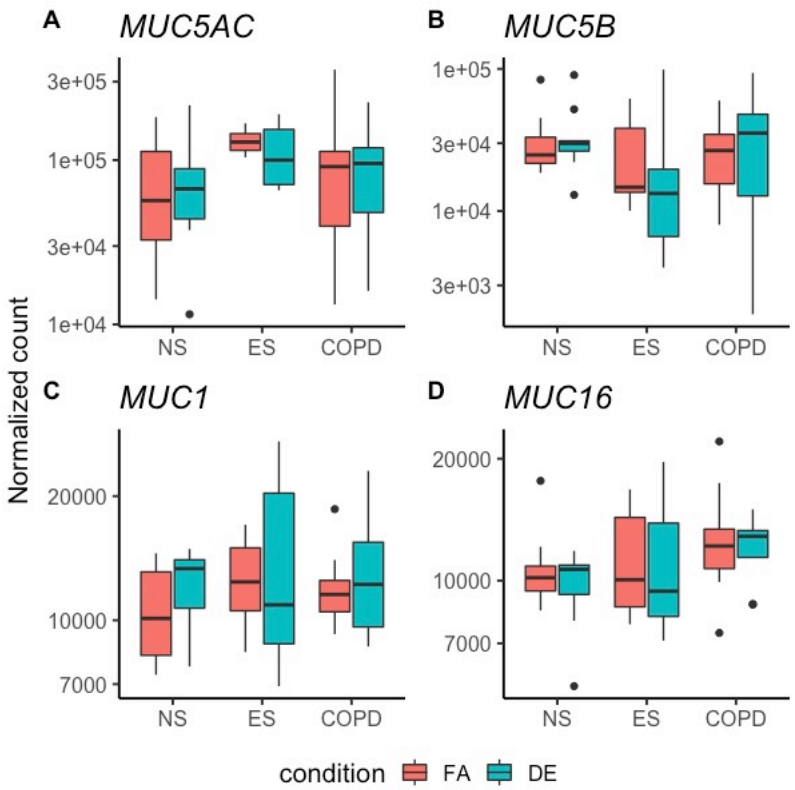

Figure 3.5. Effect of diesel exhaust exposure on mucin mRNA levels in the airway epithelium. Three groups of participants: 1) healthy never smokers without chronic obstructive pulmonary disease (COPD) (NS) group, 2) ex-smokers with >10 pack-year tobacco smoking history and without COPD (ES) group, and 3) mild to moderate COPD patients (FEV_1/FVC <0.7 and FEV_1% pred ≥50%) that are ex-smokers (COPD). Each participant was exposed to filtered air (FA) and diesel exhaust (DE) in this controlled human exposure study. Plots were generated using DESeq2 in RStudio. Boxplots show median, two hinges for 25th and 75th percentile, and two whiskers. The upper whisker extends from the hinge to the largest value no further than 1.5 x IQR from the hinge (where IQR is the inter-quartile range, or distance between the first and third quartiles). The lower whisker extends from the hinge to the smallest value no lower than 1.5 x IQR of the hinge. Data beyond the end of the whiskers are classified as outliers and are plotted individually.

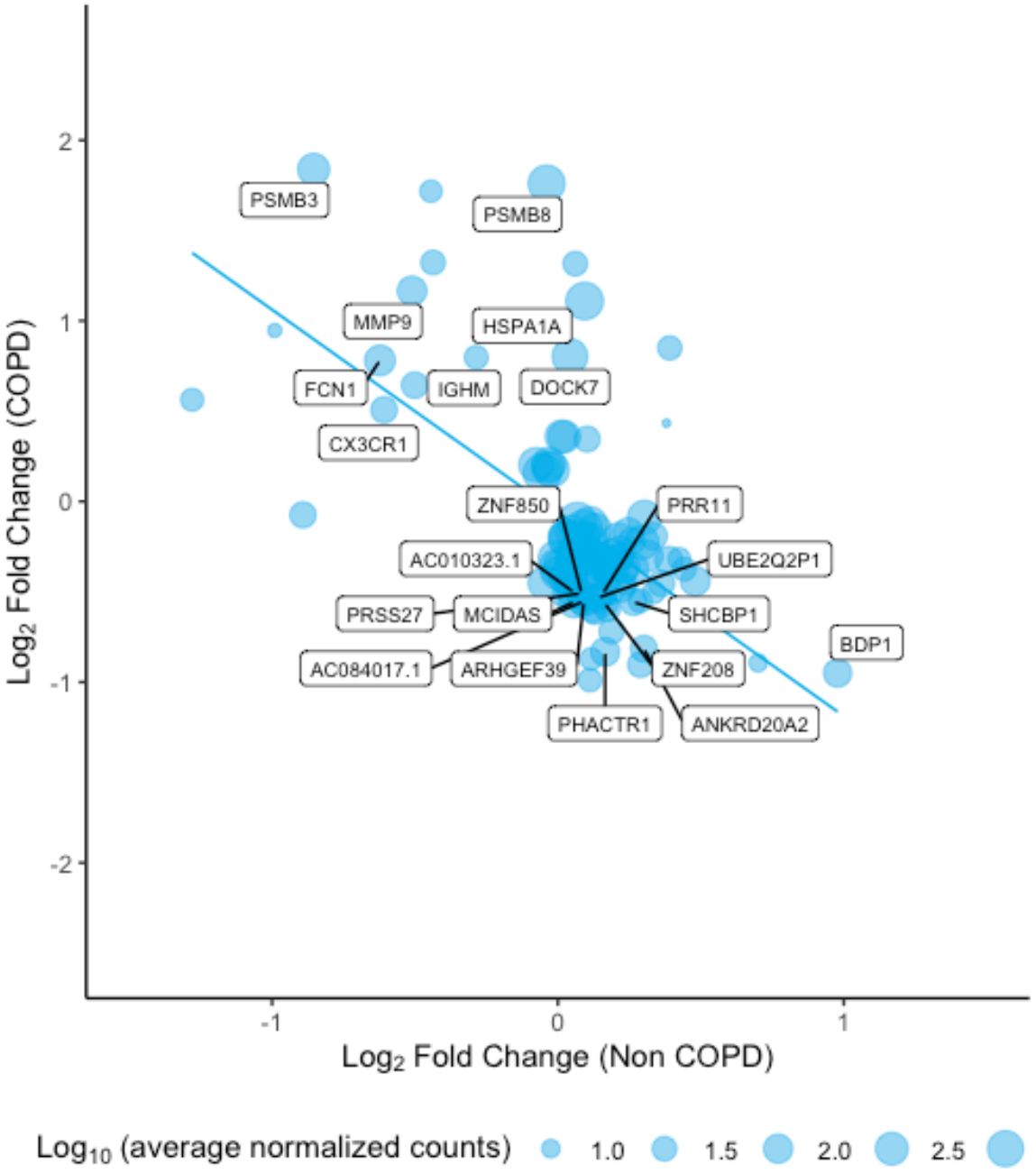

Figure 3.6. DE exposure effects on airway epithelial mRNA transcriptome according to COPD status. Scatter plot shows genes significantly modified by chronic obstructive pulmonary disease (COPD) status (p_{adj} <0.1 for exposure-by-COPD interaction). Labeled genes are |Log2 fold change (COPD)| >0.5 and average normalized count in >25% quantiles. Log_2 fold change and average normalized counts were calculated using DESeq2 package in RStudio. Graph was generated using the Tidyverse (v.1.3.0) package.

Chapter 4. Effect of exposure to traffic-related air pollution is modified by the airway microbiome

4.1 Introduction

Many chronic respiratory diseases arise in large part due to exposure to toxic inhalants or respiratory microbes. In recent years, the mechanisms through which the respiratory microbiota exerts functional effects on the pathogenesis, exacerbation, and comorbidities of chronic respiratory diseases, including chronic obstructive pulmonary disease (COPD), have become better understood.[84] Methods to characterize human-microbial dynamics have seen significant improvement with the availability of free, open-source, collaborative bioinformatics pipelines, such as QIIME2, and human microbiome databases, such as the NIH Human Microbiome Project (https://hmpdacc.org).[139–141] Several studies to date have documented differences in the composition of respiratory microbiota in individuals living with chronic respiratory diseases (e.g., COPD, asthma, and cystic fibrosis) in comparison to healthy individuals.[84–86] It is now known that COPD exacerbation events are associated with decreased microbial alpha diversity and an increased proportion of *Proteobacteria* phylum in sputum samples.[87] Use of oral corticosteroids, a commonly used treatment for COPD exacerbations, can alter the lung microbiome and is associated with increased airway microbial richness and diversity in COPD.[84,87] Alpha diversity refers to the average species diversity in a specific area, and in the context of the airway microbiome, it refers to the diversity of microbes within each individual's airways. Bacterial richness is simply a count of the species of bacteria found in a specific area and does not take into account the total abundances of each species or their relative abundance distributions.

Remarkably, the impact of ambient air pollution exposures on health continues to increase and is now one of the top contributors to global disease burden on society as measured by disability-adjusted life-years.[1] Exposure to ambient air pollution is associated with higher incidence and exacerbations of COPD along with associated mortality.[6,25,28,31,142,143] Diesel exhaust (DE, paradigmatic of traffic-related air pollution) is a major contributor to ambient air pollution in urban areas and is linked to adverse health effects,

including a decline in forced expiratory volume in 1 second (FEV_1)[144] and airway inflammation.[145] Exposure to particulate matter (PM) from ambient air pollution has been demonstrated to increase cytokine expression and release in the nasal mucosa *in vivo*[146] and in primary human bronchial epithelial cells *in vitro.*[65,147] Exposure to a high concentration of fine PM can damage the respiratory mucosa by decreasing epithelial barrier function, exaggerating inflammatory responses at the mucosal surface, and impairing host defense to pathogens.[148]

Air pollution may also drive the pathogenesis of chronic respiratory disease by altering the respiratory microbiota, with downstream effects given that respiratory microbiome dysbiosis may lead to respiratory diseases.[84,86,149] Alternatively, or perhaps additionally, the airway microbial community may modulate lung responses to environmental insults. Additional appreciation of the influence of human-microbe interactions on disease pathogenesis must incorporate how environmental insults, such as air pollutants, interact with the respiratory microbiota in altering host immune responses. Therefore, I sought to test two hypotheses in this chapter.

I hypothesized that:

1. *Acute exposure to DE alters the alpha diversity of the respiratory microbiome.*
2. *The effect of DE exposure on lung function and cytokines is modified by the respiratory microbiome richness.*

4.2 Methods

This study was approved by the University of British Columbia clinical research ethics board (H14-00821) and Vancouver Coastal Health Research Institute (V14-00821). All participants provided written informed consent prior to inclusion. This study was registered at ClinicalTrial.gov with identification number NCT02236039.

4.2.1 Participants

Research participants included healthy never smokers and ex-smokers, with and without COPD, between 40 to 80 years old. We excluded any participants who had used antibiotics during or in the 6 months prior to study enrollment from the microbiome analysis. Data from 25 research participants who completed the study and underwent research bronchoscopies were included in the analysis. Figure 2.1 describes how the study participants were recruited and how many participants underwent and completed exposures and research bronchoscopies. Demographic information of the 25 study participants who were included in the analysis of this chapter are summarized in Table 4.1. These were participants who underwent bronchoscopies and collected samples passed quality control.

All participants self-reported ≥6 months of smoking cessation prior to study enrolment, which continued throughout the study. Serum cotinine test was used to exclude active and passive tobacco smoking. Participants abstained from short- and long-acting bronchodilator and inhaled corticosteroid use for 48 hours, and caffeine for 12 hours prior to exposures. Visits were rescheduled if participants exhibited signs of an upper respiratory tract infection (i.e., Common Cold Questionnaire score >3).[97]

4.2.2 Controlled human exposure study

We conducted a randomized, double-blinded, crossover, controlled exposure study to DE using the facility and protocol as previously published.[20,75,96] Briefly, DE was generated using a 6.0 kW Coliseum GY6000 generator with a 406 cc Yanmar L 100 EE 4-stroke diesel engine (EPA Tier 3-compliant). A constant load of 2.5 kW was applied throughout the exposure, and the exposure booth air flow was controlled in real-time by the staff engineer to maintain a level of particulate matter of less than or equal to 2.5 microns in aerodynamic diameter [$PM_{2.5}$] at a nominal concentration of 300 $\mu g/m^3$. During the filtered air (FA; control) condition, the generator was in operation with the same load conditions as the DE exposure, to simulate exposure to the participants in a manner that provides effective blinding,[150] but the exhaust was diverted from the dilution and exposure system. Incoming air to the exposure booth was

twice high-efficiency particulate air filtered room air. Each participant was exposed for 2 hours on separate occasions to DE and FA. Only the exposure facility engineers were aware of the computer-randomized exposure order assignment. There was a minimum 4-week washout period between the exposures.

4.2.3 Lung function testing

Spirometry was performed in accordance with American Thoracic Society's guidelines.[151] On day 1 of each study visit, spirometry was performed before (baseline), during, and immediately after the 2-hour exposure. Spirometry was reassessed the following day (24 hours post-exposure) prior to bronchoscopy. $\Delta\Delta FEV_1$ was defined as the difference between the change in FEV_1 post-DE exposure (FEV_1 % (24 h post-DE) - FEV_1 % (DE baseline)) and the change in FEV_1 post-FA exposure (FEV_1 % (24 h post-FA) - FEV_1 % (FA baseline).

4.2.4 Endobronchial brush and bronchoalveolar lavage collection

At 24 hours after exposure, flexible bronchoscopy was performed to collect endobronchial brushing samples and bronchoalveolar lavage (BAL). Sampling was done on the opposite side of lung for each exposure visit. Endobronchial brushes (cytology brush) were collected from 4-5th generation bronchioles. Each brush sample was placed in 1 mL of sterile CytoLyt® solution (Hologic Inc) and immediately flash frozen on dry ice and stored at -80ºC until processing.

To collect lavage samples, the bronchoscope was wedged at 4-5th generation bronchus, and 2 x 20 mL instillation of sterile saline (0.9% NaCl) was suctioned back and labeled as bronchial wash samples. Subsequently, 2 x 50 mL of saline was instilled and suctioned to comprise the BAL. BAL was filtered with a sterile mesh filter (40 μm) and centrifuged for 15 minutes at 475 relative centrifugal force at 4ºC. Supernatant was separated and aliquoted for storage at -80ºC.

4.2.5 Microbiome sequencing

DNA was extracted from lung cytology brushes and controls (saline channel wash, sterile saline, blank Cytolyt®, and extraction controls) using the PureLink Microbiome DNA Purification kits (Thermo Fisher Scientific) following the manufacturer's protocol after the conclusion of the study. DNA quality was assessed using an Agilent 2100 Bioanalyzer. The V4 region of the 16S rRNA gene was then amplified by PCR and sequenced using an Illumina MiSeq sequencer. PCR blank and water samples were included as negative controls to account for potential contamination, while a mock community was used as a positive control.

4.2.6 Microbiome analysis

Bioinformatic analysis of microbiome 16S rRNA sequencing data was performed using QIIME2.[140] The raw data was demultiplexed and quality filtered using the q2-demux plugin followed by de-noising with DADA2.[152] Amplicon sequence variants (ASVs) which did not align against the SILVA database (80% cut-off) using the QIIME quality-control evaluate-seqs plugin and ASVs whose relative abundance in controls were higher compared to those in samples were removed from the analysis. Filtered ASVs were then aligned with MAFFT (via q2-alignment)[153] and used to construct a phylogeny with fasttree2 (via q2-phylogeny).[154] Taxonomy was assigned to ASVs using the q2-feature-classifier[141] (classify-sklearn: a naïve Bayes taxonomy classifier)[141] against the SILVA database.[155] Alpha diversity metrics (richness, Shannon diversity index, Faith's phylogenetic diversity, and evenness) were then exported to RStudio for further statistical analysis.For some analyses, subjects were stratified, based on median richness, into low richness (N=13; richness $\leq$ 79) and high richness (N=12; richness $>$79) groups. All interaction analyses were performed with richness represented by number of ASVs.

4.2.7 Electrochemiluminescence multiplex assay

The V-PLEX Human Cytokine 30-Plex Kit (Meso Scale Diagnostics) was used to assay the following analytes in BAL: eotaxin, eotaxin-3, granulocyte-macrophage colony-stimulating factor (GM-CSF),

interferon gamma (IFN-γ), interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12/IL-23p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, IFN-γ-induced protein 10 kDa (IP-10), monocyte chemoattractant protein (MCP)-1, MCP-4, macrophage-derived chemokine (MDC), macrophage inflammatory protein (MIP)-1α, MIP-1β, thymus and activation regulated chemokine (TARC), tumour necrosis factor (TNF)-α, TNF-β, and vascular endothelial growth factor (VEGF)-A. The assays were performed per the manufacturer's protocol with 2-fold (cytokine panel 1 and proinflammatory panel 1) and 4-fold (chemokine panel 1) dilution of BAL in assay diluent. The lower limit of detection (LLOD) was set at the signal intensity which was 2.5 standard deviations above the background noise in the blank. For statistical comparisons, values below the LLOD were replaced with ½ of the respective LLOD value.

4.2.8 Statistical analyses

Effects of DE exposures on the study endpoints were assessed using linear mixed-effects (LME) models (nlme package, v.3.1-148) in R (v.4.0.3, The R) implemented in RStudio (v.1.3.1093). Confidence intervals (CI) of effect estimates of LME models were computed using the gmodels package (v.2.181) in RStudio. When necessary, to normalize the data distribution, data was $\log_{10}$-transformed. Any effect estimates and associated CI based on $\log_{10}$-transformed data are presented as back-log transformed values. For each participant, the lower respiratory microbiome was analyzed from two endobronchial brush samples collected on two different occasions (following FA and DE; the two exposures and subsequent endobronchial brush collections were separated by a minimum of 4-week washout) and richness for each participant was defined by the average of values measured in the two endobronchial brushes collected after a given exposure.

In our primary LME model, exposure (FA and DE) was set as a fixed effect and participant ID was set as a random effect. Four models, each with the following fixed effects interaction terms (exposure-by-richness, exposure-by-COPD, exposure-by-group, and exposure-by-age), were run to evaluate whether the DE effect was modified by the following: 1) lung microbial richness (number of ASVs), 2) COPD status, 3) group (healthy, ex-smokers without COPD, and ex-smokers with COPD), and 4) participant

age. Order effects were assessed using exposure order as a fixed effect in a separate model. P-values ≤0.05 were considered statistically significant.

4.3 Results

4.3.1 Participant characteristics

Table 4.1 summarizes participant characteristics and the alpha diversity metrics (richness, Shannon diversity index, Faith's phylogenetic diversity, and evenness) of their respiratory microbiomes.

4.3.2 No detectable effect of DE exposure on bacterial richness and alpha diversity in the airways

There was no detectable DE exposure effect on bacterial richness as estimated by the number of ASVs (fold change [95% CI]: 1.1 [0.9 to 1.3]), Shannon diversity score (1.0 [0.96 to 1.07], Faith's phylogenetic diversity (1.07 [0.97 to 1.18]), or evenness (0.99 [0.94 to 1.04]) (Figure 3.1). There was also no significant change in 16S copy number (copies/ng of total extracted DNA) (1.9 [0.74 to 4.91]) due to the DE exposure. There were no significant exposure-by-group, exposure-by-COPD status, or exposure-by-age interactions for any of the outcomes above.

4.3.3 The effect DE exposure on IL-6 was modified by airway bacterial richness

The following cytokines in BAL were in the detection range of the electrochemiluminescence multiplex assay: GM-CSF, IL-1α, IL-6, IL-7, IL-8, IL-12/IL-23p40, IL-15, IL-16, IP-10, MCP-1, MDC, MIP-1β, TARC, and VEGF-A. At 24h post-exposure, we found no detectable effects of DE exposure on any of these cytokines. However, there was a significant exposure-by-richness interaction for IL-6 (p=0.03), such that participants with a lower bacterial richness experienced a larger increase in IL-6 after DE exposure, when compared to those with higher bacterial richness. Observing the low richness group alone, DE exposure significantly increased IL-6 (p=0.05), IL-7 (p=0.03), and IL-15 (p=0.03) in BAL (Figure 4.2).

4.3.4 Airway bacterial richness significantly modified the effect of DE exposure on airflow

There was no significant impact of DE on the FEV_1 % (DE effect: +1.26% [-1.8 to 4.3]). There were also no significant exposure-by-group, exposure-by-COPD, or exposure-by-age interactions. However, there was a significant exposure-by-richness interaction (p=0.01) on FEV_1 %, such that participants with lower bacterial richness had a greater decrease in FEV_1 % following DE exposure compared to those with higher airway bacterial richness. Furthermore, there was a significant positive correlation between changes in FEV_1 attributable to DE ($\Delta\Delta FEV_1$) and richness (Pearson r = 0.54; p=0.007) (Figure 4.3).

4.3.5 Airway bacterial richness, Shannon index, and phylogenetic diversity were lower in those living with COPD compared to healthy never-smokers

Figure 4.4 summarizes the difference in alpha diversity indices of three participant groups: never-smokers, ex-smokers, and COPD.

4.4 Discussion

To the best of my knowledge, this is the first controlled human exposure study that examined the relationship between traffic-related air pollution exposure, the respiratory microbiome, and host immune responses in the human airway. Moreover, this is one of the largest controlled human exposure studies to include an older adult population with mild-moderate COPD. In this unprecedented study, we revealed that individuals with lower bacterial richness in their airways experienced a greater decline in FEV_1 and a greater increase of IL-6 in BAL due to DE exposure compared to those with higher bacterial richness. Thus, our results support the notion that the composition of the bacterial community in the lower respiratory tract may alter the host response to air pollution exposure. Lower airway microbial richness may be a modifiable biomarker of one's increased susceptibility to the negative impacts of air pollution exposure.

IL-6 is a proinflammatory cytokine that is released in response to inflammatory stimuli, and elevated systemic levels of IL-6 are associated with increased cardiovascular morbidity and mortality.[156] High

levels of ambient air pollution are associated with an increased plasma IL-6 level,[157,158] and chronically elevated levels of IL-6 are a marker of increased cardiovascular risk.[156] In COPD patients, the level of IL-6 in the airways rises, along with sputum neutrophils count and plasma fibrinogen, throughout the course of COPD progression.[102] A high level of sputum IL-6 in COPD patients is associated with a faster decline in FEV_1.[102] Exposure to DE leads to an increase in IL-6 in blood and sputum in young healthy adults,[70,159,160] but no previous controlled human exposure study has looked at this biomarker in the lower airways of older adults following exposure to air pollution.

In this study, participants with lower bacterial richness in their airways showed an increase in IL-6 in BAL 24 hours after exposure to DE, while those with high bacterial richness showed no change. Likewise, there were significant exposure-by-airway bacterial richness interactions for IL-6 increase attributable to the DE exposure. In other words, a lower bacterial richness of the airways was associated with a greater increase in IL-6 attributable to the air pollution exposure. These same participants with lower microbial richness showed a DE-induced increase of IL-7 and IL-15, which are mediators of the innate immune responses. Moreover, FEV_1 change attributable to DE exposure was modified in those participants with a lower airway microbial richness, suggesting that these individuals may experience greater airflow limitation following DE exposure, compared to those with higher airway microbial richness. Interestingly, the DE effect was not modified by COPD status, group, or age for the outcomes reported here (IL-6, IL-7, IL-15, and change in FEV_1%). Our findings suggest that those individuals with lower airway microbial richness may be more susceptible to the negative health effects of air pollution exposure.

Several factors are known to alter microbial diversity in the airways. COPD exacerbation events are associated with decreased microbial diversity and an increased proportion of Proteobacteria in sputum.[87] Use of steroids, a treatment for COPD exacerbations, can alter the lung microbiome and is associated with increased airway microbial richness and diversity in COPD.[84,87] Alpha diversity of the airway microbiome was lower in Global Initiative for Chronic Obstructive Lung Disease stage 4 COPD patients, as compared

to controls without COPD, and a decline in microbial diversity was associated with infiltration of neutrophils, eosinophils, and B-cells into the lungs.[161] Microbiome dysbiosis can arise from exposure to air pollution (reviewed in [149]), although it is still not clear to what extent acute and chronic exposures to air pollution can impact the airway microbiome. A limited number of published studies have empirically probed the effects of air pollution on the airway microbiome. In our experimental setup, acute air pollution exposure had no detectable impact on airway microbial richness, Shannon alpha diversity index, phylogenetic diversity, and Pielou's evenness.

Some emerging evidence from population studies suggests a link between air pollution and alteration of the airway microbiome. For example, $PM_{2.5}$ exposure was associated with changes in the airway microbiome, and bacterial load in the airway was associated with FEV_1 and $PM_{2.5}$ exposure in COPD patients.[162] $PM_{2.5}$ exposure level was also associated with a decrease in the relative class abundances of *Prevotella, Veillonella, Fusobacterium, Camphylobacter and Capnocytophaga Porphyromonas, Peptostreptococcus, and Moraxella,* while *Firmicutes, Proteonacteria, and Actinobacteria* were increased in participants who were exposed to higher levels of $PM_{2.5}$.[163] These effects seen at the population level are more likely to be the effect of chronic exposure to $PM_{2.5}$ than short-term exposures. While our short-term exposure did not demonstrate DE effect on respiratory microbiome, our work extends the findings of epidemiological studies and provides experimental evidence that the airway microbiome may modify the impact of air pollution exposure on host responses relevant to COPD. However, a more in-depth evaluation of the impact of air pollution on bacterial communities at the species level is needed. Moreover, future studies carefully dissecting the impact of air pollution on the bacterial transcriptome and lung metabolome may be more revealing of the impact that acute exposures have on the airway microbial function, rather than compositions.

One limitation of our study is that we did not extensively explore the potential for DE exposure to alter the composition of the airway microbiome. Rather, we focused on alpha diversity indices as quantifiable metrics to test the exposure-by-microbiome interactions in our crossover study design. Bacterial richness

is simply a count of species of bacteria and does not take into consideration the total abundances of each species or their relative abundance distributions. Our choice of only selecting a few simple quantifiable metrics of the airway microbial community was intentional as we would have needed a much larger sample size to accommodate multiple statistical comparisons. Having said that, we did not correct for multiple comparisons since this work was exploratory in nature. Nevertheless, given that this is the first study in humans to experimentally assess the relationship between the airway microbiome, air pollution exposure, and host responses to air pollution exposure, our novel work opens the door to many more mechanistic studies to understand the functional impact of the microbiota in the development and exacerbation of chronic respiratory diseases. Exacerbation and development of chronic respiratory diseases are mediated in part through complex interactions between environmental factors, respiratory pathogens, and host immune responses. If the effects of air pollution exposure on airflow and airway cytokines are modified by airway microbiome richness, as we hypothesize, this would support attempts to modify that richness to benefit those inevitably exposed. Moreover, improved understanding of the mechanisms by which the microbiome may interact with the host and environment could empower patients and their clinicians to manage chronic respiratory illnesses and pave a path to the development of novel therapeutic strategies.

4.5 Tables

Table 4.1 Participant characteristics and airway microbial alpha diversity scores

	Participant characteristics						Airway microbiome alpha diversity indices			
	Age	**Sex**	**FEV_1 /FVC**	**FEV_1 %**	**Smoking History**	**Years Since Quitting Smoking**	**Richness**	**Shannon**	**Faith's PD**	**Pielou**
1	63	M	70	96.7	47	2	48.5	3.57	6.17	0.64
2	50	M	79	106	15	16	52	4.35	5.18	0.82
3	66	F	44	65.8	5	2	57.5	4.61	5.96	0.79
4	73	M	86	120	30	42	59	5.09	5.94	0.87
5	80	M	54	97.3	10	30	61	4.85	7.02	0.83
6	65	M	69	102	3	35	61.5	5.13	7.09	0.87
7	68	F	69	97	46	27	65	4.51	7.17	0.75
8	47	F	80	118	0	N/A	67	5.10	7.47	0.85
9	70	M	72	93	90	19	67.5	4.70	6.75	0.79
10	66	F	54	86	24	16	68	4.28	7.00	0.70
11	70	F	48	88.3	12	33	77	5.26	8.27	0.84
12	72	F	80	162	0	N/A	79	4.73	7.25	0.75
13	66	M	62	93	67.5	7	79	4.67	8.10	0.74
14	67	M	49	76.2	52.5	11	85.5	4.80	8.20	0.75
15	55	F	74	111	0	N/A	88	5.37	8.22	0.83
16	75	M	67	116	7	41	92	4.52	7.95	0.69
17	51	M	77	106	0	N/A	93	5.19	8.95	0.80
18	61	M	81	108	92.5	25	96.5	5.20	8.97	0.80
19	52	M	80	120	0	N/A	98.5	4.92	9.35	0.75
20	56	M	74	113	50	8	102.5	5.37	9.08	0.80
21	45	M	76	109	10.5	0.83	113	4.74	10.45	0.69
22	70	M	64	88.6	19.5	16	116.5	5.10	9.76	0.74
23	63	M	78	127	0	N/A	122.5	5.31	10.73	0.77
24	58	M	75	133	0	N/A	127.5	5.54	10.81	0.80
25	54	M	79	120	0	N/A	131.5	4.87	10.88	0.69
						Median	79.0	4.9	8.1	0.8

Abbreviations: FEV_1/FVC = the ratio of the forced expiratory volume in the first 1 second to the forced vital capacity; FEV_1 %= forced expiratory volume in the first 1 second percent predicted, Faith's PD = Faith's phylogenetic diversity. Smoking history was measured in pack-years; each pack year is equivalent to smoking one pack of tobacco cigarettes every day for one year.

4.6 Figures

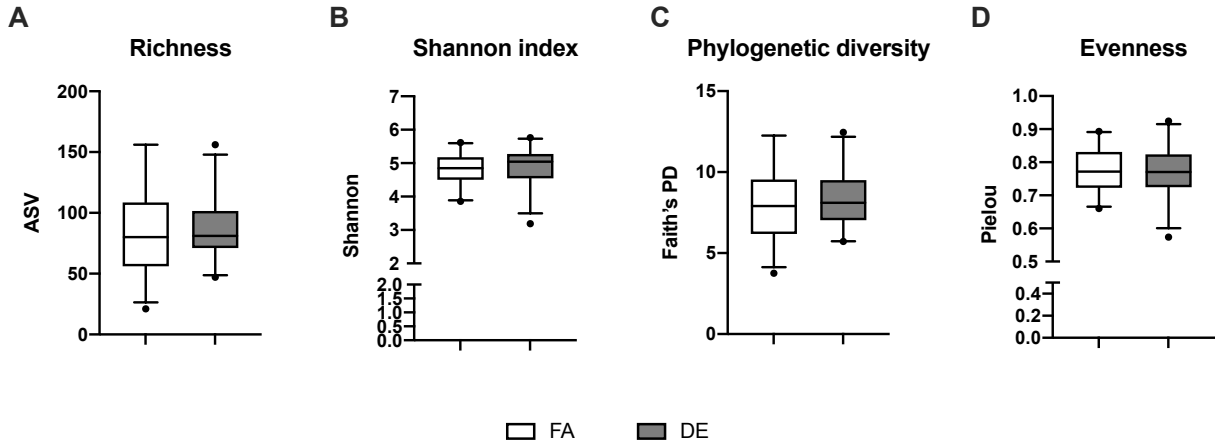

Figure 4.1. Effect of diesel exhaust exposure on the airway microbiome community. Research participants were exposed to diesel exhaust (DE) and filtered air (FA) in an order randomized controlled exposure study with a minimum of 4-week washout. Endobronchial cytology brushes were collected from 4-5th generation bronchioles 24 hours after each exposure. DNA from each cytology brushes was extracted, and the V4 region of the 16S rRNA gene was then amplified by PCR and sequenced using an Illumina MiSeq sequencer. (A) Richness, (B) Shannon alpha diversity index, (C) Faith's phylogenetic diversity index, and (D) Pielou's index were calculated using QIIME2 bioinformatics tool. Boxplots show median with 25th and 75th percentiles; whiskers extend below and above to 5th and 95th percentiles, respectively. Data was analysed with linear mixed-effect models. Definitions of abbreviations: ASV = amplicon sequence variants; Faith's PD = Faith's phylogenetic diversity.

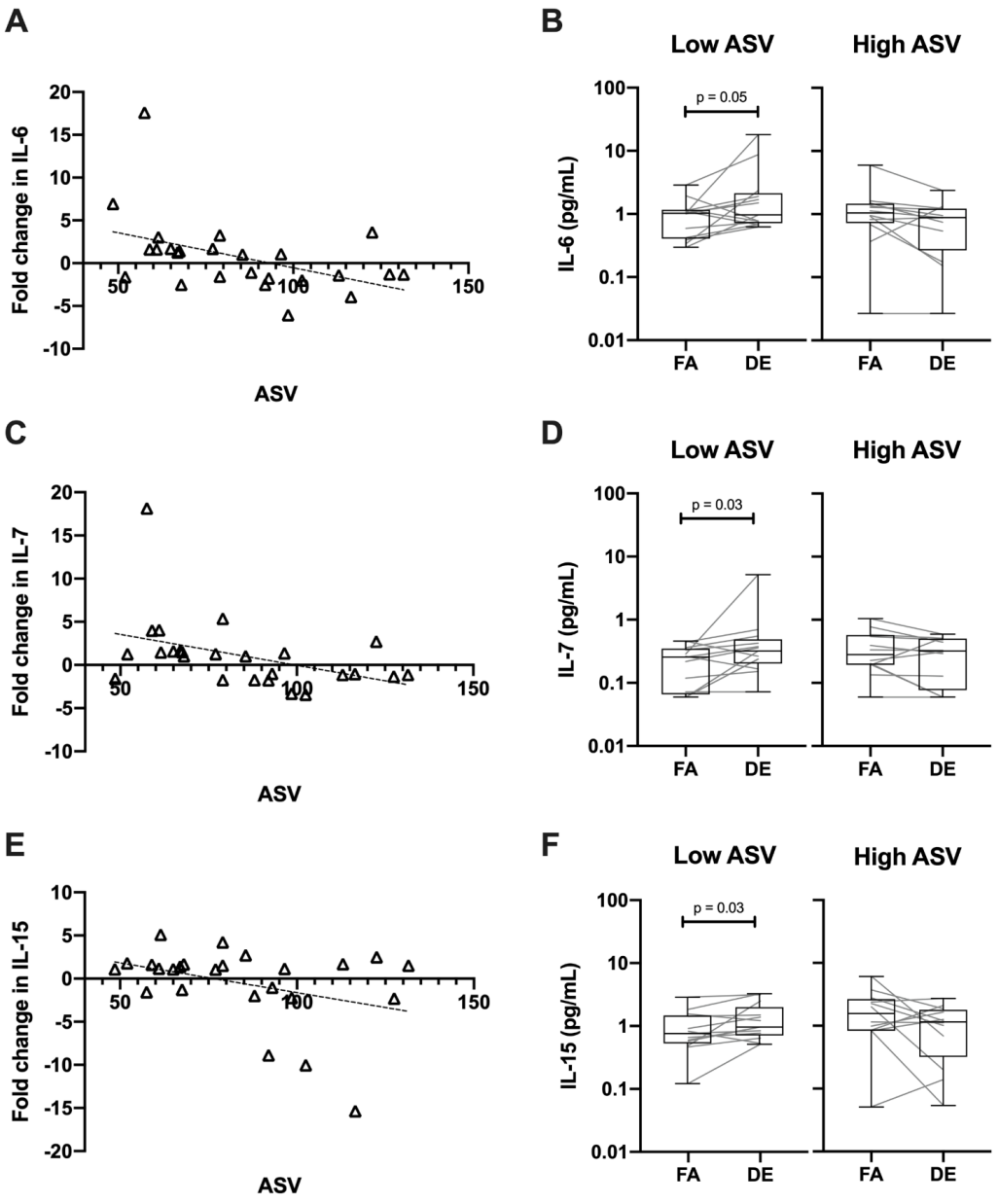

Figure 4.2. Effects of diesel exhaust exposure on interleukin (IL) -6, -7 and -15 in bronchoalveolar lavage. Interleukins (ILs) were measured in BAL collected 24 hours after 2-hour exposures to diesel exhaust (DE; $PM_{2.5}$ = 300 μg/m^3) and filtered air (FA) (N=25). Endobronchial cytology brushes were collected at the same time and analyzed for alpha diversity of the airway microbiome. (A, C, E) graphs plots the fold change (DE – FA) due to DE exposure in IL-6, -7 and -15 against richness. Dotted lines show linear regression lines. (B, D, F) boxes show median with 25th and 75th percentiles; whiskers extend below and above to 5th and 95th percentiles, respectively. Each line between FA and DE boxplots connects values measured in the same individual. Low richness group (N=13) had an average richness smaller than or equal to 79. High richness group (N=12) had an average richness greater than 79. Data was analysed with linear mixed-effect models. Exposure and richness were the fixed effects, and participant ID was a random effect in the model. Definitions of abbreviations: ASV = amplicon sequence variants; IL = interleukin.

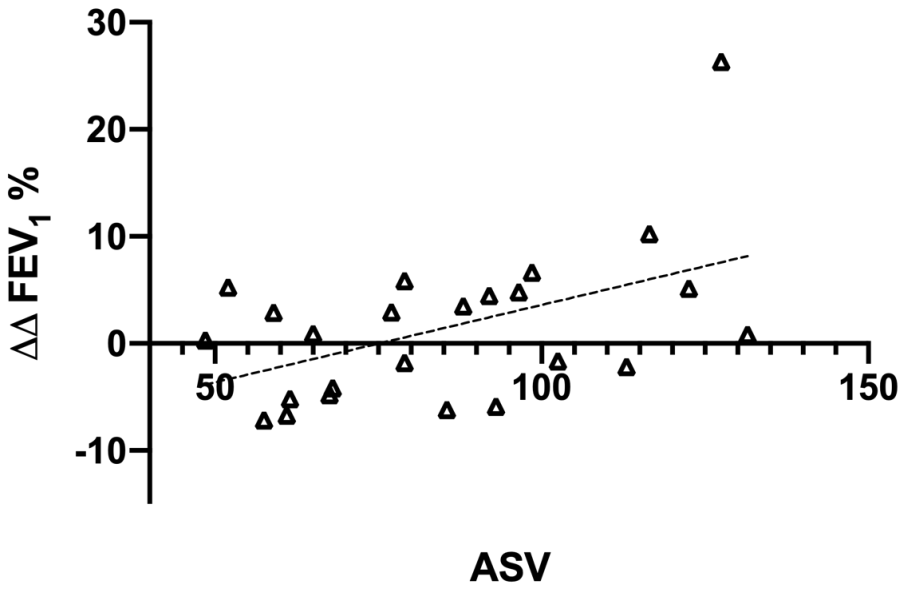

Figure 4.3. Correlation between the microbial richness of the airways and the change in FEV_1 attributable to diesel exhaust in humans. Twenty-five research participants were exposed to DE and filtered air (FA) for 2 hours in this randomized, double-blinded, crossover study. Endobronchial brushes were collected 24 hours after each exposure and DNA was extracted. Bacterial ribosomal DNA was amplified and sequenced. Forced expiratory volume in 1 second percent predicted (FEV_1 %) was measured at baseline (before) and 24 hours after each exposure. $\Delta\Delta FEV_1$ % = (FEV_1 % (24 hours post-DE) - FEV_1 % (DE baseline)) - (FEV_1 % (24 hours post-FA) - FEV_1 % (FA baseline). Dotted line is the regression line. Microbial richness was significantly correlated with $\Delta\Delta FEV_1$ (Pearson r = 0.53 [0.17 to 0.77]; p=0.007). Data was additionally analysed with linear mixed-effect (LME) models, and there was significant exposure-by-ASV interaction on change of FEV_1 attributable to DE exposure (p=0.01), indicating that those with lower ASV experienced a greater decrease in FEV_1 % following the DE exposure. Definitions of abbreviations: ASV = amplicon sequence variants.

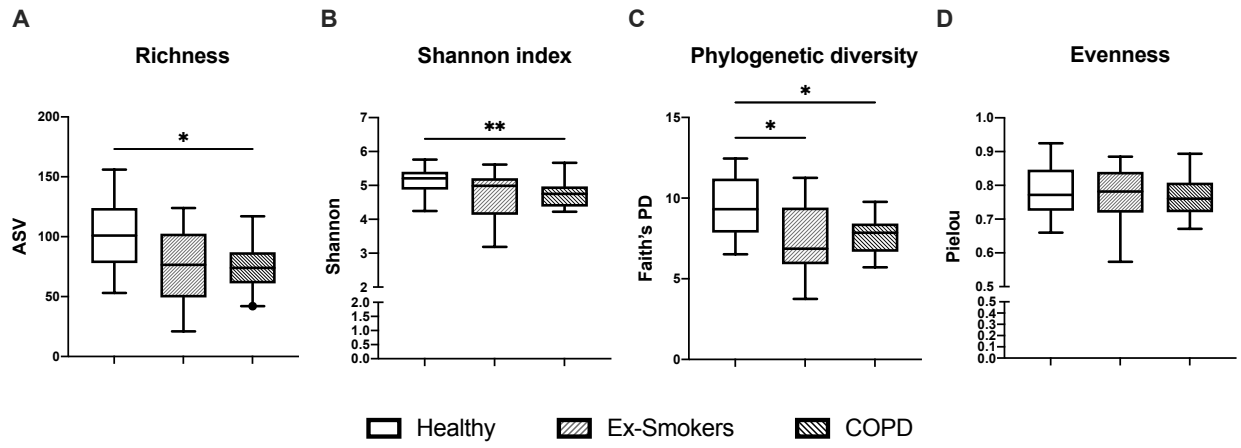

Figure 4.4. Difference in microbial alpha diversity indices of airway microbiome between the three study groups. Endobronchial cytology brushes were collected from 4-5th generation bronchioles from 25 research participants. DNA from each cytology brushes was extracted, and the V4 region of the 16S rRNA gene was then amplified by PCR and sequenced using an Illumina MiSeq sequencer. (A) Richness, (B) Shannon alpha diversity index, (C) Faith's phylogenetic diversity index, and (D) Pielou's index were calculated using QIIME2 bioinformatics tool. Boxplots show median with 25th and 75th percentiles; whiskers extend below and above to 5th and 95th percentiles, respectively. Kruskal-Wallis test was performed, and Dunn's multiple comparisons test was performed as post-hoc test for statistical difference relative to healthy controls: * $p<0.05$, ** $p<0.01$. Definitions of abbreviations: ASV = amplicon sequence variants; Faith's PD = Faith's phylogenetic diversity.

Chapter 5. Conclusion

5.1 Summary of salient findings

To the best of my knowledge, 'COPD originates in polluted air' study is the first documented study using controlled human exposure to air pollution that includes COPD patients and healthy participants and includes examination of the lower airways. Results of this crossover study of short-term exposure to moderate concentrations of traffic-related air pollution (TRAP) suggest that people living with COPD may be more susceptible to harmful impact of air pollution exposure.

In chapter 2, I tested the hypothesis that *DE exposure increases inflammatory proteins and cells in the airways and in circulation, and this increase would be greater in those with COPD.* We demonstrated in our study that exposure to DE increased airway markers of inflammation (C-reactive proteins, serum amyloid A, and vascular cell adhesion protein 1), exclusively in those with COPD. In COPD participants, we also demonstrated that in addition to the inflammatory protein markers, DE exposure increased other proteins relevant in COPD pathophysiology—matrix metalloproteinases and alpha-1 antitrypsin concentration in bronchoalveolar lavage. DE exposure led to an increase in lymphocyte count in blood irrespective of COPD status or age. However, participants with COPD showed a greater DE-attributable increase in the proportion of helper T cells in the blood than those without COPD. Our findings strongly support the notion that exposure to short-term moderate concentrations of TRAP may be more harmful to individuals with COPD compared to those without COPD, given the differential response in relevant markers as detailed in chapter 2.

Chapter 3 extended the findings from chapter 2 by evaluating the airway mucosal transcriptomic response to TRAP. I first hypothesized that *DE exposure would lead to an upregulation of genes involved in the antioxidant response*. We found that in healthy participants (with and without cigarette smoking history), exposure to DE elicited a transcriptional response with increases in genes involved in cellular antioxidant response. Specifically, we detected activation of the nuclear factor erythroid 2-related factor 2 (Nrf2)

pathway. This transcriptional response was attenuated in participants with COPD, in part because some antioxidant genes were upregulated at baseline in some COPD patients. Next, I tested the hypothesis that *DE exposure leads to upregulation of mucin genes in the airway epithelium*. Contrary to previous *in vitro* experiments that suggested upregulation of mucin genes by DE exposure,[122,129] we did not observe any significant changes in *MUC5AC*, *MUC5B*, *MUC1*, and *MUC16* expression levels in response to DE exposure. Next, I tested the hypothesis that *COPD epithelium will mount different transcriptional responses to DE exposure compared to healthy epithelium*. DE exposure elicited a divergent transcriptional response by COPD airway epithelium compared to the non-COPD epithelium. We identified that COPD status modified DE effects on 156 genes. Notably, DE exposure induced an increase in *PSMB3* (which codes for proteasome subunit beta type-3 and is involved in the ubiquitin-proteasome system) only in COPD epithelium following DE exposure. Taken all together, results from chapter 3 showed that the transcriptomic response of COPD epithelium to TRAP was different from that of healthy airway epithelium. This difference in mucosal transcriptomic response to TRAP may be the basis for increased airway inflammation observed in COPD participants following DE exposure.

Chapter 4 examined the relationship between traffic-related air pollution exposure, the respiratory microbiome, and host immune responses in the human airway. I first tested the hypothesis that *acute exposure to DE alters the alpha diversity of the respiratory microbiome*. In our study, we detected no DE effect on the bacterial richness and alpha diversity. Next, I tested the hypothesis that *the respiratory microbiome richness modifies the effect of DE exposure on lung function and cytokines*. We revealed that individuals with lower bacterial richness in their airways experienced a greater decline in forced expiratory volume in one second and a greater increase of interleukin-6 in bronchoalveolar lavage due to DE exposure compared to those with higher bacterial richness. Moreover, we found that, on average, the bacterial richness and diversity were lower in those with COPD, which was consistent with the findings in the literature. These findings support the notion that the airway microbiome may be an important aspect of COPD airway that predisposes them to the increased impact of air pollution.

While epidemiological studies demonstrate a relationship between TRAP and COPD morbidity and mortality, our study adds mechanistic evidence to support this concept, demonstrating that COPD patients and older adults are more susceptible to airway inflammation following acute exposure to TRAP. Our study is critical in providing key mechanistic insights to support public health regulations and legislation oriented to protecting susceptible populations such as older adults with COPD.

As highlighted by the Lancet Commission on Pollution and Health report, research exploring emerging causal links between pollution, disease and subclinical impairment is key to combatting the global burden of pollution-related disease.[2] In the global fight against pollution, human exposure studies such as ours are critical in providing key mechanistic insights to support legislation for public health protection and shed light on future prevention and therapeutic approaches to protect those who are more susceptible to harmful effects of air pollution.

5.2 Limitations

Our study's strength lies in utilizing the robust quantitative cellular and molecular measurements in a randomized, double-blinded, crossover study. Since individuals served as their baseline values, we were able to focus on the intraindividual changes in observed cellular and molecular marker levels following exposures, enabling the detection of statistically significant changes with thirty research participants. Moreover, the crossover design allowed for testing of effect modifications by age and COPD status. However, because I performed multiple statistical comparisons of multiple endpoints, there are multiplicity issues that may have potentially inflated the type 1 error rate as a result of multiple testing. Therefore, I adjusted p-values for multiple comparisons in RNA-seq results where thousands of multiple comparisons from a single assay were performed. However, results are presented unadjusted for multiple comparisons for other endpoints. It should be highlighted that we used multiple independent molecular assays to evaluate the impact of exposure and showed that the impact of DE on COPD participants was different from that on healthy participants.

Another limitation of the study is that the COPD group was older on average than the groups without COPD, reflecting our difficulty in recruiting younger individuals with COPD who were not active tobacco users (excluded as this would potentially overpower DE exposure effects). Also, it was difficult to recruit older 'healthy' participants who were free of potentially significant underlying health conditions (e.g., cardiovascular diseases). However, arguing against the supposition that the COPD-specific results are confounded by age, there were no exposure-by-age interactions in any of the markers increased disproportionately in COPD other than MMP-10. This suggests that age alone confers some susceptibility (perhaps through increase in abundance of MMP-10) independent of COPD status, another important finding that was not an initial focus of my study.

5.3 Implications of the findings: Impact beyond legislations

Legislators and regulatory bodies around the world depend on sound scientific evidence to formulate policies regarding permissible industrial and vehicle emissions and standards for ambient air pollution. The US Environmental Protection Agency, for example, must by law consider sensitive subpopulations when ensuring an adequate margin of safety to protect public health.[94] Epidemiological studies have linked chronic TRAP exposure to increased COPD morbidity and mortality, but detailed mechanistic support for this association has been relatively lacking, aside from limited animal models. Our study has demonstrated that people with COPD may experience greater inflammatory effects of air pollution compared to healthy individuals, supporting the notion that those living with COPD are sensitive to the adverse effects of air pollution exposure and may accordingly need focused attention to the prevention of adverse outcomes in this context. Beyond the potential impact on future air pollution legislation, I believe that our findings may be important for patients with COPD and their health care providers. In line with epidemiological findings of increased morbidity and mortality of COPD-associated acute air pollution exposures, our finding supports the notion that people living with COPD should be advised to limit their exposure to a high level of air pollution.

Beyond potentially impacting legislation and patient care, this thesis enhances our understanding of molecular changes occurring in our airways following TRAP and other air pollution exposures. We employed several state-of-the-art methodologies such as transcriptomics and microbiome analysis. We are planning on making this data available to the research community. Thus, other researchers will be able to expand and interrogate novel research questions with the dataset we generated in this novel study. In addition, our research group is in the process of acquiring additional omics data, including DNA methylation and metabolome from the airways. Integrating multi-omics data to detail the response of human airways to environmental insults *in vivo* is likely to lead to the identification of new hypotheses to be tested in the future.

Because particulate matter air pollution is not unique to TRAP, this thesis can inform about the potential toxicity of PM from other sources. One prominent example is woodsmoke particulate matter pollution arising from forest fires. Increased mortality due to COPD is associated with acute wildfire smoke exposures.[164] Since forest fires also generate fine particulate matters in large quantities, COPD patients may be at an increased risk of adverse health impacts from forest fires. However, because the composition of DE is different from that of smoke from forest fires, much more research is needed to define which air pollutant (e.g. PM, NO_2, and O_3) is responsible for the negative impact on COPD airways. Such investigation can be accomplished in several ways. The first way is to perform a human exposure study to woodsmoke in COPD patients. The second way is to remove PM from DE and compare the impact of particle-depleted DE with DE and assess whether the impact of DE can be eliminated by the removal of PM from DE. Defining which common air pollutant is responsible for the negative health impact in COPD will aid in determining intervention to reduce harms for COPD patients due to pollution exposures.

5.4 Future directions

There are several exciting areas of future research arising from my thesis work. First, the impact of air pollution exposure on oxidative stress markers in COPD should be explored in more detail. Our results

suggest that there would be increased oxidative stress damage arising from DE and the subsequent inflammatory response seen in COPD. Oxidative stress damage and antioxidant response can be measured using various assays quantifying malondialdehyde (oxidative stress damage on lipids), carbonylated protein (oxidative stress damage on proteins), 8-hydroxy-20-deoxyguanosine (oxidative stress damage on DNA), superoxide dismutase (antioxidant enzyme), and glutathione status (i.e. glutathione disulfide/reduced glutathione ratio).[65] Urinary isoprostanes can be measured to assess the oxidative stress damage in the human body, and our group have begun analyzing samples from this study using HPLC-MS/MS.[165]

Second, future research should explore the epigenetic regulation of the Nrf2 pathway in COPD in the context of air pollution exposures. I would test the hypothesis that epigenetic modification leading to alterations of genes in the Nrf2 pathway leads to increased oxidative damage from air pollution exposure. Our group has shown that gene-environmental interaction plays an important role in determining one's susceptibility to air pollution effects.[17,166] Gene-environment interaction should be explored more in the future as this may have implications in understanding COPD pathophysiology in the context of air pollution exposures.

Last, it would be important to characterize the changes in cellular composition and functionality in respiratory mucosa that may explain the difference in tissue response to air pollution in COPD and healthy. With single-cell RNA-seq, researchers can detect the changes in gene expression profiles in different groups of cells over time. This approach would help understand what gene expression changes in which cell population may be the primary driver of the impact that air pollution has on COPD exacerbation and worsening. Such discovery may lead to an identification of therapeutic or preventative measures to lower the risk of COPD exacerbation arising from air pollution exposures.

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